Characterization of Four New Mouse Cytochrome P450 Enzymes of the CYP2J Subfamily^S

Joan P. Graves, Matthew L. Edin, J. Alyce Bradbury, Artiom Gruzdev, Jennifer Cheng, Fred B. Lih, Tiwanda A. Masinde, Wei Qu, Natasha P. Clayton, James P. Morrison, Kenneth B. Tomer, and Darryl C. Zeldin

Laboratory of Respiratory Biology (J.P.G., M.L.E., J.A.B., A.G., J.C., D.C.Z.), Laboratory of Structural Biology (F.B.L., K.B.T.), and the Cellular and Molecular Pathology Branch (T.A.M., N.P.C.), National Toxicology Program Laboratory (W.Q.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina; and Charles River Laboratories-Pathology Associates, Durham, North Carolina (J.P.M.)

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ABSTRACT

The cytochrome P450 superfamily encompasses a diverse group of enzymes that catalyze the oxidation of various substrates. The mouse CYP2J subfamily includes members that have wide tissue distribution and are active in the metabolism of arachidonic acid (AA), linoleic acid (LA), and other lipids and xenobiotics. The mouse *Cyp2j* locus contains seven genes and three pseudogenes located in a contiguous 0.62 megabase cluster on chromosome 4. We describe four new mouse CYP2J isoforms (designated CYP2J8, CYP2J11, CYP2J12, and CYP2J13). The four cDNAs contain open reading frames that encode polypeptides with 62–84% identity with the three previously identified mouse CYP2Js. All four new CYP2J proteins were expressed in *Sf21* insect cells. Each recombinant protein metabolized AA and LA to epoxides and hydroxy derivatives. Specific antibodies, mRNA probes, and polymerase chain

reaction primer sets were developed for each mouse CYP2J to examine their tissue distribution. CYP2J8 transcripts were found in the kidney, liver, and brain, and protein expression was confirmed in the kidney and brain (neuropil). CYP2J11 transcripts were most abundant in the kidney and heart, with protein detected primarily in the kidney (proximal convoluted tubules), liver, and heart (cardiomyocytes). CYP2J12 transcripts were prominently present in the brain, and CYP2J13 transcripts were detected in multiple tissues, with the highest expression in the kidney. CYP2J12 and CYP2J13 protein expression could not be determined because the antibodies developed were not immunospecific. We conclude that the four new CYP2J isoforms might be involved in the metabolism of AA and LA to bioactive lipids in mouse hepatic and extrahepatic tissues.

Introduction

Cytochromes P450 (P450s) are a large gene superfamily of over 500 distinct isoforms that encode heme-thiolate proteins. P450s catalyze the metabolism of a wide range of xenobiotics, including drugs, carcinogens, and environmental pollutants (Nelson et al., 1996; Nebert and Russell, 2002). Certain P450s are also active in the metabolism of endogenous compounds such as arachidonic acid (AA) to bioactive eicosanoids (Nelson et al., 1996; Kroetz and Zeldin, 2002). AA, a polyunsaturated fatty acid present in mammalian cell membranes, is metabolized by multiple P450s into epoxyeicosatrienoic acids (EETs), midchain hydroxyeicosatetraenoic acids (HETEs), and ω -terminal HETEs (Capdevila et al., 2000; Kroetz and Zeldin, 2002; Nebert and Russell, 2002). EETs have substantial vasodilatory (Campbell et al., 1996; Larsen et al., 2006), anti-inflammatory (Node et al., 1999), fibrinolytic (Node et al., 2001), cardioprotective (Seubert

et al., 2004; Seubert et al., 2006), and angiogenic (Wang et al., 2005) effects both in vitro and in vivo, and provide protection from ischemia in brain, heart, and lung models (Seubert et al., 2007; Zhang et al., 2008; Townsley et al., 2010). EET levels are regulated by both synthesis and breakdown. Epoxide hydrolases such as EPHX2 hydrolyze EETs to their dihydroxyeicosatrienoic acids, which are physiologically less active (Fleming, 2001).

CYP2J subfamily members have been identified in many species, including rabbits, rats, mice, and humans (Kikuta et al., 1991; Zhang et al., 1997; Nelson, 2009). The majority of P450s are primarily expressed in the liver, but the mouse CYP2J subfamily includes members that have a wide tissue distribution. For example, mouse CYP2J5 transcripts are detected most prominently in the kidney and liver (Ma et al., 1999), mouse CYP2J6 is most abundantly expressed in small intestine but is present at lower levels in other tissues (Ma et al., 2002), and mouse CYP2J9 is predominantly expressed in the brain but also is present in the kidney (Qu et al., 2001). Human CYP2J2 is highly expressed in the heart, liver, kidney, and other tissues (Wu et al., 1996). Interestingly, there is a single human CYP2J subfamily gene (*CYP2J2*), but there are seven murine CYP2J genes (*Cyp2j5*, *Cyp2j6*, *Cyp2j8*, *Cyp2j9*, *Cyp2j11*, *Cyp2j12*, and *Cyp2j13*)

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ABBREVIATIONS: AA, arachidonic acid; bp, base pair; CYPOR, cytochrome P450 oxidoreductase; DiHOME, dihydroxyoctadecamonoenoic acid; EET, epoxyeicosatrienoic acid; EpOME, epoxyoctadecamonoenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecaenoic acid; LA, linoleic acid; Mb, megabase; ps, pseudogene; P450, cytochrome P450; RT-PCR, reverse-transcription polymerase chain reaction.

and three pseudogenes (*Cyp2j7-ps*, *Cyp2j14-ps*, and *Cyp2j15-ps*) (Wu et al., 1996; Nelson et al., 2004). CYP2J members have been shown to be active in the metabolism of AA and linoleic acid (LA) as well as various xenobiotics (Ma et al., 1999; Qu et al., 2001).

Mouse models are increasingly important in understanding the physiologic function of genes; however, using the mouse as a model of human physiology is more challenging when mouse and human genes lack clear orthologous relationships. Characterization of all the mouse CYP2J subfamily isoforms is an important initial step to understanding the functional significance of human CYP2J2. Based on the exonic sequences of known mouse, rat, and human CYP2Js and with the use of the Celera Discovery System and the National Center for Biotechnology Information databases, we identified all the mouse *Cyp2j* genes and pseudogenes. We then cloned the cDNAs for five new subfamily members designated CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13. CYP2J7 lacked an open reading frame and, based on sequence analysis, would be expected to produce a non-functional protein, so it was designated a pseudogene. The remaining CYP2J isoforms were expressed in *Sf21* insect cells. Each of the new isoforms was shown to be active in the metabolism of AA and LA, albeit with different catalytic efficiencies and product profiles. We also determined the tissue distribution of each new CYP2J isoform at both the mRNA and protein levels using isoform-specific probes.

Materials and Methods

Reagents. AA and LA were purchased from Cayman Chemical (Ann Arbor, MI). NADPH tetrasodium salt hydrate, isocitrate dehydrogenase, and isocitric acid were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides were synthesized by BioServe Biotechnologies (Laurel, MD). Restriction enzymes were purchased from New England BioLabs (Beverly, MA). All other chemicals, reagents and kits were purchased from Sigma-Aldrich unless otherwise specified.

In Silico Gene Identification. Basic Local Alignment Search Tool (BLAST) searching and physical map assembly were accomplished using the Celera Discovery System (assembly R26). Alignments of known mouse, rat, and human CYP2J cDNAs to the mouse genomic sequence were used to identify putative exons of new members of this P450 subfamily. An identity cutoff of 55% was used in this analysis (Nelson et al., 1996). The nine exons for each of the three known mouse *Cyp2j* genes (*Cyp2j5*, *Cyp2j6*, and *Cyp2j9*) were found on two continuous contigs on chromosome 4 (GA_x6K02T2-QEEQ:17000001.17500000 and GA_X6K02T2QEEQ:17500001.18000000). We identified four new *Cyp2j* genes and three new *Cyp2j* pseudogenes on these contigs by combining the putative exonic sequences. A physical map of the mouse *Cyp2j* locus was then assembled using this information (Fig. 1). Each of the new mouse *Cyp2j* genes and pseudogenes was given a formal name by the Committee on Standardized P450 Nomenclature (see <http://drnelson.uthsc.edu/CytochromeP450.html>).

Cloning of cDNAs for the Novel CYP2J Subfamily Members. Total RNA was prepared from C57BL/6 mouse tissues using the RNeasy Midi Kit from Qiagen (Valencia, CA) following the manufacturer's instructions. Based on the RNA sequences derived from the Celera Discovery System analysis, primer pairs (Table 1) were designed to amplify the coding regions of the CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13 cDNAs. For CYP2J11, CYP2J12, and CYP2J13, the ProSTAR Ultra HF RT-PCR (reverse-transcription polymerase chain reaction) System from Stratagene (La Jolla, CA) was used for RT-PCR cloning. Briefly, first-strand cDNA was synthesized from 200 ng of tissue total RNA using StrataScript reverse transcriptase with oligo(dT) priming. The PCR amplifications were performed with 1.0 μ l of cDNA template, 0.4 μ M of each primer, 0.2 μ M dNTPs, and 2.5 U of *Pfu* Turbo DNA polymerase in a total volume of 50 μ l. The PCR conditions were as follows: 95°C for 1 minute; 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 68°C for 5 minutes; the final extension was at 68°C for 10 minutes. For the RT-PCR cloning of CYP2J7 and CYP2J8, the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity from Invitrogen (Carlsbad, CA) was used. We mixed 400 ng of template RNA with 25 μ l 2X Reaction Mix, 0.2 μ M concentration of each primer, and 1 μ l of the SuperScript III RT/Platinum Taq High Fidelity Enzyme Mix in a total volume of 50 μ l. The conditions were as follows: 1 cycle of 50°C for 30 minutes; 94°C for 2 minutes; and 40 cycles of 94°C for 15 seconds, 52°C for 30 seconds, and 68°C for 2.5 minutes; the final extension was at 68°C for 5 minutes. The PCR products were TA-cloned into the pCR2.1 vector from Invitrogen. Positive clones were identified by blue-white selection and were sequenced using the ABI Prism Big Dye DNA Sequencing Kit from Applied Biosystems (Foster City, CA).

Expression of New Mouse CYP2J Recombinant Proteins. The open reading frames of the four new mouse CYP2Js were subcloned downstream of pRC201, the polyhedron promoter of the pAUw51-CYPOR (cytochrome P450 oxidoreductase) baculovirus expression vector (Ma et al., 1999). The pRC201 vector coexpresses the human CYPOR, which is necessary for enzymatic activity of most P450 proteins. Takara LA Taq Polymerase (Takara Mirus Bio, Madison, WI) was used to amplify the open reading frame of the new CYP2Js, and the final vectors were verified by sequencing. The expression vectors were cotransfected with BaculoGold DNA into *Sf9* insect cells using the BD BaculoGold Transfection Kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions and were maintained as high-titer viral stocks. *Sf21* insect cells were infected at a multiplicity of infection of 6, 6, 10, and 8, respectively, with the stocks of CYP2J8, CYP2J11, CYP2J12, and CYP2J13 along with 100 μ M 5-aminolevulinic acid and 100 μ M iron citrate. Infected cells were harvested 72 hours after infection and lysed in a buffer of 0.25 M sucrose, 10 mM Tris (pH 7.5), 0.1 μ g/ml of leupeptin, 0.04 U/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μ g/ml pepstatin. Differential centrifugation was used to collect the microsomal fraction, which was resuspended in 50 mM Tris (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA (pH 7.5), and 20% glycerol. P450 concentrations were determined by carbon monoxide (CO) difference spectra (Fig. 3) using a Beckman DU 640 Spectrophotometer (Beckman Coulter, Fullerton, CA) (Omura and Sato, 1964). Microsomal P450 concentrations were determined (and expressed as nmol

Fig. 1. Organization of the mouse *Cyp2j* subfamily on chromosome 4. Exon sequences of known mouse, rat, and human *Cyp2j* subfamily members were used to BLAST search the mouse genome in the Celera Discovery System and the National Center for Biotechnology Information databases. Seven *Cyp2j* genes (black arrows) and three pseudogenes (gray arrows) were mapped to the negative strand in a 0.62-Mb cluster on chromosome 4.

TABLE 1

Primer sequences used for the cloning of mouse CYP2J cDNAs

Primer pairs were designed based on the cDNA sequences derived from the Celera Discovery System and National Center for Biotechnology Information database analysis. The primer pairs were designed to amplify the coding regions of the five new CYP2Js. Corresponding positions are relative to the ATG initiating codon designated as +1.

cDNA and Direction	Primer Sequence 5'–3'	Corresponding Position
CYP2J7		
Forward	GGGCCAATAAGGATAAAGTCA	–146 to –126
Reverse	GGCCAGAAGATTCAGAGCATA	+1551 to +1571
CYP2J8		
Forward	AGGGCTAAAAGTACTGTCTCAGG	–46 to –26
Reverse	TTTCTTAGCCCAATTCTTCA	+1628 to +1648
CYP2J11		
Forward	GTGGCCAGTAGGAACAGAGTG	–149 to –129
Reverse	GTGATGGCCCCATTACTTGAGG	+1873 to +1893
CYP2J12		
Forward	GACTCTCATCATGCTTGCTAC	–10 to +11
Reverse	ACTTGGGCGGCAGGGTGTATG	+1676 to +1696
CYP2J13		
Forward	GTGCTCAAGCGCTCAAGTGCC	–25 to –5
Reverse	GTCTCATCTTGGGCACGAACC	+2115 to +2135

P450/mg total microsomal protein) so that metabolic studies could be performed with equal P450 concentrations.

Arachidonic and Linoleic Acid Metabolism by Recombinant CYP2Js.

Recombinant CYP2J proteins were incubated with either AA or LA as previously described elsewhere (Ma et al., 1999) with the modifications that nonradioactive AA and LA were used and that the sample analyses were performed by liquid chromatography/mass spectrometry. Briefly, a reaction mixture of 0.05 M Tris-Cl (pH 7.5), 0.15 M KCl, 0.01 M MgCl₂, 8 mM sodium isocitrate, 0.5 U/ml isocitrate dehydrogenase, 0.25 μ M recombinant protein, and 100 μ M AA or LA was equilibrated to 37°C. Previously we had determined that the K_m for CYP2J enzymes for AA is $\sim$ 80 μ M (Ma et al., 1999). We used a higher amount of substrate to ensure saturation of the enzyme (i.e., the substrate was not limiting). Buffers, lipid concentrations, and incubations times were selected to be consistent with prior work from our group and others (Ma et al., 1999; DeLozier et al., 2004). The reaction was started by the addition of 1 mM NADPH and incubated at 37°C for 1 hour with gentle shaking. Control reactions with microsomes from vector-infected cells, without lipid addition, with boiled microsomes, or without NADPH addition were used to determine that conversion of AA and LA was not due to endogenous proteins in the *Sf21* cell lysates. Samples were extracted with diethyl ether 3 times and then dried under nitrogen. The organic metabolites generated during the incubation were analyzed by liquid chromatography on an Agilent 1200 Series capillary high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA), then separated on a Phenomenex Luna C18(2) column (5 μ m, 150 $\times$ 1 mm; Phenomenex, Torrance, CA) as previously described elsewhere (Edin et al., 2011). Sample analyses by negative ion electrospray ionization tandem mass spectrometry were performed in triplicate on a MDS Sciex API 3000 equipped with a TurbolonSpray source (Applied Biosystems). CYP2J2 recombinant protein was used as a reference.

RT-PCR Analysis. Specific primers to the new CYP2J cDNAs (Tables 2 and 3) were designed for RT-PCR analysis. Qiagen's OneStep RT-PCR Kit was used to determine the tissue distribution at the mRNA level. Thirty-five cycles of amplification were performed with an annealing temperature of 55°C for 30 seconds for CYP2J8, 33 cycles with annealing at 62°C for 30 seconds for CYP2J11, 35 cycles with annealing at 58°C for 30 seconds for CYP2J12, and 35 cycles with annealing at 64°C for 30 seconds for CYP2J13. The mouse β -actin RT-PCR Primer Set (Agilent Technologies) was used as a control for cDNA quantity. PCR products were analyzed on 1.2% agarose gels stained with ethidium bromide.

Immunoblotting. By use of the Peptide Structure program of the GCG Wisconsin Package (Accelrys, Inc., San Diego, CA) and sequence alignment of the CYP2J subfamily members (Fig. 2), the polypeptides specific to the deduced amino acid sequences of CYP2J8, CYP2J11, CYP2J12, and CYP2J13 were designed. The specific peptides were synthesized, purified, and then coupled to keyhole limpet hemocyanin via a C-terminal cysteine by Research

TABLE 2

Primer sequences used to subclone new mouse CYP2J cDNAs into the pRC201 expression vector

Underlined bases were added to subclone into an expression vector.

Transcript	Primer Sequence (5'–3')
CYP2J8	
Forward	AGCTAGCGGGATTGGTCCAGCTCAGACTCTC
Reverse	<u>GTAGTCGACT</u> CCTCTCATACGAGCAAGGCCTTG
CYP2J11	
Forward	AGCTAGCGAGGGCTGTCTCAGTACTGTCTCAGGG
Reverse	<u>GTAGTCGACT</u> TATGTGAGCAAGGCCAAGAATC
CYP2J12	
Forward	TGGATCCACTCTCATCATGCTTGCTACTGA
Reverse	<u>GTAGTCGACT</u> GGAGAAGTGTGAGGCCATTTC
CYP2J13	
Forward	TGGATCCTCAAGCGCTCAAGTGCCAGCC
Reverse	<u>GTAGTCGACT</u> ATATGGGCAGTGGCTCAGTGAC

Genetics (Huntsville, AL). These specific peptides were then used to raise polyclonal antibodies (α -CYP2J8pep1, α -CYP2J11pep1, α -CYP2J12pep1, and α -CYP2J13pep1) in New Zealand white rabbits at Covance Research Products, Inc. (Denver, PA). C57BL/6 mouse tissue microsomes were prepared by differential centrifugation as previously described elsewhere (Ma et al., 1999). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Tissue microsomes (40 μ g/lane) or whole tissue lysates (50 μ g/lane), and recombinant CYP2Js (0.5 pmol/lane) were separated by electrophoresis on 12% Tris-glycine gels from Invitrogen, transferred onto nitrocellulose membranes, and immunoblotted using the CYP2J antibodies. The secondary antibody used was goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and blots were visualized by Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Anti-CYP2J8pep1 was used at a dilution of 1:8000 in 5% milk/phosphate-buffered saline containing 0.1% Tween 20 (Bio-Rad Laboratories) overnight at 4°C, and the secondary goat anti-rabbit antibody at a 1:10,000 dilution for 30 minutes at room temperature. We purified α -CYP2J11pep1, α -CYP2J12pep1, and α -CYP2J13pep1 with the Protein A IgG Purification and the AminoLink Plus Immobilization Kits from Pierce in an attempt to increase specificity. A dilution of 1:1000 of α -CYP2J11pep1 in 1% bovine serum albumin/phosphate-buffered saline with 0.1% Tween 20 was used overnight at 4°C, with the secondary antibody at 1:10,000 dilution for 45 minutes at room temperature. Attempts to optimize blotting conditions for α -CYP2J12pep1 and α -CYP2J13pep1 failed; the antibodies were not immunospecific for the intended recombinant protein targets.

Immunohistochemistry. Immunohistochemical staining was performed for CYP2J8 in mouse brain and for CYP2J11 in mouse kidney and heart tissues using the standard avidin-biotin-peroxidase technique, using methods described previously (Zeldin et al., 1996). Formalin-fixed, paraffin-embedded tissues were deparaffinized and unmasked with heat-induced epitope retrieval in 0.01 M citrate buffer, pH 6.0 (Biocare Medical, Concord, CA), performed in

TABLE 3

Sequences of gene-specific primers for reverse-transcription polymerase chain reaction

Transcript	Sequence (5'–3')	Product Size (bp)
CYP2J8		
Forward	GAGGCATCAAAGGCGTTAAT	891
Reverse	GGGCATATACCATGAATTCTCA	
CYP2J11		
Forward	ATTGGAGTATGCCCTGAAGAT	200
Reverse	GTGATGGCCATTACTTGAGG	
CYP2J12		
Forward	AATGACATGCACCTTCAAACC	100
Reverse	ACTTGGGCGGCAGGGTGTATG	
CYP2J13		
Forward	GTCAGTGAAGCCACTGCCCATATA	568
Reverse	GTCTCATCTTGGGCACGAACC	

1					50					301					SRS-4					350									
2j5pep	MIMFLSSLVT	TFWEALHLKT	LVLAVVTLFL	LINILRSRHP	KNYPPGPWRL	2j5pep	EENLISTTLD	LFLGGTETTS	STLRWALLYM	SSYPEIQENV	QAEIDRVIGH	2j5pep	EENLISTTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j5pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j5pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
2j6pep	MLAATGCSLLA	TIWAALHPT	LLVAAVTFL	LADYFKNRRP	KNYPPGPWGL	2j6pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j6pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j6pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j6pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
2j8pep	MFATIGCLVS	NICSEIHLWT	LLLAALTLFL	LVDYIKNRRP	KNYPPGPWRL	2j8pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j8pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j8pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j8pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
2j9pep	MLAAAGSLVA	AIWAALHLRI	LLLSAVTFL	LADFLKNRRP	KNYPPGPWRL	2j9pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j9pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j9pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGQ	2j9pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
2j11pep	MLAIATCLVA	NICSAIHLWT	LLLTLLTLL	LADYLNRRP	KNYPPGPWRL	2j11pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j11pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j11pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j11pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
2j12pep	MLATEDTFT	AIWALLHFKT	LVLAAVTFL	LAHYLKTQRP	KNYPPGPWRL	2j12pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j12pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j12pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j12pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
2j13pep	MLEAADSLAA	AFWAALHLRT	LLLAATLFL	LADYLNRRP	KNYPPGPWRL	2j13pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j13pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j13pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH	2j13pep	EENLICSTLD	LFFAGTETTS	TTLRWALLYM	ALYPEVQEKV	QAEIDRVIGH
					*****										*****														
51					100					351					SRS-5					400									
2j5pep	PFVGNFFQ..	IDTKQTHLVL	QQFVKYGNV	FSLELGQSPV	VVVSGLPLIK	2j5pep	KRQVSLSDRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j5pep	KRQVSLSDRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j5pep	KRQVSLSDRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j5pep	KRQVSLSDRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
2j6pep	PFVGNIFQ..	LDVGQPHLSI	QPLVKKYGNV	FSLNLGDITS	VVITGLPLIK	2j6pep	KRAARLADRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j6pep	KRAARLADRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j6pep	KRAARLADRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j6pep	KRAARLADRE	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
2j8pep	PFVGNLFQFD	LDVSHLHLGI	QPFVKYGNV	ISLDFGTIPS	VIISGEPLIK	2j8pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j8pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j8pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j8pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
2j9pep	PFVGLCFH..	LDPKQPHLSL	QQFVKYGNV	LSLDFANIPS	VVITGMPLIK	2j9pep	ARQPNLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j9pep	ARQPNLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j9pep	ARQPNLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j9pep	ARQPNLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
2j11pep	PFVGNLFQFD	LDVSRHLGI	QPFVKYGNV	ISVNFYIIS	VIISGLPLIK	2j11pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j11pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j11pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j11pep	GRHPTIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
2j12pep	PFVGNLFQ..	LDLEQPHVVI	QQFVKYGNV	ISLDFGNIPS	VIITGLPLIK	2j12pep	GRQPSIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j12pep	GRQPSIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j12pep	GRQPSIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j12pep	GRQPSIDDRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
2j13pep	PFVGNISFQ..	MDLTQSHLAV	QQFVKYGNV	ISLDFGKISA	VIISGLPLIK	2j13pep	SKHPSLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j13pep	SKHPSLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j13pep	SKHPSLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK	2j13pep	SKHPSLADRD	SMPTNAVIH	EVQRMGNIVP	LNSSREVIVD	TKFNGFHLPK
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101					SRS-1					150					401					450									
2j5pep	EMFTHLDQNF	VNRFMTPVRE	RITGKNGLV	SSGQTWKEQR	RLALMALRNF	2j5pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j5pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j5pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j5pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA
2j6pep	EALTMQEQNI	MNRPLSVMQE	RISNKNGLIF	SSGQIWKEQR	RFALMTLRNF	2j6pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j6pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j6pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j6pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA
2j8pep	EALTCMQQNF	LKRPLLAARQ	HIFKNNGIIS	SSGQTWKEQR	RFALMTLRNF	2j8pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j8pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j8pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j8pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA
2j9pep	EIFTQMEHNF	MNRPVTLARK	HLFNKNGLIF	SSGQTWKEQR	RFALMTLRNF	2j9pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j9pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j9pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j9pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA
2j11pep	EAITQMEQNF	LKRPSLAARQ	HVFKNNGIIV	SSGQTWKEQR	KFALMTLRNF	2j11pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j11pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j11pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j11pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA
2j12pep	EVFTNMEQNF	LNRPIPIRQ	RVFNNGLII	SSGQTWKEQR	RFALMTLRNF	2j12pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j12pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j12pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j12pep	GTMLTNLTA	LHRDPKEWAT	PEVFNPEHFL	ENGQFKKRES	FLPFSMGKRA
2j13pep	EAFSHLDENF	INRRIFPLQR	QIFNGNGLIF	SSGQTWKEQR	RFALMTLRNF	2j13pep	RSVMTNLTA	LHMDPKEWAT	PDIFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j13pep	RSVMTNLTA	LHMDPKEWAT	PDIFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j13pep	RSVMTNLTA	LHMDPKEWAT	PDIFNPEHFL	ENGQFKKRES	FLPFSMGKRA	2j13pep	RSVMTNLTA	LHMDPKEWAT	PDIFNPEHFL	ENGQFKKRES	FLPFSMGKRA
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151					200					451					SRS-6					500									
2j5pep	GLGKKSLEER	IQEETHLVE	AIREEGGQPF	NPHKLINAV	SNIIICSVTFG	2j5pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j5pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j5pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j5pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC
2j6pep	GLGKKSLEER	MQEASHLVE	AIREEGGQPF	NPHKSINAV	SNIIICSVTFG	2j6pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j6pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j6pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j6pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC
2j8pep	GLGKKSLEQR	IQEAAHLVE	AIAEKGQPF	DPHLMINAV	SNIIICSVTFG	2j8pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j8pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j8pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j8pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC
2j9pep	GLGKKSLEER	IQEAAHLVE	AIRDEGGQPF	DPHFNINAV	SNIIICSVTFG	2j9pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j9pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j9pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j9pep	CLGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC
2j11pep	GLGKKSLEQR	IQEAAHLVE	AIGEEKGQPF	DPHFRINAV	GNIIICSVTFG	2j11pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j11pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j11pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j11pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC
2j12pep	GLGKKSLEQR	IQEADHLVE	AIGEEKGQPF	DPHFKINSAV	SNIIICSVTFG	2j12pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j12pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j12pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC	2j12pep	CPGEQLAKSE	LFIFFTALMQ	KFTFKPPINE	KLSLKFRMGL	TLSPASYRIC
2j13pep	GLGKKSLEHR	IQEAAHLVE	AIGEEGGQPF	DPHFKIYSAV	SNIIICSVTFG	2j13pep	CLGEQLAKSE	LFIFFTALVQ	KFTFKPPANE	NLSLKFRMGL	TLSPASYRIC	2j13pep	CLGEQLAKSE	LFIFFTALVQ	KFTFKPPANE	NLSLKFRMGL	TLSPASYRIC	2j13pep	CLGEQLAKSE	LFIFFTALVQ	KFTFKPPANE	NLSLKFRMGL	TLSPASYRIC	2j13pep	CLGEQLAKSE	LFIFFTALVQ	KFTFKPPANE	NLSLKFRMGL	TLSPASYRIC
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201					SRS-2					250					501					550									
2j5pep	ERFDYEDCQF	QELLQLLDET	MHLMGSS.AG	QLYNGFPCIM	KYLPGPHQKI	2j5pep	ERFDYEDCQF	QELLQLLDET	MHLMGSS.AG	QLYNGFPCIM	KYLPGPHQKI	2j5pep	ERFDYEDCQF	QELLQLLDET	MHLMGSS.AG	QLYNGFPCIM	KYLPGPHQKI	2j5pep	ERFDYEDCQF	QELLQLLDET	MHLMGSS.AG	QLYNGFPCIM	KYLPGPHQKI	2j5pep	ERFDYEDCQF	QELLQLLDET	MHLMGSS.AG	QLYNGFPCIM	KYLPGPHQKI
2j6pep	ERFDYHDSRF	QELLRLLEDE	MYL.ETTMIS	QLYNIFPMIM	KYIPGSHQKV	2j6pep	ERFDYHDSRF	QELLRLLEDE	MYL.ETTMIS	QLYNIFPMIM	KYIPGSHQKV	2j6pep	ERFDYHDSRF	QELLRLLEDE	MYL.ETTMIS	QLYNIFPMIM	KYIPGSHQKV	2j6pep	ERFDYHDSRF	QELLRLLEDE	MYL.ETTMIS	QLYNIFPMIM	KYIPGSHQKV	2j6pep	ERFDYHDSRF	QELLRLLEDE	MYL.ETTMIS	QLYNIFPMIM	KYIPGSHQKV
2j8pep	ERFEYEDNQF	QELLKLADET	LCL.EASKAL	MLYNVFPISF	KYLPGPHQKL	2j8pep	ERFEYEDNQF	QELLKLADET	LCL.EASKAL	MLYNVFPISF	KYLPGPHQKL	2j8pep	ERFEYEDNQF	QELLKLADET	LCL.EASKAL	MLYNVFPISF	KYLPGPHQKL	2j8pep	ERFEYEDNQF	QELLKLADET	LCL.EASKAL	MLYNVFPISF	KYLPGPHQKL	2j8pep	ERFEYEDNQF	QELLKLADET	LCL.EASKAL	MLYNVFPISF	KYLPGPHQKL
2j9pep	ERFDYHDSQF	QELLRLLEDE	MCL.ESSMMC	QLYNIFPRIL	QYLPGSHQTL	2j9pep	ERFDYHDSQF	QELLRLLEDE	MCL.ESSMMC	QLYNIFPRIL	QYLPGSHQTL	2j9pep	ERFDYHDSQF	QELLRLLEDE	MCL.ESSMMC	QLYNIFPRIL	QYLPGSHQTL	2j9pep	ERFDYHDSQF	QELLRLLEDE	MCL.ESSMMC	QLYNIFPRIL	QYLPGSHQTL	2j9pep	ERFDYHDSQF	QELLRLLEDE	MCL.ESSMMC	QLYNIFPRIL	QYLPGSHQTL
2j11pep	ERFEYDDNQF	QELLKLADEI	ICS.EASMM	QLYNVFPISF	KYLPGPQOKL	2j11pep	ERFEYDDNQF	QELLKLADEI	ICS.EASMM	QLYNVFPISF	KYLPGPQOKL	2j11pep	ERFEYDDNQF	QELLKLADEI	ICS.EASMM	QLYNVFPISF	KYLPGPQOKL	2j11pep	ERFEYDDNQF	QELLKLADEI	ICS.EASMM	QLYNVFPISF	KYLPGPQOKL	2j11pep	ERFEYDDNQF	QELLKLADEI	ICS.EASMM	QLYNVFPISF	KYLPGPQOKL
2j12pep	ERFEYEDSHF	HELLRLLEDE	TCL.EASMM	QLYNVFPISII	QYVPGSHQTV	2j12pep	ERFEYEDSHF	HELLRLLEDE	TCL.EASMM	QLYNVFPISII	QYVPGSHQTV	2j12pep	ERFEYEDSHF	HELLRLLEDE	TCL.EASMM	QLYNVFPISII	QYVPGSHQTV	2j12pep	ERFEYEDSHF	HELLRLLEDE	TCL.EASMM	QLYNVFPISII	QYVPGSHQTV	2j12pep	ERFEYEDSHF	HELLRLLEDE	TCL.EASMM	QLYNVFPISII	QYVPGSHQTV
2j13pep	ERFEYHDSQF	QELLQLLDKA	MYLGTTSMLV	QLYNIFPWIT	KHLPQGHQTL	2j13pep	ERFEYHDSQF	QELLQLLDKA	MYLGTTSMLV	QLYNIFPWIT	KHLPQGHQTL	2j13pep	ERFEYHDSQF	QELLQLLDKA	MYLGTTSMLV	QLYNIFPWIT	KHLPQGHQTL	2j13pep	ERFEYHDSQF	QELLQLLDKA	MYLGTTSMLV	QLYNIFPWIT	KHLPQGHQTL	2j13pep	ERFEYHDSQF	QELLQLLDKA	MYLGTTSMLV	QLYNIFPWIT	KHLPQGHQTL
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251					SRS-3					300																			
2j5pep	FRNWKKLKL	VSHIVKKHEK	DWNPDPRDF	IDAFLIEMQK	DPDR.TTTSFN	2j5pep	FRNWKKLKL	VSHIVKKHEK	DWNPDPRDF	IDAFLIEMQK	DPDR.TTTSFN	2j5pep	FRNWKKLKL	VSHIVKKHEK	DWNPDPRDF	IDAFLIEMQK	DPDR.TTTSFN	2j5pep	FRNWKKLKL	VSHIVKKHEK	DWNPDPRDF	IDAFLIEMQK	DPDR.TTTSFN	2j5pep	FRNWKKLKL	VSHIVKKHEK	DWNPDPRDF	IDAFLIEMQK	DPDR.TTTSFN
2j6pep	FRNWKKLKL	VSCMIDHRK	DWNPDPRDF	IDAFLEKMTK																									

testis, 12 had a 16-bp sequence from a splice junction in intron 3 inserted between exons 3 and 4, and the remaining 7 clones had a 62-bp insert from a splice junction from intron 1 between exons 1 and 2 (Supplemental Fig. 1). All three splice variants of CYP2J7 result in early truncation of the predicted protein. Each of the cDNAs for CYP2J8, CYP2J11, CYP2J12, and CYP2J13 contains an open reading frame that encodes polypeptides of 503, 504, 502, and 503 amino acids, respectively. Domains present in all P450s are conserved in these new CYP2J isoforms, including a putative heme-binding peptide with an invariant cysteine at position 451, a proline-rich region between residues 40 to 51, and six putative substrate recognition sites (Gotoh, 1992) (Fig. 2). The four new and three previously identified mouse CYP2J isoforms, all encoding full-length proteins, are 62–84% identical at the amino acid level (Table 4).

Heterologous Expression and Enzymatic Characterization of the New CYP2Js. CYP2J8, CYP2J11, CYP2J12, and CYP2J13 were coexpressed with CYPOR in *Sf21* insect cells using the baculovirus expression system with the modified pRC201 vector, as described previously elsewhere (Ma et al., 1999). CYP2J isoform expression levels varied between 0.05 and 0.2 nmol P450/mg of microsomal protein. All four new CYP2Js displayed typical b-type cytochrome P450-reduced CO difference spectra with Soret maxima at ~450 nm (Fig. 3). In the presence of NADPH and CYPOR, the four novel CYP2J enzymes metabolized both AA and LA, albeit with different catalytic efficiencies and product profiles (Fig. 4; Tables 5–7). For comparison, a similar analysis was performed on CYP2J2, CYP2J5, CYP2J6, and CYP2J9. These enzymes showed similar epoxigenase and hydroxylase activities as previously published, and they metabolized AA and LA with similar regiochemical specificity (Wu et al., 1996; Ma et al., 1999, 2002; Qu et al., 2001).

CYP2J5, CYP2J8, CYP2J11 and CYP2J12 showed little substrate preference for AA compared with LA. In contrast, CYP2J13 metabolized LA at a rate that was 2.5-fold higher than AA. Of these enzymes, CYP2J8 had the highest catalytic turnover, metabolizing AA and LA at 167 and 137 pmol/nmol/min, respectively. CYP2J6, which had previously been shown to be an unstable P450 (Ma et al., 2002), had the lowest turnover of the CYP2J enzymes for AA at 22 pmol/nmol/min, which was approximately 7.5 times lower than that for CYP2J8. CYP2J9 had the lowest turnover of the CYP2J enzymes for LA at 10 pmol/nmol/min, which was approximately 14 times lower than that for CYP2J8. CYP2J5, CYP2J6, and CYP2J9 were primarily AA epoxigenases, with 74, 81, and 66% of total products being EETs, respectively. In contrast, CYP2J12 was primarily an AA hydroxylase, with 67% of total products being HETEs. CYP2J8, CYP2J11, and CYP2J13 showed comparable AA epoxigenase and AA hydroxylase activity, producing nearly equal mixtures of EETs (53, 48, and 43%, respectively) and HETEs (47, 52, and 57%, respectively).

The seven mouse CYP2J enzymes were also active in the metabolism of LA to epoxyoctadecamonoenoic acids (EpOMEs) and hydroxyoctadecanoic acids (HODEs) (Table 5). CYP2J6, CYP2J8, and CYP2J12 primarily metabolized LA to EpOMEs (93, 70, and 80% of total products, respectively), while CYP2J9 exclusively metabolized LA to HODEs. CYP2J5, CYP2J11, and CYP2J13 metabolized LA to mixtures of EpOMEs (45, 46, and 43%, respectively) and HODEs (55, 54, and 57%, respectively).

Regiochemical analysis of EETs formed during incubations with the recombinant CYP2Js and AA revealed a preference for the formation of 14,15-EET (43–72% of total EETs) for all the CYP2J isoforms (Table 6). Formation of 11,12-EET was lower (9–20% of total EETs), except for CYP2J8 (34% of total EETs). All CYP2Js produced lower amounts of 8,9-EET (6–14%) and 5,6-EET (5–13%). The regioisomer distribution of EpOME products formed during incubations of

TABLE 4

Amino acid sequence identity of the four new mouse CYP2J subfamily isoforms with known mouse CYP2Js

	CYP2J5	CYP2J6	CYP2J8	CYP2J9	CYP2J11	CYP2J12	CYP2J13
CYP2J5	100	73.2	68.6	70.7	66.5	70.5	70.6
CYP2J6		100	72.4	78.2	71.8	74.2	74.7
CYP2J8			100	70.3	83.7	75.9	69.3
CYP2J9				100	70.6	73.9	74.5
CYP2J11					100	75.3	68.9
CYP2J12						100	71.4
CYP2J13							100

recombinant CYP2J5, CYP2J6, CYP2J9, and CYP2J13 with LA showed a mixture of 12,13-EpOME (52–59%) and 9,10-EpOME (41–48%) (Table 6). Incubation of CYP2J8 with LA revealed a preference for 9,10-EpOME (71%), whereas CYP2J11 showed a slightly higher preference for 12,13-EpOME (61%) over 9,10-EpOME (39%).

The seven murine CYP2Js produced two patterns of HETE regioisomers. CYP2J5, CYP2J6, CYP2J9, CYP2J11, and CYP2J12 generated predominantly 15-, 8-, and 5-HETE. In contrast, CYP2J8 and CYP2J13 generated 19-HETE (28% of hydroxylated compounds for both) as their major hydroxylated metabolite. In addition, CYP2J8 and CYP2J13 produced detectable amounts of 20-HETE (7 and 22%, respectively), which was similar to that observed for human CYP2J2 (Table 7).

Expression Pattern of the New CYP2J mRNAs. To determine the relative abundance of the new CYP2J transcripts, RT-PCR (Fig. 5) using CYP2J isoform-specific primer pairs (Table 3) was performed with RNA extracted from various C57BL/6 mouse tissues. There were prominent CYP2J8 transcripts detected in the kidney, liver, small intestine, and brain by RT-PCR. The RT-PCR analysis of CYP2J11 showed prominent transcripts in the kidney and heart, with fainter

Fig. 3. CO difference spectra of the new recombinant mouse CYP2J proteins. All the new CYP2J recombinant proteins have Soret maxima at approximately 450 nm. The scale for the absorbance for each of the CYP2Js for 0.001 OD (optical density) is as follows: CYP2J8 is 0.44 mm, CYP2J11 is 2.1 mm, CYP2J12 is 3.6 mm, and CYP2J13 is 1.9 mm.

Fig. 4. Liquid chromatography–tandem spectrometry (LC-MS/MS) profiles for the metabolism of AA and LA by the recombinant CYPJs. A representative chromatogram of the incubation of CYP2J8 with AA and with (top) or without (bottom) NADPH is shown. Detection of individual AA metabolites is indicated. Experiments were performed in triplicate for each P450 enzyme.

transcripts seen for liver, ovary, and other tissues. CYP2J12 RT-PCR analysis revealed a prominent transcript in the brain, with fainter transcripts for all of the other tissues examined including the liver.

CYP2J13 transcripts were present in all the mouse tissues examined but were more prominently expressed in the kidney. The CYP2J13 transcript was also seen to be more highly expressed in the male

TABLE 5

Metabolism of arachidonic acid (AA) and linoleic acid (LA) by recombinant mouse CYP2J enzymes

Total metabolic rates, percentage of epoxigenase, and percentage of hydroxylase metabolites formed during incubations of recombinant mouse CYP2J isoforms with AA and LA were quantified by liquid chromatography–tandem mass spectrometry (as described under Materials and Methods). Data for human CYP2J2 are shown for comparison.

Protein	Rates (pmol/nmol/min)		Distribution (% Total)			
	AA	LA	AA Epoxy	AA Hydroxyl	LA Epoxy	LA Hydroxyl
CYP2J2	254	399	72	28	85	15
CYP2J5	76	85	74	26	45	55
CYP2J6	22	29	81	19	93	7
CYP2J8	167	137	53	47	70	30
CYP2J9	37	10	66	34	0	100
CYP2J11	37	62	48	52	46	54
CYP2J12	35	42	33	67	80	20
CYP2J13	78	193	43	57	43	57

kidney as compared with the female kidney. The CYP2J13 transcript expression pattern in the kidney is similar to that of CYP2J5, which is higher in male versus female kidneys (Ma et al., 1999). The majority of P450s are expressed in the liver, so it is not surprising that transcripts for the four new CYP2Js were present in the liver, albeit at low levels. It is not clear whether these liver transcripts reflect expression of the new CYP2Js or whether they represent cross-reactivity to other P450s in mouse liver.

Tissue Distribution of the New CYP2J Proteins. Unique peptides, chosen by aligning the amino acid sequences of all members of the CYP2J subfamily (Fig. 2), were used to raise polyclonal antibodies for the new CYP2Js (α -CYP2J8pep1, α -CYP2J11pep1, α -CYP2J12pep1, and α -CYP2J13pep1). Immunoblotting with the mouse CYP2J recombinant proteins to examine the specificity of the new polyclonal antibodies revealed that α -CYP2J8pep1 and α -CYP2J11pep1 are highly immunospecific for their respective recombinant proteins (Fig. 6). In contrast, α -CYP2J12pep1 recognized all the recombinant mouse CYP2J proteins and also had cross-reactivity with multiple CYP2C recombinant proteins (unpublished data); therefore, this antibody was not useful to determine the CYP2J12 tissue distribution at the protein level. The α -CYP2J13pep1 was also not useful to determine the tissue distribution of CYP2J13 at the protein level, as it recognized both the CYP2J13 and CYP2J5 proteins (unpublished data). This cross-reactivity is not surprising in light of the ~80% identity of the deduced CYP2J5 and CYP2J13 amino acid sequences (Table 4). The 62–84% amino acid identity between the mouse CYP2J subfamily members makes it difficult to identify unique peptide stretches to raise isoform-specific polyclonal antibodies.

CYP2J8 protein expression was analyzed by immunoblotting of whole tissue lysates using the α -CYP2J8pep1 antibody (Fig. 6). The

α -CYP2J8pep1 antibody detected two proteins in brain: one at 58 kDa corresponding to CYP2J8 and another higher molecular weight protein. We did not detect an immunoreactive protein band in the kidney with α -CYP2J8pep1, despite the presence of a CYP2J8 transcript in the kidney (Fig. 5). CYP2J11 protein expression was analyzed by immunoblotting of microsomal fractions of various mouse tissues using α -CYP2J11pep1 (Fig. 6). A prominent ~57 kDa band was observed in the kidney, liver, and stomach, with a slightly higher molecular weight protein in the heart. The difference in electrophoretic mobility between the recombinant CYP2J11 protein and endogenous CYP2J11 in the heart may possibly be explained by the endogenous protein being posttranslationally modified, or having cross-reactivity to a related, slightly higher molecular weight protein that is highly expressed in the heart. A slightly lower molecular weight protein was detected in the testis and lung, and it likely represents cross-reactivity with an unknown protein given the lack of CYP2J11 transcripts in the testis or lung (Fig. 5). Further investigation will be needed to account for these minor differences in electrophoretic mobility between the CYP2J11 recombinant protein and endogenous CYP2J11.

Localization of CYP2J8 and CYP2J11 Protein Expression in Mouse Tissues. Formalin-fixed, paraffin-embedded mouse brain sections were stained with either α -CYP2J8pep1 or normal rabbit serum (negative control) to determine the cellular distribution of CYP2J8 protein in the brain (Fig. 7, A and B). Diffuse staining was present in the neuropil of the gray matter throughout the brain, including the forebrain and the globus pallidus; this staining may represent expression of CYP2J8 protein in neuronal processes such as dendrites and unmyelinated axons. White matter tracts were devoid of staining. Staining of large neurons was nonspecific; similar staining patterns were observed in the negative control sections.

TABLE 6

Regiochemical distribution of epoxyeicosatrienoic acid (EET) and epoxyoctadecamonoenoic acid (EpOME) products formed during incubation of recombinant mouse CYP2Js with arachidonic acid and linoleic acid

Data for human CYP2J2 are shown for comparison.

Protein	Regioisomer Distribution (% Total EET)				Regioisomer Distribution (% Total EpOME)	
	14,15-EET	11,12-EET	8,9-EET	5,6-EET	12,13-EpOME	9,10-EpOME
CYP2J2	71	18	6	5	58	42
CYP2J5	53	20	14	12	52	48
CYP2J6	78	9	7	5	59	41
CYP2J8	43	34	13	10	29	71
CYP2J9	75	11	6	7	58	42
CYP2J11	73	14	7	7	61	39
CYP2J12	63	15	9	13	53	47
CYP2J13	72	14	6	7	55	45

TABLE 7

Regiochemical distribution of hydroxyeicosatetraenoic acid (HETE) products formed during incubation of recombinant CYP2Js with arachidonic acid

Data for human CYP2J2 are shown for comparison.

Protein	Regiochemical Distribution (% Total HETEs)						
	20-HETE	19-HETE	15-HETE	12-HETE	11-HETE	8-HETE	5-HETE
CYP2J2	17	19	14	7	10	20	13
CYP2J5	1	1	18	0	10	23	47
CYP2J6	2	1	34	8	11	25	20
CYP2J8	7	28	9	18	8	21	9
CYP2J9	3	1	33	11	12	25	14
CYP2J11	11	1	28	2	11	24	23
CYP2J12	2	1	35	4	13	21	26
CYP2J13	22	28	13	3	7	13	14

To determine the cellular distribution of CYP2J11, formalin-fixed, paraffin-embedded mouse heart and kidney sections were stained with either α -CYP2J11pep1 or the normal rabbit serum (Fig. 7, C–F). Diffuse staining was observed in cardiomyocytes and renal proximal tubules; glomeruli and distal tubules did not stain. There was little or no background staining with normal rabbit serum. Fixed liver sections were also stained with α -CYP2J11pep1 or normal rabbit serum (unpublished data). No differences were detected between the α -CYP2J11pep1 and normal rabbit serum with respect to staining in the liver.

Discussion

P450 enzymes have many physiologically relevant functions including regulating vascular tone in the heart (Campbell and Harder, 1999; Fisslthaler et al., 1999), mediating ion transport in the kidney (Zou et al., 1996), anti-inflammatory properties in the vasculature (Node et al., 2001), and controlling the secretion of pancreatic peptide hormones (Falck et al., 1983). CYP2J2, the lone human CYP2J subfamily member, plays an active role in the metabolism of endogenous fatty acids (Wu et al., 1996). Previously cloned and characterized mouse CYP2J isoforms (CYP2J5, CYP2J6, and CYP2J9) have been shown to actively metabolize AA and LA (Ma et al., 1999, 2002; Qu et al., 2001). Herein, we report the cloning, enzymatic characterization, and tissue distribution of four new members of the mouse CYP2J subfamily, CYP2J8, CYP2J11, CYP2J12, and CYP2J13. In addition, we report that *Cyp2j7*, which had previously

been thought to be a mouse *Cyp2j* gene (Nelson et al., 2004), is a pseudogene.

The mouse *Cyp2j* genes have been mapped to chromosome 4 and are located in a 0.62 Mb region, in close proximity to the mouse *Cyp4a* gene cluster (Nelson et al., 1996; Ma et al., 1998). Members of the CYP4A subfamily have been shown to be active in the metabolism of AA to 20-HETE, and may be involved in regulation of renal vascular tone and ion transport (Capdevila, 2007; Fidelis et al., 2010). This is similar to the human *CYP2J2* gene, which is in close proximity to the *CYP4A* gene cluster on chromosome 1 (Nelson et al., 1996; Ma et al., 1998). Mouse CYP2J subfamily members are highly homologous at the amino acid level, with 62–84% identity. Individual mouse CYP2J isoforms likely arose by gene duplication events (Nelson et al., 1996). Alignment of the mouse CYP2J isoforms reveals that the sequence is most divergent within the six putative substrate recognition sites. These alterations are likely responsible for the varied substrate specificity and catalytic properties of the mouse CYP2J proteins.

Each of the new CYP2Js is active in the metabolism of AA, with CYP2J8 having the highest rate; however, the rates are lower than the AA metabolism rate of human CYP2J2. CYP2J11, CYP2J12, and CYP2J13 showed a 4- to 5-fold preference for metabolism of AA to 14,15-EET compared with 11,12-, 8,9-, or 5,6-EETs. This preference for 14,15-EET production is similar to human CYP2J2 and the previously reported mouse CYP2J enzymes that also show a preference for 14,15-EET production. In contrast, many CYP2C epoxigenases show a preference for 11,12-EET production (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004). CYP2J8 showed nearly equal preference for both 11,12-EET and 14,15-EET.

All of the new mouse CYP2J enzymes are also active in the metabolism of LA. The distribution of LA epoxides and alcohols among the new CYP2Js differs, with both CYP2J8 and CYP2J12 having epoxides as the principle products while CYP2J11 and CYP2J13 have similar amounts of epoxide and hydroxyl products. Notably, CYP2J9 has a strong preference for LA hydroxylation, which has not been reported previously. The regioisomeric distribution of EpOME products from LA also varies between the CYP2J enzymes. It is interesting to note that although the CYP2J8 and CYP2J11 isoforms have the highest amino acid identity among the mouse CYP2Js (Table 4), they show different metabolic rates and preferences for LA metabolites and EET regioisomers. Although the metabolism of LA by the CYP2J subfamily members into biologically active products has been previously reported (Zeldin et al., 1995; Bylund et al., 1998), the function of DiHOMEs (dihydroxyoctadecamonoenoic acids) in the kidney, brain, and heart is less clear. Increased DiHOMEs are associated with reduced recovery from ischemia/reperfusion injury in the heart (Edin et al., 2011). In addition, soluble epoxide hydrolase (EPHX2) inhibitors improve recovery from ischemic injury in the kidney, which is associated with increased EpOME to DiHOME ratios (Lee et al., 2012). These toxic effects of LA metabolites may be mediated through induction mitochondrial dysfunction (Moran et al., 2000). This suggests a possible role for CYP2J-derived LA metabolites in the pathogenesis of ischemia/reperfusion injury in kidney and other tissues.

We also report the presence of CYP2J8 in the brain, similar to the previously reported expression of mouse CYP2J9 in the Purkinje cells (Qu et al., 2001). Immunohistochemistry with the α -CYP2J8pep1 revealed abundant staining of the neuropil throughout the brain. The neuropil in the brain consists mainly of unmyelinated axons, dendrites, and glial cell processes. Because EETs are potent vasodilators and have been shown to be neuroprotective (Zhang et al., 2008), neural CYP2J8 expression may be involved in

Fig. 5. Tissue distribution of the new CYP2J transcripts by RT-PCR. Total RNA extracted from multiple mouse tissues was analyzed by a one-step RT-PCR with sequence-specific primer pairs (Table 3). All tissues, unless specified or female-specific, were from males. Primer specificity was determined using the mouse CYP2J cDNAs as templates. A mouse β -actin primer pair was used as a control for RNA quantity. The results are representative of three independent experiments.

Fig. 6. Specificity and tissue distribution of the new CYP2Js by immunoblotting. (A) CYP2J recombinant proteins isolated from *Sf21* insect cells infected with baculovirus stocks (0.5 pmol of P450/lane) were immunoblotted with α -CYP2J8pep1, α -CYP2J11pep1, α -CYP2J12pep1, or α -CYP2J13pep1. (B) Total tissue lysates (50 μ g protein/lane) were prepared from various mouse tissues and immunoblotted with α -CYP2J8pep1. Microsomal fractions (40 μ g protein/lane) prepared from various mouse tissues were immunoblotted with α -CYP2J11pep1. The results are representative of three independent experiments.

neuroprotective mechanisms. CYP2J12 transcripts have also been identified in the brain.

Regulation of kidney P450s is not fully understood, but some data suggest that renal EETs may play a pivotal role in the pathogenesis of hypertension in both humans and rodents. Renal P450 epoxygenases

are under regulatory control by dietary salt intake, and their inhibition leads to salt-dependent hypertension (Holla et al., 1999; Oyekan et al., 1999). In women with pregnancy-induced hypertension, the urinary level of excreted epoxygenase metabolites is increased (Catella et al., 1990). There is also evidence that the CYP2J subfamily is involved in

Fig. 7. Immunohistochemical staining of CYP2J8 protein in the brain and CYP2J11 protein in the heart and kidney. Sections of the brain were immunostained with (A) α -CYP2J8pep1 or (B) normal rabbit serum as described in *Materials and Methods*. Diffuse staining for CYP2J8 was present in the neuropil of the gray matter of the brain with no staining of the white matter. Sections of (C and D) heart and (E and F) kidney were immunostained with (C and E) α -CYP2J11pep1 or (D and F) normal rabbit serum. Staining for CYP2J11 protein was present in cardiomyocytes and renal proximal tubules.

the development of hypertension. Renal CYP2J expression has been localized to sites in the nephron where EETs regulate the actions of hormones that affect blood pressure (Ma et al., 1999). CYP2J2, the single human ortholog of the mouse CYP2J subfamily, is abundantly expressed in the kidney (Wu et al., 1996). Mouse CYP2J5 is abundant in the convoluted and straight portions of the proximal tubules in the kidney and is active in the metabolism of AA to EETs (Ma et al., 1999). CYP2J5 expression appears to be beneficial, as CYP2J5-deficient mice develop spontaneous hypertension (Athirakul et al., 2008). CYP2J11, like CYP2J5, is expressed in the kidney, is localized to the renal proximal convoluted tubules, and is active in the metabolism of AA to EETs, primarily 14,15-EET. Renal proximal tubules and collecting ducts have high levels of EETs (Omata et al., 1992). Together, this suggests that CYP2J11 products may also help moderate fluid/electrolyte transport in the kidney. Furthermore, there may be a redundancy in the functional roles of CYP2J5 and CYP2J11 due to overlapping expression and metabolic profiles. CYP2J13 transcripts were also present in the kidney, with expression levels varying between male and female mice.

EETs have significant biologic effects on the cardiovascular system. Cardiac tissue has high levels of EETs, both in humans and rodents (Wu et al., 1996, 1997). Single nucleotide polymorphisms that decrease CYP2J2 expression and EET synthesis are associated with a higher prevalence of coronary heart disease (Spiecker et al., 2004). Cardiomyocyte-specific expression of human CYP2J2 in the mouse results in improved postischemic recovery of contractile function (Seubert et al., 2004). We found CYP2J11 to be present in cardiomyocytes. Like CYP2J2, CYP2J11 metabolizes AA with a preference for 14,15-EET. This suggests that CYP2J11-derived AA metabolites may play a role in cardiac function after ischemia.

In summary, all the mouse *Cyp2j* genes are located in a ~0.62 Mb cluster on chromosome 4. We report that *Cyp2j7* is most likely a pseudogene, as all cloned cDNAs lacked open reading frames. We cloned the cDNAs of the four new CYP2J enzymes (CYP2J8, CYP2J11, CYP2J12, and CYP2J13) and demonstrated that the recombinant proteins are active in the metabolism of both AA and LA, albeit with different catalytic rates and product profiles. In addition, our data show each of the new P450s is expressed in extrahepatic tissues. Although none of the individual mouse *Cyp2j* genes can be conclusively shown to be the homolog of human *CYP2J2*, together the murine CYP2Js show similar tissue expression patterns to CYP2J2. Like human CYP2J2, these four new CYP2J enzymes might be involved in fatty acid metabolism and may influence many cardiovascular and renal processes.

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Authorship Contributions

Participated in research design: Graves, Edin, Zeldin.

Conducted experiments: Graves, Edin, Bradbury, Lih, Masinde, Clayton, Tomer.

Contributed new reagents or analytic tools: Qu.

Performed data analysis: Graves, Edin, Clayton, Morrison, Zeldin.

Wrote or contributed to the writing of the manuscript: Graves, Edin, Gruzdev, Cheng, Zeldin.

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Address correspondence to: Dr. Darryl C. Zeldin, NIH/NIEHS, 111 T.W. Alexander Drive, Building 101, Room A214, Research Triangle Park, NC 27709.
E-mail: zeldin@niehs.nih.gov