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Cloning and Expression of Three Rabbit Kidney cDNAs Encoding Lauric Acid ω-Hydroxylases[†,‡]

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ABSTRACT: cDNAs encoding three cytochrome P-450 enzymes were cloned from a rabbit kidney cDNA library. These three cDNAs exhibit >90% nucleotide sequence identity across the coding region. This degree of sequence identity is also seen with P450IVA4, an enzyme that catalyzes the ω-hydroxylation of prostaglandins and that is elevated during pregnancy and induced by progesterone in rabbit lung. The 3′ untranslated regions of the three cDNAs display very little sequence identity, suggesting that they are the products of distinct genes. The predicted amino acid sequences derived from each cDNA and for P450IVA4 exhibit about 85% identity. Each cDNA was inserted into an expression vector for transient transfection of COS-1 cells. The transfected cells each expressed a protein recognized by antibodies to P450IVA4. Microsomes isolated from the cells transfected with each cDNA efficiently catalyzed the ω-hydroxylation of lauric acid with rates that greatly exceed that catalyzed by microsomes isolated from the host cell line. One of the cDNAs encodes an enzyme that ω-hydroxylates prostaglandin A_1; however, the specific activity was 2 orders of magnitude lower than that for lauric acid. Our results indicate that the substrate selectivity of the kidney P-450s encoded by these cDNAs is distinct from that of the lung P450IVA4 and that multiple enzymes comprise P-450 class IVA in the rabbit.

An unusual aspect of some forms of cytochrome P-450 is their capacity to preferentially oxidize primary rather than secondary or tertiary carbon–hydrogen bonds. This is particularly evident for fatty acid substrates where the hydroxylation of the terminal carbon is referred to as ω-hydroxylation (Kupfer, 1980). The ω-hydroxylated fatty acids are subsequently oxidized further to dicarboxylic acids, which are elevated in man and other species during ketotic states such as starvation or diabetes.

The cytochrome P-450 enzymes that catalyze ω-hydroxylation reactions appear to comprise a distinct family of P-450[1] proteins. A cDNA corresponding to a rat liver lauric acid ω-hydroxylase, P-450 LAω (P450IVA1), has been cloned and characterized (Hardwick et al., 1987; Earnshaw et al., 1988). A comparison of the derived amino acid sequence of the rat P-450 LAω with that of the rabbit lung enzyme (Matsubara et al., 1987), which catalyzes the ω-hydroxylation of prostaglandins E_1, E_2, A_1, A_2, and $F_{2\alpha}$, indicates that they are members of the P450IVA gene family (Nebert et al., 1989). The latter enzyme, termed either P-450 PGω (Williams et al., 1984) or p-2 (Yamamoto et al., 1984), is designated as P450IVA4 in a uniform system of P-450 nomenclature (Nebert et al., 1989).

Rabbit liver and kidney express lauric acid ω-hydroxylases (Lu et al., 1969; Kusunose et al., 1981; Yamamoto et al., 1986; Kusunose et al., 1985), but the kidney enzyme exhibits rela-

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[‡] The nucleic acid sequences in this paper have been submitted to GenBank under Accession Numbers M28655-KDB3.seq, M28656-KDA6.seq, and M28657-KDB18.seq.
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[1] P-450 is used as a generic term for forms of cytochrome P-450. Rat P-450 LAω and rabbit P-450 p-2 or PGω are designated as P450IVA1 and P450IVA4, respectively, in the uniform system of nomenclature (Nebert et al., 1989). This nomenclature is used for other forms of P-450 discussed in the text. Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high-pressure liquid chromatography.

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tively low ω-hydroxylase activity for prostaglandins E_1 and $F_{2\alpha}$, which are substrates of the lung enzyme, P450IVA4 (Powell, 1980). Moreover, preparations of P450IVA4 exhibit negligible lauric acid ω-hydroxylase activity (Williams et al., 1984; Yamamoto et al., 1984). This suggests that class IVA P-450s, distinct from P450IVA4, may be expressed in the rabbit. This prompted us to screen a rabbit kidney cDNA library with a partial cDNA for the rabbit lung P450IVA4 isolated in our laboratory in order to identify additional cDNAs that might encode ω-hydroxylases. Three cDNAs identified in this manner were inserted into expression vectors and then used to transiently transfect COS-1 cells in order to characterize the activity of the P-450 enzyme expressed from each cDNA.

Experimental Procedures

Cloning and Characterization of cDNAs. A rabbit kidney cDNA library in λZAP (Stratagene), kindly provided by Dr. Robert Ryan and Dr. Richard Philpot, NIEHS, Research Triangle, NC, was screened by hybridization with a ^{32}P-labeled probe derived from a cDNA for P450IVA4. The probe was labeled by random primer extension (Pharmacia) with [^{32}P]dCTP (Amersham) to a specific activity of 10^9 cpm/μg. Positive plaques were subjected to several rounds of repeated cloning, and the cDNA was rescued in the plasmid pBS from the cloned phage by use of the helper phage R408 as described by the supplier (Stratagene).

The plasmid-borne cDNAs and deletion clones constructed from them were sequenced directly with Sequenase (U.S. Biochemical) by the dideoxy chain termination procedure using strand-specific primers obtained from Stratagene. Both strands were sequenced. Restriction enzymes were obtained from commercial suppliers and were used according to the recommendations of the vendor.

Transfection of COS-1 Cells. For expression of P-450 enzymes from the cloned cDNA, each insert was excised from the pBS plasmid with restriction enzymes that cut the polylinker of pBS, leaving the insert intact. These fragments were ligated into compatible restriction sites of each of two expression vectors, pSVL (Pharmacia) and pCMVI (Dr. Mark Stinski, University of Iowa). Both vectors harbor the SV40 and pBR322 origins of replication and a gene conferring resistance to ampicillin. The pSVL vector utilizes the SV40 late promoter, a polyadenylation signal, and transcription termination sequences for the expression of the inserted cDNA. The pCMVI vector employs a promoter-enhancer derived from human cytomegalovirus as well as transcription termination and polyadenylation signals derived from the human growth hormone gene. The pCMVI vector was constructed by Dr. David Russell, The University of Texas Southwestern Medical Center (Andersson et al., 1989), using a portion of the human cytomegalovirus provided by Dr. Mark Stinski (University of Iowa).

Each construct was propagated in *Escherichia coli* SCS-1 (Stratagene) and isolated by alkaline lysis and centrifugation in $CsCl_2$ (Garger et al., 1983). COS-1 cells (provided by Dr. Michael Waterman, University of Texas Southwestern Medical Center, Dallas) were transfected with each plasmid, 10 μg/T-75 flask, by the DEAE-dextran procedure (Zuber et al., 1986; Cullen, 1987). Two days following transfection, the cells were assayed for activity by supplementing the medium with radiolabeled substrate, and/or the cells were harvested for the preparation of microsomes by dislodging the cells from the flask with a rubber policeman.

Microsomes were prepared by homogenization of COS-1 cells harvested from 4 to 10 T-75 flasks in 1 mL of 0.25 M sucrose containing 1 mM EDTA with a Potter Elvehjam glass/glass homogenizer. Following centrifugation at 10000*g* for 5 min, the microsomes were pelleted from the supernatant by centrifugation at 150000*g* for 20 min at 4 °C in a Beckman TL100 centrifuge. The pellet was resuspended in a minimal volume of 50 mM potassium phosphate, pH 7.5, containing 0.2 mM EDTA and 20% glycerol.

Protein concentrations were estimated by the BCA procedure (Bio-Rad). Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate employed a discontinuous buffer system (Laemmli, 1970). Immunoblotting procedures were performed essentially as described (Towbin et al., 1979) using sequentially a goat polyclonal antibody to P450IVA4 (Muerhoff et al., 1987), a pig anti-goat IgG antibody, and a final incubation with ^{125}I-labeled protein A (Amersham).

Immunoaffinity Purification of Class IVA Enzymes Using a Monoclonal Antibody to P450IVA4. Balb/c mice were immunized with purified P450IVA4 (Williams et al., 1984) for the preparation of monoclonal antibodies. Procedures for the immunization of the mice and the development of monoclonal antibodies were essentially as outlined in an earlier publication (Reubi et al., 1984). The myeloma SP2/0 was used as the fusion partner, and positive clones were identified by ELISA with the immunization antigen bound in the wells of microtiter plates. The monoclonal antibody, 6C10, used in this study was purified from ascites fluid by chromatography on protein A–Sepharose, and the purified antibody was linked covalently to CNBr-activated Sepharose (Pharmacia). This antibody resin was used to immunopurify proteins for electrophoretic analysis as described (Reubi et al., 1984). The monoclonal antibody did not exhibit cross-reactivity toward the following purified forms of rabbit P-450 as judged by ELISA: IA1, IA2, IIB4, IIC3, IIC5, IIE1, IIIA6, and IVB1.

Enzyme Assays. Microsomal reactions were performed at 37 °C in 0.5-mL mixtures containing 100 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.03 unit of isocitrate dehydrogenase, 30 mM sodium isocitrate, and either 20 μM [1-^{14}C]lauric acid (0.15 μCi) or 10 μM [5,6-^{3}H]-prostaglandin A_1 (0.17 μCi). Reactions were initiated with the addition of NADPH (1 mM). Lauric acid was incubated with 50 μg of microsomal protein, and the reaction was terminated after 60 or 90 min by the addition of 0.1 mL of 1 N HCl. Prostaglandin A_1 was incubated with 150 μg of microsomal protein, and the reaction was terminated after 120 or 180 min by adjusting the pH to 3.5 with ~0.05 mL of 1 N HCl. Each reaction mixture was then extracted twice with 3 mL of ethyl acetate. The ethyl acetate was evaporated under N_2, and the dried samples were stored at −25 °C until analyzed by reverse-phase high-pressure liquid chromatography (HPLC).

For the measurement of product formation from lauric acid by intact cells, the culture media was supplemented with 1 μM [1-^{14}C]lauric acid (1 μCi, Amersham). At time intervals indicated later, 2-mL aliquots were withdrawn for analysis. Aliquots of the culture media were stored frozen at −25 °C and then thawed and titrated to pH ~3.5 with 1 N HCl. Lauric acid and its metabolites were extracted from each aliquot with 6 mL of ethyl acetate. The ethyl acetate was evaporated under N_2, and the dried samples were stored at −25 °C until analysis by reverse-phase HPLC could be performed.

The products of each reaction were analyzed by reverse-phase HPLC with either a Varian 5020 or a Varian 5060 instrument (Varian, Palo Alto, CA). Each was fitted with a Bio-Sil ODS-5S column (150 × 4 mm, Bio-Rad, Richmond, CA) and a guard column (40 × 4.6 mm) connected to a

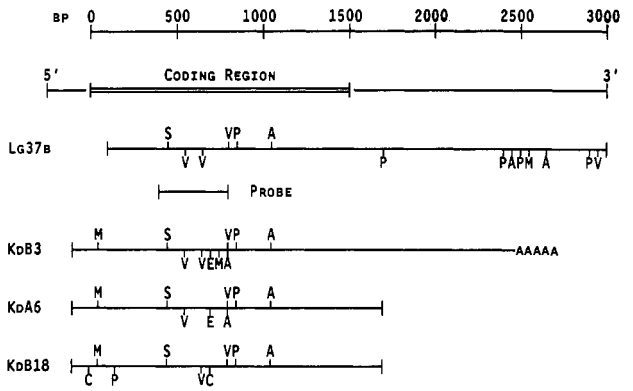

FIGURE 1: Restriction maps. Restriction maps determined for the three kidney cDNAs, KDB3, KDA6, and KDB18, characterized in this study are displayed together with that based on the sequence reported for the lung 37b clone for P450IVA4 (Matsubara et al., 1987). Shared restriction sites are indicated above the map of each cDNA, whereas those that are distinct appear below the map. The segment of the cDNA for P-450 PGω used to identify the kidney clones is also shown. The following symbols denote restriction endonucleases: A, *Ava*I; C, *Sac*I; E, *Eco*RI; M, *Sma*I; P, *Pst*I; V, *Pvu*II.

Radiomatic Flo-One Model HP detector (Radiomatic, Tampa Bay, FL). [^{3}H]Prostaglandin A_1 and its metabolites were eluted from the column isocratically with 40% aqueous acetonitrile containing 0.2% acetic acid and 0.2% benzene (Okita et al., 1987). Elution times for 20-hydroxyprostaglandin A_1 and prostaglandin A_1 were 4.1 and 16.9 min, respectively. [^{14}C]Lauric acid and its hydroxylated products were eluted with a step gradient. Isocratic elution with 62% aqueous methanol containing 0.2% acetic acid was applied for 17 min followed by 100% methanol as described (Okita & Chance, 1984). Elution time for 11-hydroxylauric acid, 12-hydroxylauric acid, and lauric acid are given in the figure legends for the specific HPLC system employed in each assay.

RESULTS

Identification of cDNA Clones That Hybridize with a Partial cDNA for P450IVA4. A rabbit kidney cDNA library constructed in λZAP was screened with a probe derived from a cDNA for P450IVA4 isolated in our laboratory (unpublished). The probe used corresponded to nucleotides 364–764 of the cDNA designated 37b, for P450IVA4 reported by Matsubara et al. (1987). A total of 15 phage that hybridized to the probe were plaque purified. Restriction mapping revealed that three distinct classes of cDNAs were present. Examples of each, KDB3, KDA6 and KDB18, Figure 1, that appeared to span the coding region as judged by comparison to the cDNA for P450IVA4 were selected for sequence determination.

The nucleotide sequence of the longest clone, KDB3, is displayed in Figure 2. The other two cDNAs, KDA6 and KDB18, are closely related to KDB3, and only those nucleotides differing from KDB3 are shown. A long open-reading frame terminates for each of the cDNAs at nucleotide 1534 for KDB3 and KDB18 and at 1531 for KDA6, which has one less codon than the other two cDNAs. The cDNAs exhibit >90% sequence identity over the coding region with each other and with the lung cDNA 37b (Matsubara et al., 1987), which corresponds to P450IVA4. The sequences of the three kidney clones diverge in the 3′ untranslated region. Neither a consensus polyadenylation signal sequence nor a poly(A) sequence is evident for KDA6 and KDB18. Thus, the two cDNAs are probably truncated at the 3′ end. The two cDNAs are also shorter than KDB3. KDB3 terminates 16 bp beyond a consensus polyadenylation signal sequence with the sequence AA. A longer poly(A) sequence was found in an independent clone that contained a 5′ terminus that corresponds to residue 290 of KDB3.

The lung 37b cDNA for P450IVA4 is reported (Matsubara et al., 1987) to hybridize to a single band of mRNA in kidney, lung, and several other tissues. On the basis of the sequence similarity reported here, it is likely that this "single" band includes mRNAs corresponding to KDB3, KDB18, and KDA6. Although the lung 37b clone for P450IVA4 (Matsubara et al., 1987) exhibits a 3′ untranslated sequence that is considerably longer than that of KDB3, 1459 versus 811 bp, the lung 37b clone did not contain a poly(A) sequence or polyadenylation signal site. Lung and liver cDNAs for P450IVA4 isolated in our laboratory differ from 37b in that they diverge from the latter sequence at nucleotide 2313 of 37b and terminate 273 residues later with a poly(A) sequence and a polyadenylation signal sequence. This was observed for three independent lung cDNA clones and one liver cDNA. Thus, the 3′ untranslated region of mRNAs corresponding to P450IVA4 are likely to be only 300 nucleotides longer than that corresponding to KDB3, and mRNAs coding for both might contribute to the reported hybridization (Matsubara et al., 1987).

All three kidney cDNAs exhibit long open-reading frames that correspond closely to that of P450IVA4. The predicted amino acid sequences of the three rabbit kidney cDNAs and that of P450IVA4 are displayed in Figure 3. The N-terminal sequence reported for purified preparations of the P-450 p-2 protein (Matsubara et al., 1987) is identical with that predicted for KDB3 and begins with the fifth amino acid, Ala. The predicted N-terminal sequences of KDA6 and KDB18 are similar, differing at only 2 and 4 of the 25 N-terminal amino acid residues reported for the P-450 p-2 protein. The complete amino acid sequences predicted from the cDNA are closely related, >85% identity, indicating that, like P450IVA4, KDB3, KDA6, and KDB18 are members of the same class. As such, the three proteins are designated as P450IVA5, P450IVA6, and P450IVA7 in a recent update of a systematic nomenclature for cytochrome P-450 enzymes (Nebert et al., 1989). The degree of divergence among the four rabbit class IVA enzymes is greatest at segments 65–120 and 170–240 of the amino acid sequences. These regions are often divergent for other classes of closely related P-450s. In contrast, a long segment of almost complete identity is evident from amino acid 244 to amino acid 388.

Expression of the P-450 Enzymes from Their Corresponding cDNAs. In order to ascertain whether the cDNAs identified in this study encode ω-hydroxylases, each cDNA was inserted into the expression vectors pSVL and pCMVI for transient transfection of COS-1 cells. Microsomes, isolated from COS-1 cells transfected with each of the cDNAs in either the pSVL or the pCMVI expression vector, express proteins that are recognized by a polyclonal antibody to P-450 PGω (Muerhoff et al., 1987). No immunoreactive proteins are detected with this antibody in microsomes from mock-transfected cells, as is shown in Figure 4. The electrophoretic mobility of these microsomal proteins is essentially the same as that of a rabbit kidney protein that was immunoaffinity purified with a monoclonal antibody to P-450 PGω, Figure 4. On the basis of comparisons of the intensity of the protein band detected by immunoblotting for COS cells transfected with these constructs and purified P450IVA4, the microsomal concentration of each protein is estimated at roughly 50 pmol/mg of microsomal protein for the pCMVI constructs, which gave roughly 2-fold higher levels of expression than the

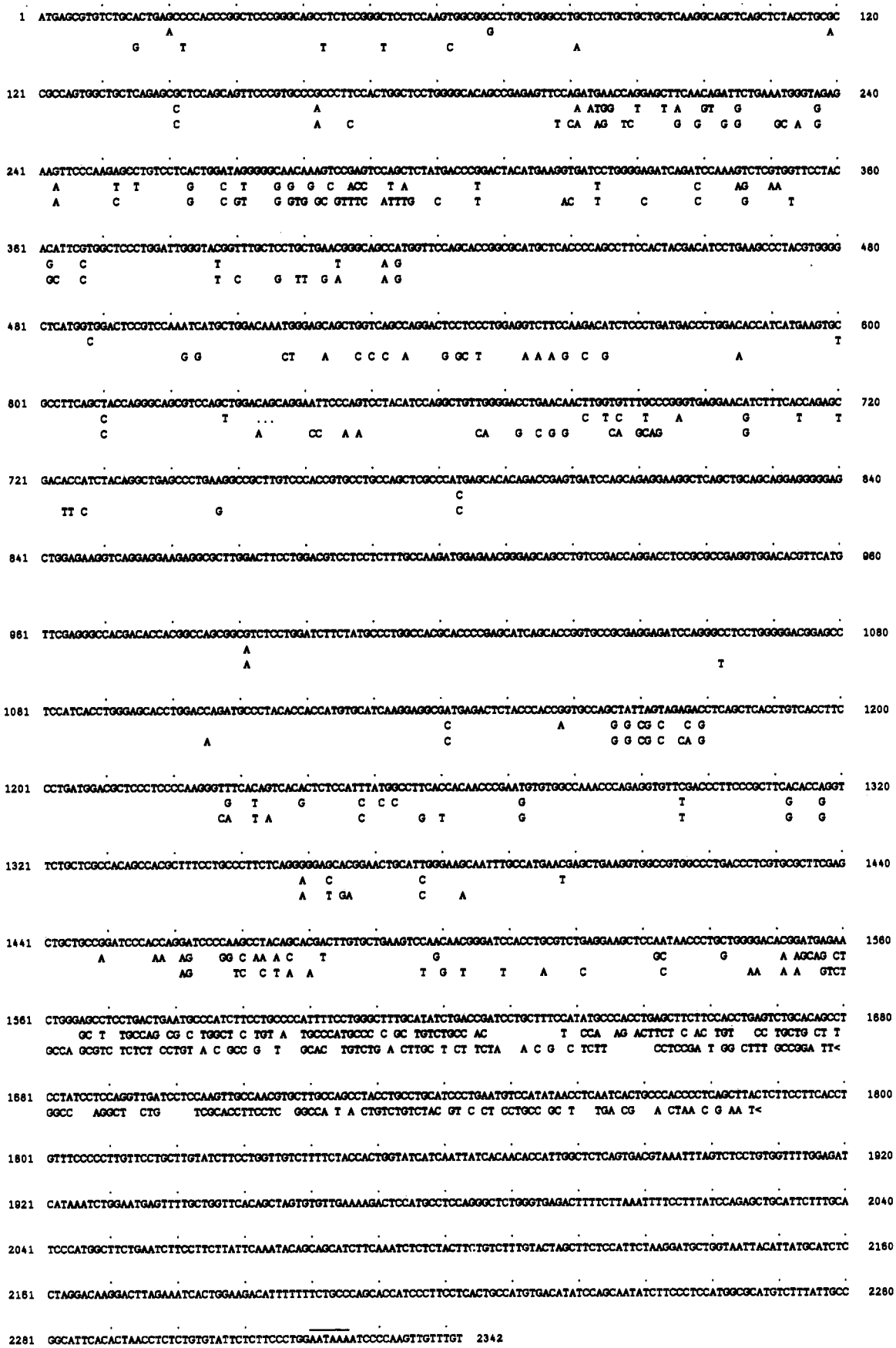

FIGURE 2: Nucleotide sequences. Nucleotide sequence determined for the cDNA KDB3 is shown beginning at the putative initiation codon. A consensus polyadenylation signal sequence is overlined. Differences in the nucleotide sequences of KDA6 and KDB18 are shown below that of KDB3. Their 3′ terminii are indicated by (<). A long open-reading frame terminates for each of the cDNAs at nucleotide 1534, corresponding to codon 511 of KDB3.

```
                10                  30                  50
                 .         .         .         .         .         .
KdB3    MSVSALSPTRLPGSLSGLLQVAALLGLLLLLLKAAQLYLRRQWLLRALQQFPCPPFHWLL
KdA6          N               G                H
KdB18          S      F  F  A                                  S
Lg37b                      >                   H

                70                  90                 110
                 .         .         .         .         .         .
KdB3    GHSREFQMNQELQQILKWVEKFPRACPHWIGGNKVRVQLYDPDYMKVILGRSDPKSRGSY
KdA6           NGH   VM        S   R LW SRAHLLI                AQ
KdB18         PIDS    V  R     S   R LW SELFLIC       T        A V
Lg37b          ND   ER Q       G   W LS   A LLV     L          APRN

               130                 150                 170
                 .         .         .         .         .         .
KdB3    TFVAPWIGYGLLLLNGQPWFQHRRMLTPAFHYDILKPYVGLMVDSVQIMLDKWEQLVSQD
KdA6    R L              T                        A
KdB18   S L           E  T                             V    L K ARK
Lg37b   KLMT          D  T                                 R    I

               190                 210                 230
                 .         .         .         .         .         .
KdB3    SSLEVFQDISLMTLDTIMKCAFSYQGSVQLDSRNSQSYIQAVGDLNNLVFARVRNIFHQS
KdA6                           H       .                F S    V
KdB18   AP  IYEHV     E        H      E  T K      RE SD ALQ    V
Lg37b       I  HV                      .   H     IN       Y A  V

               250                 270                 290
                 .         .         .         .         .         .
KdB3    DTIYRLSPEGRLSHRACQLAHEHTDRVIQQRKAQLQQEGELEKVRRKRRLDFLDVLLFAK
KdA6
KdB18    FL
Lg37b    FL         F

               310                 330                 350
                 .         .         .         .         .         .
KdB3    MENGSSLSDQDLRAEVDTFMFEGHDTTASGVSWIFYALATHPEHQHRCREEIQGLLGDGA
KdA6                                  I
KdB18                                 I
Lg37b

               370                 390                 410
                 .         .         .         .         .         .
KdB3    SITWEHLDQMPYTTMCIKEAMRLYPPVPAISRDLSSPVTFPDGRSLPKGFTVTLSIYGLH
KdA6                        L       GVG Q                VI      A
KdB18           K           L       GVGSK                III
Lg37b                       L       SVT Q  K             VILF

               430                 450                 470
                 .         .         .         .         .         .
KdB3    HNPNVWPNPEVFDPSRFTPGSARHSHAFLPFSGGARNCIGKQFAMNELKVAVALTLVRFE
KdA6       K             A                P
KdB18      K             A                S
Lg37b   Y  K  Q       F  A D  Y                      R

               490                 510
                 .         .         .
KdB3    LLPDPTRIPKPTARLVLKSNNGIHLRLRKLQ
KdA6         K V DQKP      S          R
KdB18          V I IT      K          H
Lg37b            I I  V    K          H
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FIGURE 3: Amino acid sequences. Predicted amino acid sequences corresponding to the long open-reading frame of each of the cDNAs noted in Figure 2 are displayed. The predicted sequence of KdB3 is shown in its entirety, whereas only differences are shown where they occur for KdA6 and KdB18 and for the sequence of P450 PGω reported by Matsubara et al. (1987). The start of the sequence derived from lung clone 37b is indicated by (>).

Table I: Rates of Hydroxylation of Prostaglandin A_1 and Lauric Acid by Microsomes from COS-1 Cells

	pmol min^{-1} mg^{-1}		
transfection	20-OH-PGA_1[a]	12-OH-laurate	11-OH-laurate
mock	*b*	*b*	*b*
KdA6	*b*	175 ± 21	*b*
KdB3	*b*	200 ± 14	4.7 ± 2.8
KdB18	1.5 ± 0.7	320 ± 0	17 ± 14
DEHP treated[c] rat liver	ND[d]	4860	520

[a] Incubations were performed with 10 μM [^{3}H]prostaglandin A_1 (PGA_1) and 150 μg of microsomal protein for 120 or 180 min or with 20 μM [^{14}C]laurate and 50 μg of microsomal protein for 60 or 90 min. The mean of two values is shown. [b] Rate was below the limit of detection, which for PGA_1 was 0.4 pmol min^{-1} mg^{-1} and for laurate was 4 pmol min^{-1} mg^{-1}. [c] Liver microsomes were isolated from a rat treated with bis(2-ethylhexyl) phthalate to induce lauric acid ω-hydroxylation as described (Okita & Chance, 1984). [d] Not determined.

corresponding pSVL constructs for each insert DNA.

The catalytic activity of microsomes isolated from COS-1 cells transfected with plasmids bearing each cDNA was examined with [^{14}C]lauric acid and [^{3}H]prostaglandin A_1 as substrates. The products of each incubation were analyzed by reverse-phase chromatography. As is shown for KdB18 harbored in pCMVI in Figure 5, a single, major metabolite corresponding to 12-hydroxylauric acid was observed for microsomes isolated from transfected cells for each of the

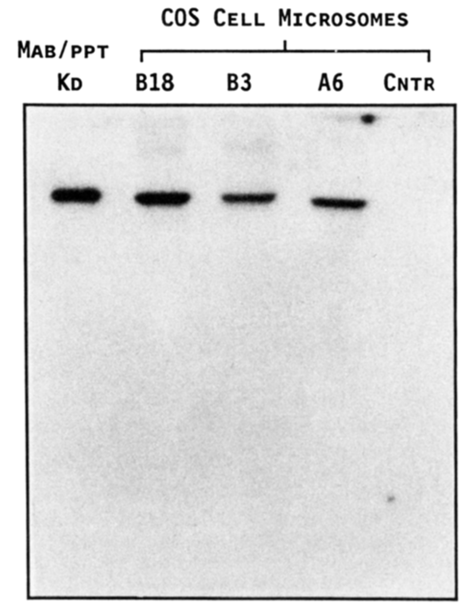

FIGURE 4: Detection by immunoblotting of P-450s related to P-450 PGω in microsomes from COS-1 cells transfected with each cDNA. Microsomes were isolated from COS-1 cells, solubilized with sodium dodecyl sulfate, and electrophoresed in a polyacrylamide gel (10%). Following electrophoretic transfer to nitrocellulose, proteins related to P-450 PGω were detected by sequential reaction with a goat polyclonal antibody to P-450 PGω (Muerhoff et al., 1987), pig anti-goat IgG, and ^{125}I-labeled protein A. The lanes contain microsomal proteins from mock-transfected COS-1 cells, CNTR, or from COS-1 cells transfected with the indicated cDNA inserted into the expression vector pCMVI. For comparison, a protein immunopurified from rabbit kidney with a monoclonal antibody to P450IVA4 was loaded in the adjacent lane, MAB/PPT Kd.

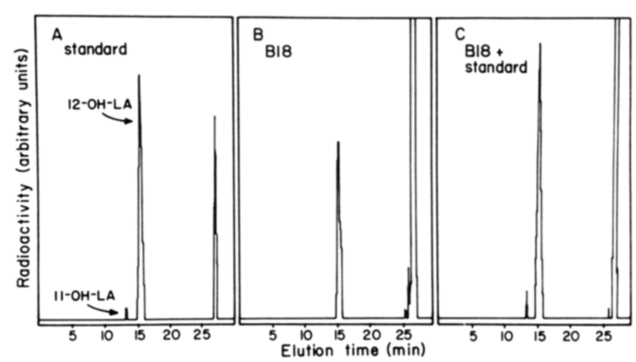

FIGURE 5: Reverse-phase chromatography of lauric acid and its metabolites. 12-Hydroxylauric acid was identified as the major metabolite produced from lauric acid by microsomes prepared from COS-1 cells transiently transfected with pCMVI harboring KdB18. (Panel A) 12-Hydroxy[1-^{14}C]lauric acid (15.6 min, 400 cpm) was prepared by incubation of [1-^{14}C]lauric acid with liver microsomes prepared from rats treated with bis(2-ethylhexyl) phthalate to induce ω-hydroxylase activity (Okita & Chance, 1984). (Panel B) Reaction product (15.6 min, 300 cpm) obtained after incubation of lauric acid with microsomes prepared from COS-1 cells transfected with KdB18 in the pCMVI expression vector. (Panel C) Coelution of the metabolite produced by microsomes from the transfected COS-1 cells (panel B) with the standard (panel A). The samples in panels A and B were mixed such that each would contribute approximately equal amounts of radioactivity in the 15.6-min peak corresponding to 12-hydroxylauric acid, 12-OH-LA (500 cpm, total). Lauric acid eluted at 27.2 min, and 11-hydroxylauric acid, 11-OH-LA, eluted at 13.8 min.

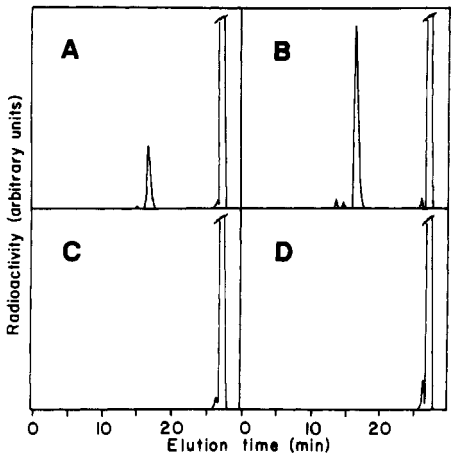

FIGURE 6: Analysis of metabolites of lauric acid appearing in the media of COS-1 cells transfected with cDNA KDA6. Elution profiles of metabolites formed from [1-^{14}C]lauric acid by COS-1 cells transfected with KDA6 in the pSVL vector (panels A and B) or COS-1 cells subjected to the transfection procedure without the inclusion of the plasmid (panels C and D). [1-^{14}C]Lauric acid was added to the medium, 1 μM, and aliquots were removed after 1 h (panels A and C) and 3 h (panels B and D) for analysis. A major metabolite was secreted into the medium by COS-1 cells transfected with KDA6 which was not evident for the mock-transfected cells. This product was identified as 12-hydroxylauric acid by coelution with a standard. The retention times differ from those in Figure 5 due to the use of different instrumentation. They are 15, 17, and 26.4 min for 11-hydroxylauric acid, 12-hydroxylauric acid, and lauric acid, respectively.

cDNAs. As summarized in Table I, the rates for each enzyme are similar and are very high when compared to those of microsomes prepared from untransfected cells, which exhibit negligible capacity to hydroxylate lauric acid. The formation of 11-hydroxylauric acid was very low for all three of the enzymes, indicating that each exhibits a high degree of specificity (>18:1) for ω-hydroxylation versus (ω - 1)-hydroxylation. The capacity of each cDNA to catalyze the ω-hydroxylation of prostaglandin A_1 was also low. Only KDB18 expressed a capacity for ω-hydroxylation of prostaglandin A_1 that was significantly above (4-fold) the limit of detection. This rate was less than 1% of that determined for lauric acid.

The capacity of COS-1 cells transfected with each of the cDNAs to effect the ω-hydroxylation of [^{14}C]lauric acid (1 μM) added to the culture medium was also evident. The appearance of 12-hydroxylauric acid was monitored in the culture medium by reverse-phase HPLC, as is shown for the example in Figure 6. The amount of ω-hydroxylated product secreted in the medium increased with time and accounted for extensive conversion of the substrate to this product. These results indicate that the enzymes encoded by each cDNA can compete effectively for fatty acid substrates with pathways of β-oxidation in the transfected cells. Additional metabolites, more polar than 12-hydroxylauric acid, were detected at longer incubation times, suggesting that further oxidation of the ω-hydroxylated fatty acid to the corresponding aldehyde and carboxylic acid by COS-1 cells transfected with the constructs had occurred.

DISCUSSION

The identification and characterization of the three cDNAs described in this paper clearly indicate that the IVA family of P-450 genes is complex. Each of the kidney cDNAs encodes an efficient lauric acid ω-hydroxylase. Despite the close structural similarity to P450IVA4, however, none of the three cDNAs encodes enzymes that efficiently catalyze the ω-hydroxylation of prostaglandin A_1, and preparations of P-450 PGω do not catalyze the ω-hydroxylation of lauric acid (Williams et al., 1984). Of the three, only KDB18 encodes an enzyme exhibiting detectable activity with prostaglandin A_1, and the turnover number appears to be much lower than that reported for purified preparations of P-450 PGω (Williams et al., 1984) or P-450 p-2 (Yamamoto et al., 1984). Additional differences in the catalytic activities of the enzymes encoded by the three kidney cDNAs are likely to become apparent when a wider range of substrates is examined, and it is interesting to note that the extent of the amino acid sequence divergence among the three kidney cDNAs is as great as their differences with P450IVA4. Rabbit kidney microsomes are reported to catalyze the ω-hydroxylation of arachidonic acid (Oliw et al., 1981; Schwartzman et al., 1986), and future experiments will address whether one or more of the enzymes described here catalyzes this reaction.

Rabbit kidney microsomes also contain a P-450 that catalyzes the ω-hydroxylation of prostaglandin A_1 when reconstituted with NADPH–cytochrome P-450 reductase (Kusunose et al., 1984). The rate of prostaglandin A_1 hydroxylation catalyzed by KDB18 appears to be too low to account for the activity of the kidney enzyme. It is also unlikely that the P-450 catalyzing prostaglandin A_1 ω-hydroxylase activity in kidney is P450IVA4 because the latter also catalyzes the ω-hydroxylation of prostaglandin E_1 and prostaglandin $F_{2\alpha}$, and these activities are not expressed in the rabbit kidney (Powell, 1980). Thus, it is likely that additional members of the IVA family of P-450 genes in rabbits will be identified. This family appears to encode a variety of enzymes with closely related amino acid sequences that exhibit differences in substrate preference while maintaining a high degree of discrimination for the hydroxylation of the terminal hydrogen–carbon bond of primary alkyl groups. The existence of these kidney ω-hydroxylase clones will make it possible to probe the structure–function relationships among these closely related gene products. Hopefully, this will lead to new insights into the basic requirements for catalysis of ω-hydroxylation reactions by these enzymes.

Registry No. DNA (rabbit clone KDB3 cytochrome P 450IVAS messenger RNA complementary), 124316-15-0; DNA (rabbit clone KDA6 cytochrome P 450IVA6 messenger RNA complementary), 124316-14-9; DNA (rabbit clone KDB18 cytochrome P 450IVA7 messenger RNA complementary), 124316-16-1; cytochrome P 450IVASa (rabbit clone KDB3 protein moiety reduced), 124316-18-3; cytochrome P 450IVA6 (rabbit clone KDA6 protein moiety reduced), 124316-19-4; cytochrome P 450IVA7 (rabbit clone KDB18 protein moiety reduced), 124316-20-7; cytochrome P450, 9035-51-2; lauric acid ω-hydroxylase, 78783-57-0; prostaglandin A_1 ω-hydroxylase, 74191-27-8.

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Characterization of Phospholipid Transfer between Mixed Phospholipid–Bile Salt Micelles†

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ABSTRACT: Concentration-dependent self-quenching of the fluorescent phospholipid *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) was used to measure the rate of *N*-NBD-PE transfer between phosphatidylcholine–bile salt mixed micelles. In a previous study using the same technique, the rate of *N*-NBD-PE transfer between phosphatidylcholine–taurocholate mixed micelles was found to be several orders of magnitude faster than its transfer between phosphatidylcholine vesicles as a result of an increased rate of transfer through the water at low micelle concentrations and an increased rate of transfer during transient micelle collisions at higher micelle concentrations [Nichols, J. W. (1988) *Biochemistry 27*, 3925–3931]. In this study we have determined the influence of bile salt structure, incorporation of cholesterol, and temperature on the rate and mechanism of phospholipid transfer between mixed micelles. We found that both transfer pathways were a common property of mixed micelles prepared from a series of different bile salts and that the rates of transfer by both pathways increased as a function of the degree of bile salt hydrophobicity. Cholesterol incorporation into phosphatidylcholine–taurocholate mixed micelles displaced taurocholate from the micelles and resulted in an increased rate of transfer through the water and a decreased rate of transfer during micelle collisions. The temperature dependence of the transfer rates was used to calculate the activation free energy, enthalpy, and entropy for both mechanisms. The activation enthalpy was the major barrier to transfer by both mechanisms. However, the observed increase in the rate of phospholipid transfer through the water between mixed micelles relative to vesicles, and the increased rate of collision-dependent transfer between mixed micelles prepared with the more hydrophobic bile salts, are both primarily the result of increased activation entropy.

Fluorescent-labeled NBD[1] phospholipids have recently been shown to transfer rapidly between phospholipid–taurocholate mixed micelles when compared to their transfer between vesicles (Nichols, 1988). This increased rate of transfer was shown to result from an increase in the rate of phospholipid dissociation into the water phase (aqueous diffusion mechanism), which is the rate-limiting step at low concentrations of micelles, and to result predominantly from collision-dependent transfer at higher concentrations of micelles. These conclusions were based on a kinetic analysis of NBD-labeled phospholipid transfer between mixed phospholipid–taurocholate micelles in which the rate of transfer was determined from the loss of self-quenching of the fluorescent phospholipid

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[1] Abbreviations: NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; *N*-NBD-PE, *N*-(NBD)diacylphosphatidylethanolamine; *N*-NBD-DLPE, *N*-(NBD)-dilaurylphosphatidylethanolamine; *N*-NBD-DMPE, *N*-(NBD)dimyristoylphosphatidylethanolamine; *N*-NBD-DPPE, *N*-(NBD)dipalmitoylphosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; imc, intermicellar concentration; cmc, critical micelle concentration; HBS, HEPES-buffered saline, 0.9% NaCl in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4.

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