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Cloning and Expression of a Pig Liver Taurochenodeoxycholic Acid 6α-Hydroxylase (CYP4A21)

A NOVEL MEMBER OF THE CYP4A SUBFAMILY*

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A cytochrome P450 expressed in pig liver was cloned by polymerase chain reaction using oligonucleotide primers based on amino acid sequences of the purified taurochenodeoxycholic acid 6α-hydroxylase. This enzyme catalyzes a 6α-hydroxylation of chenodeoxycholic acid, and the product hyocholic acid is considered to be a primary bile acid specific for the pig. The cDNA encodes a protein of 504 amino acids. The primary structure of the porcine taurochenodeoxycholic acid 6α-hydroxylase, designated CYP4A21, shows about 75% identity with known members of the CYP4A subfamily in rabbit and man. Transfection of the cDNA for CYP4A21 into COS cells resulted in the synthesis of an enzyme that was recognized by antibodies raised against the purified pig liver enzyme and catalyzed 6α-hydroxylation of taurochenodeoxycholic acid. The hitherto known CYP4A enzymes catalyze hydroxylation of fatty acids and prostaglandins and have frequently been referred to as fatty acid hydroxylases. A change in substrate specificity from fatty acids or prostaglandins to a steroid nucleus among CYP4A enzymes is notable. The results of mutagenesis experiments indicate that three amino acid substitutions in a region around position 315 which is highly conserved in all previously known CYP4A and CYP4B enzymes could be involved in the altered catalytic activity of CYP4A21.

The formation of bile acids from cholesterol in the liver involves a series of cytochrome P450-dependent hydroxylations of the steroid nucleus as well as the side chain. The two primary bile acids formed in most mammals are 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid)[1] and 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid). The domestic pig, however, is virtually unable to synthesize cholic acid. Instead, another trihydroxylated bile acid, 3α,6α,7α-trihydroxy-5β-cholanoic acid (hyocholic acid), is formed in amounts equal to that of cholic acid in other mammals. Formation of hyocholic acid is accomplished by a 6α-hydroxylation of chenodeoxycholic acid or its conjugates. Hyocholic acid is considered to be a species-specific primary bile acid in the pig. It has been speculated that the ability to synthesize hyocholic acid in pigs evolved from a need of trihydroxylated bile acids caused by changes in dietary circumstances. 6α-Hydroxylation of chenodeoxycholic acid seems to have been biochemically preferred during evolution compared with the introduction of cholic acid biosynthesis (1). In man, small amounts of hyocholic acid have been found in plasma and urine especially from patients with cholestatic liver disease (2) and also in fetal blood sample (3), indicating a different role for this bile acid in man.

In previous reports from this laboratory, the purification and characterization of the taurochenodeoxycholic acid 6α-hydroxylase from pig liver have been described (4, 5). The enzyme was shown to be a microsomal cytochrome P450 heme protein. The N-terminal amino acid sequence and sequences of peptides obtained after partial tryptic digestion suggested that this enzyme belong to the cytochrome P450 4A (CYP4A) subfamily.

In the present study, we report on the cDNA cloning and expression of a taurochenodeoxycholic acid 6α-hydroxylase from pig liver. The results confirm and extend the previous observation that this enzyme is a, so far uncharacterized, member of the CYP4A subfamily. The novel CYP4A enzyme was designated CYP4A21.[2]

EXPERIMENTAL PROCEDURES

Materials—Livers and other tissues from castrated, otherwise untreated, male pigs were obtained from the local slaughterhouse. RNeasy Midi kits, QIAprep Spin Miniprep kit, and Plasmid Maxi kit were from Qiagen. Restriction enzymes, SureClone Ligation kit, and ECL Western blotting analysis system were from Amersham Pharmacia Biotech. pBluescript II KS was purchased from Stratagene. Oligonucleotides were obtained from Life Technologies and Interactiva. *Taq* polymerase and oligo(dNTP) mix were from PE Applied Biosystems and Advanced Biotechnologies. The reverse transcription system was from Promega, and the 1.5-kb DNA ladder and 5′-rapid amplification of cDNA ends (RACE) system were from Life Technologies, Inc. T4 DNA ligase and buffer were from New England Biolabs. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies were purchased from Bio-Rad. The megaprime DNA labeling system, CYP IVA ECL Western blotting kit, and [1-^{14}C]lauric acid (53 Ci/mol) were from Amersham Pharmacia Biotech. Tauro[24-^{14}C]chenodeoxycholic acid (0.8 Ci/mol) was synthesized according to Norman (6). All chemicals used were of first grade quality.

Cloning of cDNA from Pig Liver—Total RNA isolated from pig liver using RNeasy Midi kits was reversed transcribed, and first strand cDNA was used directly for PCR (RT-PCR). Oligonucleotide primers for the initial PCR were designed from amino acid sequences obtained from

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ278474 SSC278474.

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[1] The abbreviations and trivial names used are: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; CYP, cytochrome P450; kb, kilobase pair(s); RACE, rapid amplification of cDNA ends; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); GSP, gene-specific primer; UTR, untranslated region.

[2] The sequence reported for the porcine taurochenodeoxycholic acid 6α-hydroxylase was designated CYP4A21 by the P450 nomenclature committee.

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the purified taurochenodeoxycholic acid 6α-hydroxylase (5). The forward primer was N2 (5′-CGTCCCGGCCCTGGCCAGCGT-3′), and the reverse primer was R-FEL (5′-TGGGATCTGGCGCCAGCTCGAA-3′). The PCR was performed in presence of 2.5 mM Mg^{2+} and 10% dimethyl sulfoxide. The cycles were as follows: 94 °C for 2 min, 55 °C for 1 min, 72 °C for 2 min for 30 cycles, and 72 °C for 10 min. The 1,430-bp PCR product was gel purified and cloned into pUC18 using a SureClone ligation kit and subsequently transformed in *Escherichia coli* DH5α cells. Plasmid DNA was purified using DNA minipreparation columns (Qiagen) according to the manufacturer's instruction. A plasmid containing the insert was sequenced using Dye-labeled Terminator for the sequence reaction and a ABI377 DNA sequencer for analysis. The full-length nucleotide sequence was obtained by 5′- and 3′-RACE using a 5′-RACE system according to the manufacturer's instructions and a 3′-RACE procedure according to Frohman (7). Gene-specific primers (5′-GSP1 and 5′-GSP2) used for the 5′-RACE were 5′-GSP1 (5′-TAGATGATGTCGTTCTCGAGAA-3′) and 5′-GSP2 (5′-CATGCGCCGGTGCTGGAACCA-3′). For the 3′-RACE, two gene-specific primers (3′-GSP1 and 3′-GSP2) and a $(T)_{12}$ primer were used. The sequences of the gene-specific 3′-primers were: 3′-GSP1, 5′-TTCTCGAGAACGACATCATCTA-3′, and 3′-GSP2, 5′-TCTGCTCGCCACAGCCATGCTTT-3′. The PCR products of the 5′-RACE and 3′-RACE, respectively, were gel purified and cloned into pUC18 and subsequently sequenced according to the procedure above. The nucleotide sequence of the cDNA was determined by alignment of overlapping sequences of the 1,430-bp PCR product and the sequences of the 5′-RACE and 3′-RACE.

Expression of cDNA in COS-1 Cells—A cDNA sequence for cloning into the expression vector pSVL (Amersham Pharmacia Biotech) was obtained by RT-PCR of total RNA from pig liver using two primers based on sequences in the 5′- and 3′-end, respectively, of the deduced coding region. The forward primer (Xho-N, 5′-CTCTCGAGCGTGCACCATGGGGGTCCC-3′) contained a restriction site for cloning (*Xho*I) and a conserved eukaryotic initiation site sequence as described by Kozak (8). The reverse primer contained the *Xba*I restriction site for cloning and the most 3′-nucleotides of the deduced coding sequence (Xba-C, 5′-TTTCTAGAATGGTGTCTTCCTGAGGTTCAG-3′). The PCR cycles were according to the procedure above. The PCR product was gel purified and cloned into a pSVL vector. The cloned pSVL-CYP4A21 was amplified and purified using plasmid Maxi columns (Qiagen), sequenced, and subsequently transfected into COS-1 cells by the DEAE-dextran method as described by Esser *et al.* (9) or by electroporation (settings 0.4 kV, 100 microfarads). Transfected cells were grown for 48 h at 37 °C, 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), glutamine, and antibiotics, then washed and harvested. Microsomes from COS cells were isolated by differential centrifugation according to Clark and Waterman (10), resuspended, and homogenized in 100 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA. Protein concentration was determined using BCA reagent (Pierce).

Activity Assay—^{14}C-Labeled taurochenodeoxycholic acid (25, 145, and 200 μM) was incubated with freshly prepared microsomes from transfected COS cells. In addition to microsomes and substrate, the incubation mixture contained 1 unit of NADPH-cytochrome P450 reductase from pig liver microsomes (11), 5 μM dithiothreitol, and 2 μM NADPH in a total volume of 1 ml of 100 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA. Incubations were performed at 37 °C for 20 or 30 min and terminated with 5 ml of ethanol. The incubation mixtures were prepared for thin layer chromatography as described previously (5). The chromatoplates were analyzed by radioactivity scanning. Two unlabeled authentic 6-hydroxylated chenodeoxycholic acid metabolites, 3α,6α,7α-trihydroxy-5β-cholanoic acid and 3α,6β,7α-trihydroxy-5β-cholanoic acid (Steraloids, Inc.), were used as external standards in thin layer chromatography. Hydroxylase activity toward lauric acid was assayed by incubation of microsomes from transfected COS cells with ^{14}C-labeled lauric acid (100 μM), 1 unit of NADPH-cytochrome P450 reductase, and 2 μM NADPH in a total volume of 0.2 ml of 0.1 M Tris-HCl, pH 7.5. Incubations were performed at 37 °C for 20 min and terminated by the addition of 0.1 ml of 6% acetic acid in ethanol. Samples were centrifuged and the supernatant analyzed by high performance liquid chromatography according to Okita *et al.* (12) using a Radiomatic detector.

As positive controls of the activity assays, freshly prepared pig liver microsomes were used and incubated with respective substrate according to the procedure described above except that NADPH-cytochrome P450 reductase was omitted.

Western Blotting—SDS-PAGE was carried out according to Laemmli (13) with 15% acrylamide and 0.09% bisacrylamide slab gels containing 0.1% SDS. The proteins on the gels were electrophoretically blotted onto nitrocellulose filters. Western blot analysis was performed using polyclonal antibodies raised against the purified 6α-hydroxylase (5) as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibodies (Bio-Rad). The immunoreactive protein bands were visualized with an ECL Western blotting analysis system. The filter was stripped of bound antibodies and immunodetected a second time with antibodies raised against rat CYP4A (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Northern Blot Analysis—Total RNA from pig liver (20 μg) was electrophoresed on 1.2% agarose gel containing formamide as described by Sambrook *et al.* (14) and transferred to Hybond-N membranes (Amersham Pharmacia Biotech). A nucleotide sequence corresponding to the coding sequence of the cloned cDNA was ^{32}P-labeled using a Megaprime labeling kit and used as probe. The membrane was hybridized with the ^{32}P-labeled probe in hybridization buffer containing 50% deionized formamide, 5× SSC, 100 μg/ml denatured single-stranded DNA, 100 μg/ml total RNA, and 1× PE (50 mM Tris-HCL, pH 7.5, 0.1% sodiumpyrophosphate, 1% SDS, 0.2% polyinylpyrolidine, 0.2% Ficoll, 5 mM EDTA, 0.2% bovine serum albumin). Hybridization was performed overnight at 42 °C. The membrane was washed twice in each of the following solutions, 2× SSC containing 0.1% SDS for 10 min at room temperature, 2× SSC containing 0.1% SDS for 30 min at 62 °C, and 0.2× SSC for 10 min at room temperature. The membrane was exposed to Fuji RX film.

RT-PCR for Tissue Distribution Studies—Total RNA was extracted from nine different tissues of pig (heart, muscle, intestine, spleen, thymus, lung, adrenal gland, kidney, and liver) using RNeasy Midi kits or according to Chomczynski and Sacchi (15). The quality of RNA was checked by formamide gel electrophoresis. Total RNA (1 μg) from each tissue was reversed transcribed, and the cDNA obtained was used for PCR. A reverse primer (RNON-CONS, 5′-TGGAGTCGTGACCTGCAGC-3′), which hybridizes to position 995 of CYP4A21 (see Fig. 1) was used together with the forward primer Xho-N described above. PCR was performed in presence of 2.5 mM Mg^{2+} and 10% dimethyl sulfoxide for 30 cycles (94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min) and 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis.

Point Mutations of the CYP4A21 Sequence—The sequence of CYP4A21, from the restriction site *Aat*II at position 951 to position 1578 (Fig. 1), was amplified by PCR using CYP4A21 cDNA as template. A forward primer, 5′-CTTGACGTCCGTGCCGAAGTGGACACGTTCATGTTCGAGGGTCATGACACCACAGCC-3′ containing the restriction site *Aat*II for cloning and nucleotides coding for three point mutations (A314F, A315E, S319T) was used in combination with a reverse primer, 3CB (5′-TTTCTAGAGCTTGTCCTTGTCCCCACA-3′, which hybridizes to a sequence in the 3′-untranslated region (3′-UTR). PCR was performed in presence of 2.5 mM Mg^{2+} and 10% dimethyl sulfoxide for 30 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) and 72 °C for 10 min. The 600-bp PCR product was gel-purified, digested with *Aat*II and *Xba*I, and subcloned into a pBluescript IIKS-ligated CYP4A21 sequence between positions 951 and 1578. The mutated sequence of CYP4A21 thus obtained was digested further with the restriction enzymes *Bsm*BI and *Bsu*36I, and the fragment between nucleotides 588 and 1535 (Fig. 1) was subsequently cloned into the corresponding restriction sites of the expression plasmid pSVL-CYP4A21. The mutated CYP4A21 sequence, cloned into pSVL, was verified by sequencing and used for transfection of COS cells by the DEAE-dextran method (9).

RESULTS

Cloning and Nucleotide Sequencing of the Pig Liver Taurochenodeoxycholic Acid 6α-Hydroxylase (CYP4A21)—Oligonucleotide primers for PCR were designed from peptide sequences of the purified protein (5). A 1,430-bp PCR product was produced with a forward primer based on residues in the N-terminal sequence of the purified protein and a reverse primer based on a tryptic fragment, which in an alignment with rabbit CYP4A7, was the most C-terminal sequence obtained from the protein. The DNA sequence of this PCR product was analyzed. Nucleotides in the 5′- and 3′-regions of the cDNA were obtained by use of 5′- and 3′-RACE procedures. The sequence of the full-length cDNA was finally determined by alignment of overlapping sequences.

The complete nucleotide sequence of the CYP4A21 and the deduced amino acid sequence are shown in Fig. 1. *Underlined* sequences correspond to peptides obtained by tryptic cleavage

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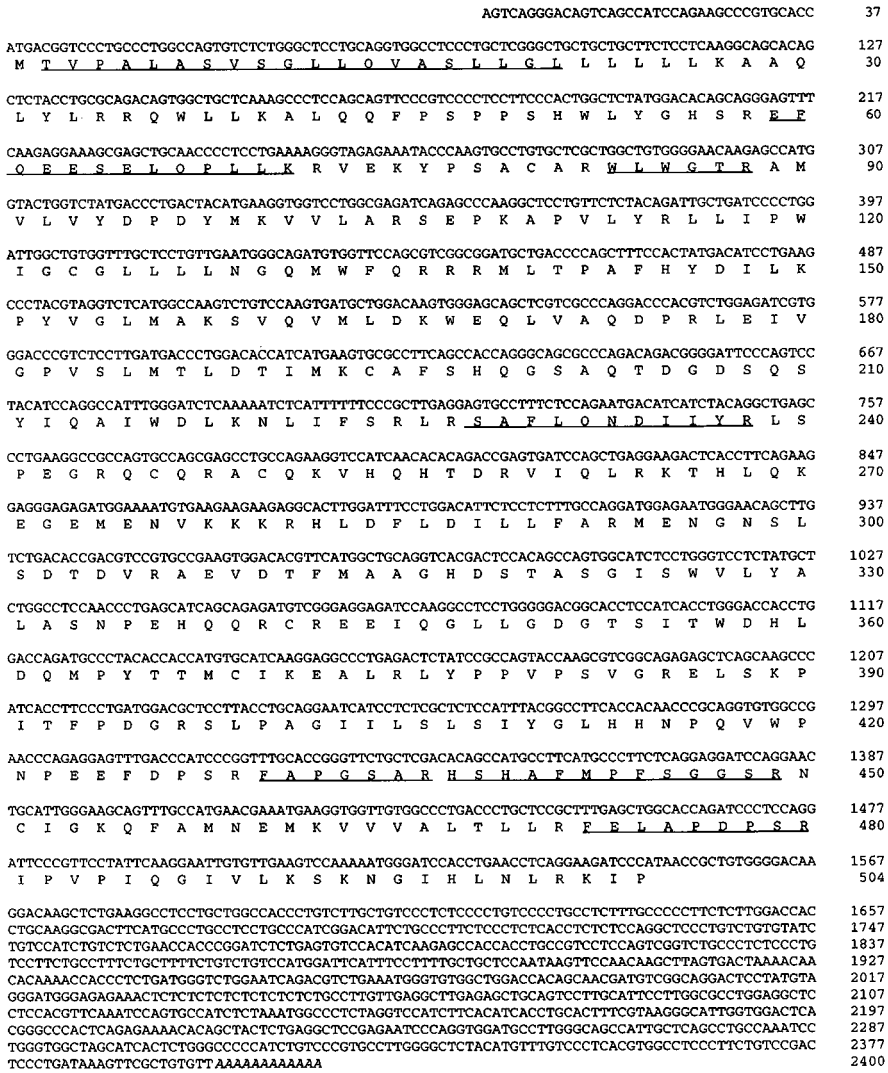

FIG. 1. **cDNA and deduced amino acid sequence of taurochenodeoxycholic acid 6α-hydroxylase from pig liver.** *Underlined* amino acid sequences correspond to the N-terminal sequence and sequences of peptides obtained previously by tryptic digestion of the purified enzyme.

and N-terminal sequencing of the purified protein as reported previously (5). All tryptic fragments have Arg and Lys in positions that permit trypsin cleavage, and the peptide sequences completely match those encoded by the cDNA. Of the 20 N-terminal residues sequenced in the purified protein, 19 were in full agreement with those of the deduced sequence. One residue, Ala in position 17, was replaced by Ser in the encoded sequence. The N terminus of the deduced sequence had an additional Met in the 5′-end compared with that of the purified protein.

The cDNA is 2,400 bp long and contains a 37-nucleotide 5′-UTR, a 1,515-bp open reading frame, and an 848-bp 3′-UTR downstream from the TAA terminal codon. The cDNA encodes a protein of 504 amino acids containing the P450 signature motif of 10 residues (444–453) which includes the invariant cysteine residue that ligates the heme iron (16). The conserved poly(A) additional site signal (17) could not be identified in the 3′-UTR, indicating that the $(T)_{12}$ primer used adhered to a poly(A)-rich sequence upstream from the poly(A) tail. The terminal nucleotides of the 3′-UTR are thus likely missing in the cDNA sequence shown in Fig. 1.

The DNA sequence and the predicted amino acid sequence exhibit between 75 and 79% overall sequence identity with the reported sequences for known members of the CYP4A subfamily from rabbit and human (18–22). It is generally considered that all cytochrome P450 enzymes share a largely conserved tertiary structure core of amino acids (23). A hydrophobic sequence of about 20 residues in the N-terminal of eukaryotic P450 enzymes is thought to represent a transmembrane anchor. In the deduced amino acid sequence of CYP4A21 this N-terminal sequence is shorter by six residues compared with other CYP4A subfamilies. A unique, highly conserved region exclusively found in members of the CYP4A and 4B subfamilies (24) is located around position 315. In CYP4A21, however, amino acid substitutions in this region are found. As shown in Fig. 2, Phe and Glu are replaced by Ala in positions 314 and 315, and Thr is replaced by Ser in position 319 of CYP4A21.

The sequences in the 5′-UTR and 3′-UTR of CYP4A21 show no overall identity with those of other members of the CYP4A subfamily. A short sequence between nucleotides 1686 and 1721 in the 3′-UTR of CYP4A21 shows 94% identity with that of a sequence in the 3′-UTR of CYP4A11 (1716–1751). The significance of this similarity is not known at present.

Expression in COS-1 Cells—To ascertain that the cloned cDNA encodes a functional enzyme, a nucleotide sequence from position 37 to 1549 (Fig. 1) was produced by PCR and inserted into the expression vector pSVL for transfection of COS-1 cells. Mock transfection with the pSVL vector without the cDNA insert was used as a control. Microsomes were isolated from transfected cells by differential centrifugation and subsequently used for the assay of hydroxylase activity and Western blot analysis.

6α-Hydroxylase activity toward taurochenodeoxycholic acid was assayed by incubating microsomes isolated from trans-

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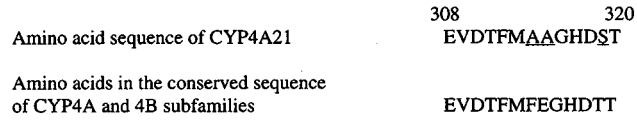

FIG. 2. **Alignment of the deduced sequences between positions 308 and 320 of CYP4A21 and corresponding residues in the conserved sequence common to CYP4A and CYP4B subfamilies.** Residues discussed in the paper are *underlined* in the CYP4A21 sequence.

fected COS cells with ^{14}C-labeled taurochenodeoxycholic acid. The conjugated substrate was chosen because previous studies with pig liver microsomes have shown that the rate of 6α-hydroxylation was five times more efficient when taurochenodeoxycholic acid was used as substrate compared with chenodeoxycholic acid (4). The incubation mixtures were subsequently analyzed by thin layer chromatography together with standards of the unlabeled authentic 6α- and 6β-hydroxylated metabolites, 3α,6α,7α-trihydroxy-5β-cholanoic acid and 3α,6β,7α-trihydroxy-5β-cholanoic acid. A single radioactive peak corresponding to the migration of the 6α-hydroxylated product (R_f = 0.22) was seen in samples incubated with microsomes from COS cells transfected with the pSVL-vector containing CYP4A21 cDNA but not in samples incubated with microsomes from the mock-transfected COS cells. The 6β-hydroxylated metabolite standard migrated with a lower R_f value (R_f = 0.16).

Microsomes of the cells transfected with CYP4A21 cDNA catalyzed 6α-hydroxylation of taurochenodeoxycholic acid at a rate between 5 and 50 pmol/mg of microsomal protein/min, using three concentrations of substrate (25, 145, and 200 μM). A similar range of activity, dependent on the substrate concentration, was found in preparations of pig liver microsomes using the same incubation procedure (Table I). A K_m of 62.9 μM has been calculated previously for the purified pig liver enzyme (5). The higher rate of 6α-hydroxylation by recombinantly expressed CYP4A21 with higher concentrations of substrate is consistent with the K_m-value for the purified enzyme and the high concentrations of bile acids in portal venous blood (50–170 μM) (25).

Microsomes isolated from COS cells transfected with CYP4A21 cDNA did not show ω- or (ω-1)-hydroxylase activity toward lauric acid. In Table I, the hydroxylase activities toward taurochenodeoxycholic acid and lauric acid, using the same preparation of microsomes from CYP4A21-transfected COS cells, are shown together with results using freshly prepared pig liver microsomes. The 6α-hydroxylase activity in microsomes from transfected COS cells and pig liver microsomes is comparable, but the ω- and (ω-1)-hydroxylase activities, present in pig liver microsomes, were not detectable with microsomes from the transfected COS cells. It has been shown in rat and human liver that formation of a ω-hydroxy metabolite of lauric acid is a marker of CYP4A enzyme activity, whereas other cytochrome P450 isoenzymes, like members of the CYP2 family, hydroxylate primarily the (ω-1)-position of lauric acid (26–29). The present results strongly indicate that 6α-hydroxylation of taurochenodeoxycholic acid and ω-hydroxylation of lauric acid in pig liver microsomes are performed by two distinct isozymes. Thus, the cloned microsomal porcine enzyme is not a pig equivalent to the CYP4A fatty acid hydroxylase enzymes found in other species.

Western Blot Analysis of Microsomes from Transfected COS Cells—Polyclonal antibodies raised against the purified taurochenodeoxycholic acid 6α-hydroxylase (5) and antibodies raised against rat CYP4A were used to detect immunoreactive proteins in microsomes of transfected COS cells. Samples from pig liver microsomes and the purified 6α-hydroxylase enzyme were used as positive controls. As shown in Fig. 3, microsomal protein from COS cells transfected with the pSVL vector containing CYP4A21 cDNA was detected by both antibodies. The electrophoretic mobility of the immunoreactive protein was the same as that of the protein recognized by antibodies in pig liver microsomes and purified protein. Microsomes from mock-transfected cells did not show immunoreactive proteins with similar electrophoretic mobility. The ability of the antibodies raised against rat CYP4A to bind expressed CYP4A21 indicates that similar epitopes are recognized in both proteins. The overall amino acid sequence identity between pig taurochenodeoxycholic acid 6α-hydroxylase and rat CYP4A is about 68%.

TABLE I

Hydroxylase activities toward taurochenodeoxycholic acid and lauric acid using microsomes from transfected COS cells and pig liver microsomes

A preparation of microsomes from COS cells transfected with CYP4A21 cDNA was used for the assay of hydroxylase activities toward both taurochenodeoxycholic acid and lauric acid. Microsomal protein (1 mg) from transfected COS cells and pig liver, respectively, was incubated with 25, 145, and 200 μM taurochenodeoxycholic acid and 100 μM lauric acid as described under "Experimental Procedures." The 6α-hydroxylase activity toward taurochenodeoxycholic acid in the transfected COS cell microsomes is comparable to that in pig liver microsomes, whereas there is a marked difference in ω- and (ω-1)-hydroxylation of lauric acid.

Preparation	Hydroxylation of	
	Taurochenodeoxycholic acid 6α	Lauric acid ω- and (ω-1)
	pmol/mg microsomal protein/min	
Microsomes from CYP4A21-transfected COS cells[a]	5 (25 μM) 28 (145 μM) 49 (200 μM)	ND[b]
Pig liver microsomes	12 (25 μM) 39 (145 μM) 56 (200 μM)	575 and 2950

[a] Microsomes from mock-transfected COS cells (pSVL vector without insert) did not show any detectable hydroxylase activity toward the two substrates.

[b] ND, not detected.

Northern Blot Analysis—Total RNA from pig liver was subjected to Northern blot analysis. A nucleotide sequence corresponding to the coding sequence of CYP4A21 was labeled and used as probe. As shown in Fig. 4, the probe hybridized with one major band corresponding to ~2.5 kb. The cloned cDNA is a nucleotide sequence of 2.4 kb, but a proper poly(A) signal sequence in the terminal 3′-UTR is missing, indicating that the mRNA is longer. An mRNA of 2.5 kb could thus correspond to the full-length mRNA.

RT-PCR for Tissue Distribution Studies—Members of the CYP4A gene subfamily have been found in various mammalian tissues including the liver, kidney, lung, intestine, brain, prostate, uterus, and placenta (30). Considering the high homology between CYP4A genes, an RT-PCR procedure was chosen for investigation of tissue expression of CYP4A21 in pig. A CYP4A21-specific reverse primer (RNON-CONS), which hybridizes to position 995 of CYP4A21 but not to the corresponding conserved sequence of CYP4A fatty acid hydroxylases, was used in combination with a forward primer (Xho-N) (see "Experimental Procedures"). As shown in Fig. 5, an intense band corresponding to the size of the expected amplified region (956 bp) was obtained using total RNA from liver. A faint band of the same size was also seen with total RNA from kidney, whereas total RNA from other tissues (heart, muscle, intestine, spleen, thymus, lung, and adrenal gland) did not generate bands of similar size. Because of the RT-PCR result using total RNA from pig kidney, the hydroxylase activity toward taurochenodeoxycholic acid was tested using pig kidney microsomes

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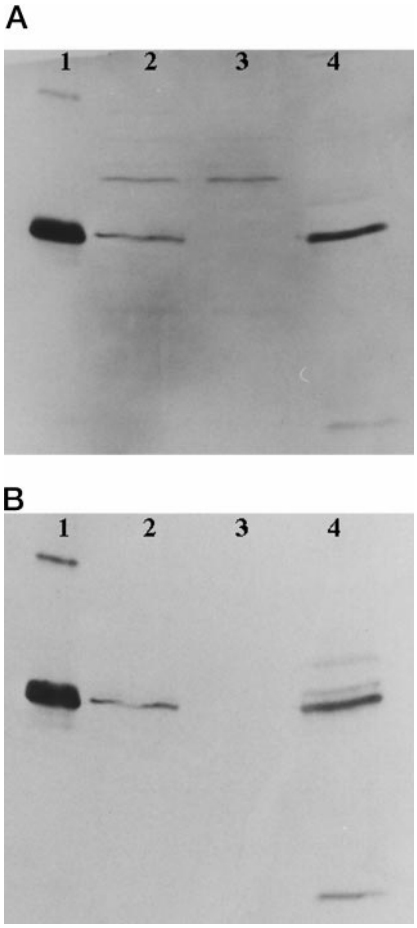

FIG. 3. **Western blot experiment with microsomes from transfected COS cells.** Microsomes from transfected COS cells and pig liver as well as purified 6α-hydroxylase from pig liver microsomes were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred from the gel to a sheet of nitrocellulose and incubated with polyclonal antibodies raised against the purified protein (*panel A*). The filter was stripped of bound antibodies and incubated a second time with antibodies against rat CYP4A (*panel B*). *Lane 1*, 0.4 μg of purified pig liver microsomal taurochenodeoxycholic acid 6α-hydroxylase; *lane 2*, 60 μg of microsomes from COS cells transfected with taurochenodeoxycholic acid 6α-hydroxylase cDNA; *lane 3*, 60 μg of microsomes from COS cells transfected with pSVL vector without insert; *lane 4*, 40 μg of pig liver microsomes. Immunoreactive bands are seen in *lane 2* with both antibodies (*A* and *B*) but not in *lane 3*. The electrophoretic mobility of the immunoreactive protein in *lane 2* corresponds to the protein detected by both antibodies in samples of pig liver microsomes and purified enzyme.

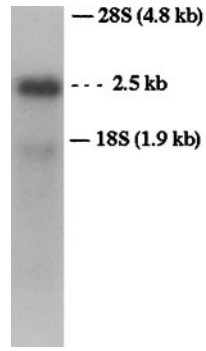

FIG. 4. **Northern blot analysis of total RNA (20 μg) isolated from pig liver.** A nucleotide sequence corresponding to the coding sequence of CYP4A21 from pig liver was labeled with ^{32}P and used as probe according to the hybridization procedure described under "Experimental Procedures." The positions of ribosomal RNA 28 S (4.8 kb) and 18 S (1.9 kb) used as built-in molecular weight markers and the estimated size of the major band detected by the probe are indicated. This band of ~2.5 kb probably corresponds to the full-length mRNA of taurochenodeoxycholic acid 6α-hydroxylase from pig liver.

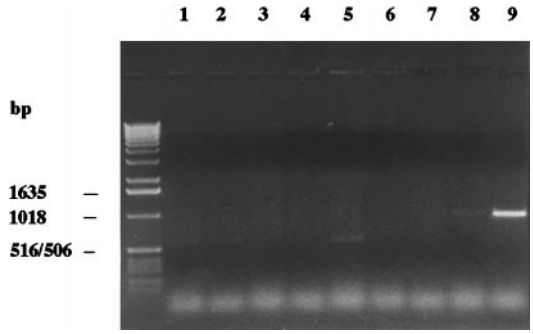

FIG. 5. **RT-PCR of total RNA for tissue distribution studies.** Total RNA from different tissues were reversed transcribed, and the first strand cDNAs were used for PCR. A CYP4A21-specific reverse primer based on the differences between CYP4A21 and CYP4A/4B in the sequence around residue 315 was used together with a forward primer (Xho-N) (see "Experimental Procedures"). The PCR products were analyzed by agarose gel-electrophoresis using a 1-kb DNA ladder for size determination. The samples were as follows: *lane 1*, heart; *lane 2*, muscle; *lane 3*, intestine; *lane 4*, spleen; *lane 5*, thymus; *lane 6*, lung; *lane 7*, adrenal gland; *lane 8*, kidney; and *lane 9*, liver.

(1 mg and 5 mg). The activity was, however, below the limit of detection. The RT-PCR and activity measurement experiments indicate a substantially lower expression of the enzyme in kidney compared with liver.

Expression of a Point-mutated Sequence of CYP4A21—To study the importance of Ala-314, Ala-315, and Ser-319 for the 6α-hydroxylase activity, a mutated sequence of the CYP4A21 was produced using a primer containing nucleotides coding for the corresponding conserved amino acids Phe-314, Glu-315, and Thr-319 (Fig. 2). The three mutations thus introduced in CYP4A21 were A314F, A315E, and S319T. The mutated sequence was cloned into pSVL vector and used for transfection of COS cells. Microsomes prepared from the transfected COS cells were used for Western blot and activity assays.

Expression of a protein in COS cell microsomes was confirmed by Western blot analysis using antibodies raised against rat CYP4A. An immunoreactive band with an electrophoretic mobility identical to that of the expressed CYP4A21 and of the purified 6α-hydroxylase was detected (results not shown). Microsomes from COS cells transfected with the mutant CYP4A21 did not show hydroxylase activity toward taurochenodeoxycholic acid or lauric acid. This indicates that the mutated amino acids in the active site of CYP4A21 are involved in the catalytic activity toward taurochenodeoxycholic acid but are not the sole determinants for the CYP4A enzyme activity toward lauric acid.

DISCUSSION

The primary structure of the taurochenodeoxycholic acid 6α-hydroxylase (CYP4A21) from pig liver described in this paper reveals it to be a member of the CYP4A subfamily. The hitherto known CYP4A enzymes catalyze ω- and (ω-1)-hydroxylations of fatty acids and/or prostaglandins and are often referred to as fatty acid hydroxylases. Despite the overall sequence similarity (74% identity) between the human fatty acid hydroxylase CYP4A11 and the present CYP4A21, the former showed no 6α-hydroxylase activity toward taurochenodeoxycholic acid (31) and the latter no hydroxylase activity toward lauric acid. A change in substrate specificity from a fatty acid to a steroid nucleus among CYP4A enzymes is notable and is probably caused by amino acid substitutions in regions involved in substrate binding and catalytic activity.

An unexpected difference between the deduced amino acid sequence of CYP4A21 and those of other hitherto cloned mem-

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bers of the CYP4A subfamily is amino acid substitutions found in the otherwise conserved sequence around residue 315 (24). Based on homology alignment (23, 32) this part of the sequence is located in the center of the I-helix close to the heme in the active site. Amino acids in this region have been reported to define the steric environment of the heme group and contribute to the preference for ω-hydroxylation of fatty acids, a unique feature of CYP4A fatty hydroxylases (33). The expressed mutated CYP4A21 (A314F/A315E/S319T) did not show 6α-hydroxylase activity toward taurochenodeoxycholic acid. Thus, introduction of the conserved amino acids in CYP4A21 abolishes the 6α-hydroxylase activity, indicating that Ala-314, Ala-315, and Ser-319 in the active site of CYP4A21 are important for the catalytic activity. Introduction of these amino acids did, however, not convert CYP4A21 into a fatty acid hydroxylase. The presence of a CYP4A fatty acid hydroxylase in pig liver was shown by the ability of pig liver microsomes to ω-hydroxylate lauric acid. It seems likely that the substrate specificity of CYP4A21 and CYP4A fatty acid hydroxylase(s), respectively, in pig liver are highly restricted and determined by residues also outside the active site. Substrate recognition sites distal to the heme moiety have been shown to influence the catalytic activity of rat and human CYP4As in studies using mutated enzymes (34–36) or lauric acid analogs (37). A number of substrate-binding residues have also been identified in the substrate access channel in a structural model of CYP4A11 (38). Those studies were, however, confined to the issue of the regiospecificity of fatty acid hydroxylation and catalytic activity toward different fatty acids. The results with CYP4A21 presented here provide new information, which should be useful in further studies on the molecular basis for CYP4A enzymes to bind and hydroxylate a steroid nucleus.

Besides structural considerations, the CYP4A21 is interesting also from an evolutionary point of view. The pig is unique in having hyocholic acid as the main trihydroxylated bile acid. The ratio between hyocholic acid and chenodeoxycholic acid in pigs is comparable to that of cholic acid and chenodeoxycholic acid in most other mammals. It has been speculated that the ancestor of the domestic pig had the ability to carry out 12α-hydroxylation and to form cholic acid as the related warthog now does, but this ability was subsequently lost. Formation of hyocholic acid by 6α-hydroxylation of chenodeoxycholic acid might have evolved as a more biochemically expedient way to fill the requirement of trihydroxylated bile acids brought about by some change in dietary circumstances (1). The CYP4 family is one of the oldest cytochrome P450 families having diverged from a common ancestor over 1.25 billion years ago (30, 39, 40). Considering the speculation that the ability to form hyocholic acid in pigs evolved late by dietary changes, the CYP4A21 is probably a more recent cytochrome P450 compared with the known fatty acid-hydroxylating members of the CYP4A subfamily. In this context, it is interesting to note that the key enzyme in formation of cholic acid, the sterol 12α-hydroxylase (CYP8B), shows a high degree of sequence identity with the prostacyclin synthase (CYP8A). In that case, it has been suggested that CYP8A might have diverged from an ancient CYP8B and subsequently acquired the novel function through extensive alteration (41).

In humans, increased amounts of hyocholic acid have been found in plasma and urine from patients with cholestatic liver disease and also in fetal blood sample. It seems likely that hydroxylations of bile acids in man, such as 6α-hydroxylation, are a way for detoxification of accumulating cytotoxic bile acids when the normal pathways for excretion are either disturbed (as in cholestasis) or poorly developed (as in the fetus). CYP3A4 has been shown to carry out 6α-hydroxylation of taurochenodeoxycholic acid in experiments with human liver microsomes and recombinantly overexpressed enzyme (31). A role for CYP3A4 in hyocholic acid formation in man is supported by the observation that 6α-hydroxylation of bile acids in humans is stimulated by rifampicin, a well known inducer of CYP3A4 (42). It is not known at present whether an additional 6α-hydroxylating enzyme, orthologous to the porcine CYP4A21, is expressed in human or in other species besides pig. The present results showing a low expression of CYP4A21 in pig kidney indicate that this enzyme has functions in addition to participating in bile acid biosynthesis in pig liver.

In conclusion, this paper describes the cloning and deduced primary structure of a novel CYP4A enzyme (CYP4A21) with the ability to 6α-hydroxylate taurochenodeoxycholic acid. In addition to its biological role in formation of hyocholic acid in the pig, this enzyme is also interesting from evolutionary and structural aspects. The porcine CYP4A21 opens up possibilities to study the evolutionary relationship between this enzyme and other members of the CYP4A subfamily and should contribute to our understanding of features important for the substrate specificity of CYP4A enzymes.

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