Cloning and expression of two novel pig liver and kidney fatty acid hydroxylases [cytochrome P450 (CYP)4A24 and CYP4A25]

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A new member of the cytochrome P450 (CYP) 4A subfamily (CYP4A21) was recently cloned by PCR from pig liver [Lundell, Hansson, and Wikvall (2001) *J. Biol. Chem.* **276**, 9606–9612]. This enzyme does not catalyse ω - or (ω –1)-hydroxylation of lauric acid, the model substrate for CYP4A enzymes. Instead, CYP4A21 participates in bile acid biosynthesis in the pig. Extensive studies, primarily conducted to verify the aberrant amino acids found in CYP4A21 within a normally conserved CYP4A motif, revealed that besides CYP4A21 two additional sequences were co-amplified by PCR. These two sequences (designated CYP4A24 and CYP4A25), generated from both pig liver and kidney, were characterized by restriction-enzyme analysis and were subsequently cloned. The deduced amino acid

sequences of CYP4A24 and CYP4A25 share extensive sequence identity (97%). Both enzymes, expressed in yeast cells, exhibit ω - and (ω –1)-hydroxylase activities towards lauric acid and palmitic acid. The positions of the variable regions between CYP4A24 and CYP4A25, which are confined to β -sheets 1 and 4, indicate a possible difference in substrate specificity or regioselectivity. The porcine CYP4A21, CYP4A24 and CYP4A25 enzymes, with an overall identity of 94%, have probably evolved from a common ancestral gene, perhaps in conjunction with species-specific habits.

Key words: CYP4A subfamily, ω -hydroxylation, yeast cell expression.

INTRODUCTION

The cytochrome P450 (CYP) 4A subfamily encodes a number of enzymes which catalyse the ω - and (ω –1)-hydroxylation of fatty acids and prostaglandins. Multiple isoenzymes of this subfamily have been cloned and characterized in rat, rabbit and mouse, whereas a single CYP4A enzyme has been identified in human [1]. Some members of the CYP2 family hydroxylate methylene groups of fatty acids [2,3], but members of the CYP4A subfamily are distinguished by their ability to hydroxylate the least reactive terminal methyl group, ω -hydroxylation [4]. The physiological significance of ω -hydroxylation of fatty acids and prostaglandins is not fully understood. A role for CYP4A enzymes in fatty acid metabolism has been proposed [5]. This is supported, in some species, by a co-ordinate regulation of CYP4A enzymes and genes that control fatty acid synthesis, storage and catabolism by the peroxisome proliferator-activated receptor [6]. Renal CYP4A enzymes have been implicated in the ω - and (ω –1)-hydroxylation of arachidonic acid. Hydroxylated products of arachidonic acid (19- and 20-hydroxyeicosatetraenoic acid) have been suggested to play a role in renal function and regulation of vascular tone [7].

A new member of the CYP4A subfamily (CYP4A21) was recently cloned from pig liver [8]. Unlike other known members of the CYP4A subfamily this enzyme does not catalyse ω - or (ω –1)-hydroxylation of fatty acids. Instead, CYP4A21 participates in bile acid biosynthesis in the pig and performs a 6 α -hydroxylation of the steroid nucleus of 3 α ,7 α -dihydroxy-5 β -cholanoic acid (chenodeoxycholic acid), generating the primary bile acid 3 α ,6 α ,7 α -trihydroxy-5 β -cholanoic acid ('hyocholic acid'). Besides being found in the liver, traces of CYP4A21 were found in kidney [8]. Pig liver and kidney microsomes show ω -

hydroxylase activity towards lauric acid, indicating the co-existence of CYP4A fatty acid hydroxylase(s) besides CYP4A21. A pig kidney ω - and (ω –1)-hydroxylase has previously been purified and characterized, but was not reported to be cloned [9].

CYP4A21 was cloned by PCR using primers designed from the purified taurochenodeoxycholic acid 6 α -hydroxylase [8,10]. Results obtained by restriction-enzyme digest analysis indicated that the PCR product contained more than one distinct sequence. Considering the high sequence identity between CYP4A enzymes within other species it seemed likely that a pig liver CYP4A sequence was co-amplified. This prompted further investigation of the PCR fragment in order to identify additional cDNAs that might encode fatty acid hydroxylase(s). The present study identifies and describes the cloning of two CYP4A enzymes from both pig liver and kidney. The predicted amino acid sequences of these two enzymes show 97% identity and the expressed enzymes exhibit ω - and (ω –1)-hydroxylase activities towards lauric acid and palmitic acid. These two enzymes were designated CYP4A24 and CYP4A25.

EXPERIMENTAL

Materials

Livers and kidneys from castrated, otherwise untreated, male pigs were obtained from the local slaughterhouse. RNeasy Midi Kits, a QIAprep Spin Miniprep Kit and a Plasmid Maxi Kit were obtained from Qiagen. Restriction enzymes were purchased from Amersham Biosciences and New England BioLabs, and pBlue-scriptIIKS was purchased from Stratagene. Oligonucleotides were obtained from Life Technologies and Thermo Hybaid

Abbreviations used: chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholanoic acid; CYP, cytochrome P450.

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(Interactiva Division, Ulm, Germany). TaqGold polymerase and oligo dNTP mix were purchased from PE Applied Biosystems, and PlatinumPfx DNA polymerase and a 1.5 kb DNA ladder were obtained from Life Technologies. A Reverse Transcription System was purchased from Promega, and T4 DNA ligase and buffer were from New England BioLabs. A Megaprime DNA labelling system, [1-¹⁴C]lauric acid (53 Ci/mol) and [1-¹⁴C] palmitic acid (56 Ci/mol) were purchased from Amersham Biosciences. All chemicals used were of first grade quality.

Restriction-enzyme digest analysis of a 1430 bp PCR product

Total RNA isolated from pig liver and kidney using RNeasy Midi Kits was reverse-transcribed and first-strand cDNAs were used for PCR (reverse transcriptase-PCR). Oligonucleotide primers for PCR were originally designed for cloning of CYP4A21 [8]: forward primer N2, 5'-CGTCCCGGCCCTGGCCAGCGT-3'; and reverse primer R:FEL, 5'-TGGGATCTGGCGCCAGCTCGAA-3'. The PCR cycles were as follows: 94 °C for 2 min, 55 °C for 1 min and 72 °C for 2 min for 30 cycles, and 72 °C for 10 min. The 1430 bp PCR products generated were gel-purified and portions of approx. 60 ng were digested with *Pst*I or *Eco*RI. The restriction-enzyme digests were electrophoresed on a 1% (w/v) agarose gel and subjected to Southern transfer to Hybond-N membranes (Amersham Biosciences). The gel was prepared for transfer by denaturation (0.5 M NaOH and 1.5 M NaCl) for 20 min and neutralization (1 M Tris/HCl and 1.5 M NaCl) for 20 min. A nucleotide sequence, corresponding to the coding sequence of CYP4A21, was ³²P-labelled using a Megaprime labelling Kit and was used as a probe in Southern hybridization. The membrane was hybridized overnight at 65 °C with the ³²P-labelled probe in modified Church buffer [11], 0.25 M phosphate buffer (pH 7.2), 7% (w/v) SDS and 1 mM EDTA. The membrane was then washed twice in 2 × SSC (where 1 × SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS for 5 min at room temperature (approx. 22 °C), and exposed to Fuji RX film.

Cloning of cDNA from pig liver and kidney

First-strand cDNAs obtained by reverse transcription of total RNA isolated from pig liver and kidney, were used for PCR. Two oligonucleotides corresponding to sequences immediately upstream of the initiation codon and downstream of the stop codon in the CYP4A21 sequence were used to generate complete coding sequences. The oligonucleotide sequences were: forward primer 5N1, 5'-CTTGGTACCAGGGACAGTCAGCCATCC-A-3', containing the restriction site for *Kpn*I; and reverse primer 3CB, 5'-TTTCTAGAGCTTGTCCTTGTCCTCCACACA-3', with the restriction site for *Xba*I. High fidelity Pfx polymerase was used for PCR and the cycles were as described above. The 1575 bp PCR products, generated from liver and kidney, were cloned into the pBluescriptIIKS vector using the restriction sites for *Kpn*I and *Xba*I, and were transformed into *Escherichia coli* DH5α cells. Plasmid DNAs from several colonies were purified using DNA miniprep columns (Qiagen) according to the manufacturer's instructions. Miniprep DNAs were classified by restriction-enzyme digest analysis using *Pst*I and *Eco*RI. Eight plasmid minipreps thus obtained from liver and kidney, characterized as potential CYP4A fatty acid hydroxylases by *Pst*I digest, and showing two distinct cleavage patterns using *Eco*RI (cleaved or not cleaved) were sequenced using Dye-labelled Terminator for the sequence reaction and a ABI377 DNA sequencer for analysis.

Expression of cDNA sequences in yeast

The two cDNA sequences, classified by *Eco*RI restriction-enzyme digestion, were cloned into the expression vector pYeDP60 (a gift from Drs Denis Pompon and Philippe Urban, Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette, France). Plasmid DNA sequences were amplified using: forward primer Nv60, 5'-TCTTCCCGGGAAAATGACGGTCCCTGCCCTGGC-3', containing the restriction site for *Xma*I followed by three adenosine nucleotides in front of the initiation codon; and the reverse primer Cv60, 5'-TCTTGGTACCTTACGGGAGCTTGCGAAGCT-3', containing the 17 nucleotides preceding the stop codon in the 3' end of both cDNAs followed by the restriction site for *Kpn*I. The PCR cycles were performed according to the procedure described above. The 1526 bp PCR products were gel-purified, digested with *Xma*I and *Kpn*I, cloned into a similarly digested pYeDP60 vector, and transformed into *E. coli* DH5α cells. The plasmid DNA was purified, sequenced and used for transformation of the *Saccharomyces cerevisiae* strain W(R) [12] (a gift from and used with the consent of Drs Denis Pompon and Philippe Urban). Transformation was performed using the lithium acetate procedure described by Gietz et al. [13]. Yeast cell microsomes were prepared according to the method described by Pompon et al. [14], and protein concentration was determined by the method of Lowry et al. [15] using BSA as a standard. Yeast microsomes prepared from transformed cells were used for activity assays, and yeast microsomes prepared from cells transformed by the pYeDP60 vector without cDNA (mock transformation) were used as controls.

Western-blot analysis

SDS/PAGE was carried out on a 7% (w/v) gel according to the method of Laemmli [16]. The proteins on the gels were electrophoretically blotted on to nitrocellulose filters, and Western-blot analysis was performed using antibodies raised against rat CYP4A (Amersham Biosciences) according to the manufacturer's instructions. Calibration of relative molecular mass was carried out using pre-stained molecular mass markers (Sigma).

Activity assay

Hydroxylase activity towards lauric acid and palmitic acid was assayed by incubation of microsomes from transformed yeast cells with ¹⁴C-labelled substrate and 2 μM NADPH in a total volume of 0.2 ml of 0.1 M Tris/HCl (pH 7.5). The final concentration of substrate was 100 μM. Incubations were performed at 37 °C for 20 min and terminated by the addition of 0.1 ml of 6% (v/v) acetic acid in ethanol (lauric acid) or 6% (v/v) acetic acid in acetonitrile (palmitic acid). Samples were centrifuged and the supernatants analysed by HPLC according to the method of Okita et al. [17], using a LichroCART 250 mm × 4 mm, RP-18 (5 μm particle diameter) column (Merck) and a Radiomatic detector (Packard Instrument Company, Meriden, CT, U.S.A.). Hydroxylase activity towards taurochenodeoxycholic acid was analysed as described previously [8].

RESULTS

Analysis of the heterogeneity of a 1430 bp PCR product

The 1430 bp PCR product (N2-R:FEL fragment) generated with total RNA from pig liver and kidney, using primers designed from the purified taurochenodeoxycholic acid 6α-hydroxylase

Figure 1 Schematic representation of restriction digest analysis of a 1430 bp PCR product (N2-R:FEL fragment) using *PstI* (A) and *EcoRI* (B)

Restriction sites and fragment sizes are outlined for two populations of sequences for each restriction enzyme. *PstI* cleaves sequences corresponding to CYP4A21 (fragment 1) at an internal position but not CYP4A fatty acid hydroxylases (fragment 2) due to a divergence from the conserved CYP4A motif in CYP4A21. *EcoRI* either cleaves (fragment 2) or does not cleave (fragment 1) two subpopulations of sequences in the N2-R:FEL fragment.

Figure 2 Southern hybridization of *PstI*- and *EcoRI*-digested 1430 bp PCR products (N2-R:FEL fragments) using ^{32}P -labelled CYP4A21 as the probe

Four N2-R:FEL fragments were generated using total RNA from two different pig livers and two different pig kidneys. Portions of 60 ng of each gel-purified PCR product were subjected to restriction-enzyme digestion, followed by agarose electrophoresis and Southern-blot analysis as described in the Experimental section. A 1 kb DNA ladder (Life Technologies) was used for size determination. Lane 1, uncleaved PCR product; lanes 2 and 3, *PstI* digest of the two pig liver fragments; lanes 4 and 5, *PstI* digest of the two pig kidney fragments; lanes 6 and 7, *EcoRI* digest of the two pig liver fragments; lanes 8 and 9, *EcoRI* digest of the two pig kidney fragments.

were found to contain more than one population of sequences when subjected to analysis by restriction-enzyme digest. As outlined in Figure 1(A), *PstI* cleaves at an internal position (at 940 bp), generating a fragment of 905 bp, in the sequence of CYP4A21 due to nucleotides coding for the aberrant amino acids (Ala³¹⁵ and Ala³¹⁶) within the otherwise conserved CYP4A motif [8] (Figure 1A, fragment 1). This position is not cleaved by *PstI* in sequences encoding the conserved CYP4A amino acids, thus a fragment of 1204 bp is generated (Figure 1A, fragment 2).

The use of *EcoRI* to differentiate between subpopulations of sequences in the N2-R:FEL fragment was based on accumulating sequencing data. A persisting difference between sequences was found in nucleotides around position 327 in the fragment. *EcoRI* cleaves one of these sequences generating a fragment of 1103 bp as indicated in Figure 1(B) (fragment 2). CYP4A21 belongs to a subpopulation of sequences, which is not digested by *EcoRI* (Figure 1B, fragment 1).

The results of the *PstI* and *EcoRI* digestion analysis of the N2-R:FEL fragments are shown in Figure 2. Labelled CYP4A21 cDNA was used to probe the Southern blot of digested fragments.

Pig liver N2-R:FEL fragments, generated using RNA from two different individuals, were equally digested by *PstI* (lanes 2 and 3). An intense band at 905 bp indicates that the major component in this PCR product is the nucleotide sequence corresponding to that of CYP4A21 (Figure 1A, fragment 1). A weaker band at 1204 bp, also hybridizable with the ^{32}P -labelled CYP4A21 cDNA, indicates the presence of a sequence with the expected cleavage pattern of CYP4A fatty acid hydroxylases (Figure 1A, fragment 2). The N2-R:FEL fragments from pig kidney (lanes 4 and 5), also generated using total RNA obtained from two individuals, are similarly digested by *PstI* as the pig liver fragments but the intensity of the 905 and 1204 bp bands respectively are inverse, indicating a low expression of CYP4A21 in kidney and a more abundant expression of CYP4A fatty acid hydroxylases. The CYP4A21 sequence is not digested by *EcoRI*. From the Southern blot in Figure 2 the pig liver N2-R:FEL fragment (lanes 6 and 7) does not appear to be significantly digested by *EcoRI*. A weak band can, however, be discerned in the 1103 bp region in lane 6. The *EcoRI*-digested pig kidney PCR products (lanes 8 and 9) clearly show the presence of an 1103 bp digested product in addition to the uncleaved N2-R:FEL fragment.

Taken together, the cleavage pattern obtained using N2-R:FEL fragments from two different pig liver and kidney samples confirm the co-amplification of a subpopulation of sequences, distinct from CYP4A21.

Cloning and sequencing

The complete coding sequences were obtained using two primers based on nucleotide sequences in the 5' untranslated region (5N1) and the 3' untranslated region (3CB) of CYP4A21. The 1575 bp PCR products (5N1-3CB fragments) generated using pig liver and kidney RNAs showed the same pattern of heterogeneity when digested by *PstI* and *EcoRI*, as was shown for the 1430 bp N2-R:FEL fragment. The 5N1-3CB fragments from pig liver and kidney were cloned into the pBluescriptIIKS vector and transformed into *E. coli* DH5 α cells. Miniprep DNAs were classified by restriction-enzyme digest using *PstI* and *EcoRI*. Plasmid DNA showing a *PstI* restriction digest pattern corresponding to the potential CYP4A fatty acid hydroxylases (Figure 1A, fragment 2) was further digested by *EcoRI*. Surprisingly, two subpopulations of plasmids, either cleaved or not cleaved by *EcoRI*, were found among both liver and kidney miniprep DNAs. Sequencing and alignment of multiple representatives of each of these two miniprep DNA subpopulations confirmed the presence of two similar, but distinct, nucleotide sequences. The two cDNAs and the predicted amino acid sequences are shown in Figure 3. The 17 nucleotides that differ between the two sequences (marked in bold) were clearly distinguished among the two populations of aligned sequences. These two sequences thus cloned from both pig liver and kidney share extensive sequence similarity (approx. 99 % overall nucleotide identity). The two cDNAs encode proteins of 504 amino acids, similar to that of CYP4A21. The overall identity between these two sequences is 97 % compared with a 94 % overall identity with CYP4A21 [8]. The amino acid differences between the two sequences were found in the N-terminal region, between residues 33-113 (90 % identity), and in the C-terminal region from residues 404-490 (90 % identity). A long segment of complete identity is evident from residue 114 through to 403. Based on secondary structure prediction using the PHD program [18] and secondary structure nomenclature adopted from Chang and Loew [19] the majority of the variable amino acids are

CYP4A24 (AA)	M T V P A L A S A S G L L Q V A S L L G L L L L L L K A A Q	30
CYP4A24 (NT)	ATGACGGTCCCTGCCCTGCGCAGTGCCTCTGGGCTCCTGCAGGTGGCTCCCTGCTCGGGTGTGCTGCTCTCTCCCAAGGCAGCACAG	90
CYP4A25 (NT)	ATGACGGTCCCTGCCCTGCGCAGTGCCTCTGGGCTCCTGCAGGTGGCTCCCTGCTCGGGTGTGCTGCTCTCTCCCAAGGCAGCACAG	90
CYP4A25 (AA)		30
CYP4A24 (AA)	L Y L H R Q W L L K A L Q Q F P S P P S H W L Y G H S R E F	60
CYP4A24 (NT)	CTCTACCTGCACAGACAGTGGTGTCTCAAGGCCCTCCAGCAGTCCCGTCCCCCTCTCCCACTGGCTCTATGGACACAGCAGGAGTTT	180
CYP4A25 (NT)	CTCTACCTGCACAGACAGTGGTGTCTCAAGGCCCTCCAGCAGTCCCGTCCCCCTCTCCCACTGGCTCTATGGACACAGCAGGAGTTT	180
CYP4A25 (AA)	R	60
CYP4A24 (AA)	Q E E S E L P P L L K R V E K Y P S A C A L W R W G T R A M	90
CYP4A24 (NT)	CAAGAGGAAAGCGAGCTGCCACCCCTCCTGAAAAGGTAGAGAAATACCAAGTGCTGTGCTCTCTGGCGGTGGGGAACAGAGCCATG	270
CYP4A25 (NT)	CAAGAGGAAAGCGAGCTGCCACCCCTCCTGAAAAGGTAGAGAAATACCAAGTGCTGTGCTCTCTGGCGGTGGGGAACAGAGCCATG	270
CYP4A25 (AA)	Q R L	90
CYP4A24 (AA)	V L V Y D P D Y M K V V L A R S D P K N S V V Y R L L I P W	120
CYP4A24 (NT)	GTACTGGTCTATGACCCCTGACTACATGAAGGTGGTCTGGCGAGATCAGACCCCAAGAATCTGTGTCTACAGATTGCTGATCCCTGG	360
CYP4A25 (NT)	GTACTGGTCTATGACCCCTGACTACATGAAGGTGGTCTGGCGAGATCAGAGCCCAAGGCTCTGTCTCTACAGATTGCTGATCCCTGG	360
CYP4A25 (AA)	E A P L	120
CYP4A24 (AA)	I G C G L L L L N G Q T W F Q R R R M L T P A F H Y D I L K	150
CYP4A24 (NT)	ATTGGCTGTGGTTTGTCTCTGTGAATGGCGAGACGTGGTTCACGCTCGCGGGATGCTGACCCACGCTTCCACTATGACATCTCTGAAG	450
CYP4A25 (NT)	ATTGGCTGTGGTTTGTCTCTGTGAATGGCGAGACGTGGTTCACGCTCGCGGGATGCTGACCCACGCTTCCACTATGACATCTCTGAAG	450
CYP4A25 (AA)		150
CYP4A24 (AA)	P Y V G L M A K S V Q V M L D K W E Q L V A Q D P R L E I V	180
CYP4A24 (NT)	CCCTACGTAGGTCTCATGGCCAAGTCTGTCCAAGTGATGCTGGACAAAGTGGGAGCAGCTCGTCGCCACGACCCACGCTCTGGAGATCGTG	540
CYP4A25 (NT)	CCCTACGTAGGTCTCATGGCCAAGTCTGTCCAAGTGATGCTGGACAAAGTGGGAGCAGCTCGTCGCCACGACCCACGCTCTGGAGATCGTG	540
CYP4A25 (AA)		180
CYP4A24 (AA)	G P V S L M T L D T I M K C A F S H Q G S A Q T D G D S H S	210
CYP4A24 (NT)	GGACCCGTCTCCTTGATGACCTGGACACCATCATGAAGTGGCCTTCAGCCACACGAGGCGAGCCAGACAGACGCGGATTCCTCACTCC	630
CYP4A25 (NT)	GGACCCGTCTCCTTGATGACCTGGACACCATCATGAAGTGGCCTTCAGCCACACGAGGCGAGCCAGACAGACGCGGATTCCTCACTCC	630
CYP4A25 (AA)		210
CYP4A24 (AA)	Y I Q A I W D L K N L F S I R T K S A F L Q N D I I Y R L S	240
CYP4A24 (NT)	TACATCCAGGCCATTGGGATCTCAAAATCTCTTTTCTATCCGCACGAAAGTGCCTTTCTCCAGATGACATCATCTACAGCTGAGC	720
CYP4A25 (NT)	TACATCCAGGCCATTGGGATCTCAAAATCTCTTTTCTATCCGCACGAAAGTGCCTTTCTCCAGATGACATCATCTACAGCTGAGC	720
CYP4A25 (AA)		240
CYP4A24 (AA)	P E G R K N H R A A R I A H Q H T D R V I Q L R K A Q L Q K	270
CYP4A24 (NT)	CCTGAAGCCGTGAAGAACCCGTCGCCCGAGATAGCCCATCAACACACAGACGAGTGCCTTCGCGAAGGCGAGCTGCAAAAG	810
CYP4A25 (NT)	CCTGAAGCCGTGAAGAACCCGTCGCCCGAGATAGCCCATCAACACACAGACGAGTGCCTTCGCGAAGGCGAGCTGCAAAAG	810
CYP4A25 (AA)		270
CYP4A24 (AA)	Q G E M E N V R K K R H L D F L D I L L L A R M E K G N S L	300
CYP4A24 (NT)	CAGGAGAGATGGAGAACCGTGAGGAAGAGAGGCACTTGGATTCTCGGACATCTCTCTCTGCGCAATGGAGAGGGGAACAGCTTG	900
CYP4A25 (NT)	CAGGAGAGATGGAGAACCGTGAGGAAGAGAGGCACTTGGATTCTCGGACATCTCTCTCTGCGCAATGGAGAGGGGAACAGCTTG	900
CYP4A25 (AA)		300
CYP4A24 (AA)	S D T D L R A E V D T F M F E G H D T T A S G I S W I L Y A	330
CYP4A24 (NT)	TCGGACACCGACCTCCGTCGCCAAGTGAGACAGTTTCATGTTTCGAGGGTTCATGACACACAGCCAGTGGCATCTCTGGATCCTCTATGCT	990
CYP4A25 (NT)	TCGGACACCGACCTCCGTCGCCAAGTGAGACAGTTTCATGTTTCGAGGGTTCATGACACACAGCCAGTGGCATCTCTGGATCCTCTATGCT	990
CYP4A25 (AA)		330
CYP4A24 (AA)	L A S H P E H Q Q R C R E E I Q G L L G D G T S I T W D H L	360
CYP4A24 (NT)	CTGGCCTCCACCCCTGAGCATCAGCAGAGATGTCGGAGGAGATCCAAAGGCTCTCTGGGGACGGCACCTCCATCACCTGGGACCACTG	1080
CYP4A25 (NT)	CTGGCCTCCACCCCTGAGCATCAGCAGAGATGTCGGAGGAGATCCAAAGGCTCTCTGGGGACGGCACCTCCATCACCTGGGACCACTG	1080
CYP4A25 (AA)		360
CYP4A24 (AA)	D Q M P Y T T M C I K E A L R L Y P P V P G V S R E L S K P	390
CYP4A24 (NT)	GACCAGATGCCCTACACCACCATGTGCATCAAGGAGGCCCTGAGACTCTATCCGCCAGTACCAGGAGTTAGCAGAGAGCTCAGCAAGCCC	1170
CYP4A25 (NT)	GACCAGATGCCCTACACCACCATGTGCATCAAGGAGGCCCTGAGACTCTATCCGCCAGTACCAGGAGTTAGCAGAGAGCTCAGCAAGCCC	1170
CYP4A25 (AA)		390
CYP4A24 (AA)	I T F P D G R S L P A G I T L S L S I Y G L H H N P Q V W P	420
CYP4A24 (NT)	ATCACCTTCCCTGATGGACGCTCCTTACCTGCGAGGAATCACCTCTCGCTCTCCATTACGGCTTCCACCAACCCGAGGTGTGGCCG	1260
CYP4A25 (NT)	ATCACCTTCCCTGATGGACGCTCCTTACCTGCGAGGAATCACCTCTCGCTCTCCATTACGGCTTCCACCAACCCGAGGTGTGGCCG	1260
CYP4A25 (AA)	I V S	420
CYP4A24 (AA)	N P E E F D P S R F A P G S A R H S H A F M P F S G G S R N	450
CYP4A24 (NT)	AAACCCAGAGGAGTTTGACCCATCCCGGTTTGACCCGGGTTCTGCTGCACACAGCCATGCCTTCATGCCCTTCTCAGGAGGATCCAGGAAC	1350
CYP4A25 (NT)	AAACCCAGAGGAGTTTGACCCATCCCGGTTTGACCCGGGTTCTGCTGCACACAGCCATGCCTTCATGCCCTTCTCAGGAGGATCCAGGAAC	1350
CYP4A25 (AA)		450
CYP4A24 (AA)	C I G K Q F A M N E M K V A V A L T L L R F E L A P D P S R	480
CYP4A24 (NT)	TGCATTGGGAAGCAGTTTGCCATGAACGAGATGAAGTGGCTGTGGCCCTGACCTGCTCCGCTTTGAGCTGGCACCAGATCCCTCCAGG	1440
CYP4A25 (NT)	TGCATTGGGAAGCAGTTTGCCATGAACGAGATGAAGTGGCTGTGGCCCTGACCTGCTCCGCTTTGAGCTGGCACCAGATCCCTCCAGG	1440
CYP4A25 (AA)		480
CYP4A24 (AA)	K P I A T P E V V L N S K N G I H L K L R K L P	504
CYP4A24 (NT)	AAGCCATTGCCATCCAGAGTCTGTGAACCTCAAAATGGGATCCACCTGAAGCTTCGCAAGCTCCCGTAA	1515
CYP4A25 (NT)	AAGCCATTGCCATCCAGAGTCTGTGTGAACCTCAAAATGGGATCCACCTGAAGCTTCGCAAGCTCCCGTAA	1515
CYP4A25 (AA)	T V I H	504

Figure 3 Sequence alignment of the cDNAs and deduced amino acid sequences of CYP4A24 and CYP4A25

The complete nucleotide (NT) sequences for CYP4A24 and CYP4A25 are shown. The nucleotides that differ between the sequences are marked as bold letters in the sequence of CYP4A25. The complete deduced amino acid (AA) sequence of CYP4A24 is shown, whereas only residues in CYP4A25 which differ from those of CYP4A24 are indicated.

positioned in β -sheet 1 (strands 1, 3 and 5) and β -sheet 4 (strands 1 and 2) as shown in Figure 4.

Expression in yeast cells

In order to examine whether the new cDNAs encode fatty acid hydroxylases each cDNA was expressed in the *S. cerevisiae* strain

W(R). Yeast microsomes from transformed cells were isolated and used for Western-blot analysis and activity assays.

Western-blot analysis of microsomes from transformed yeast cells, probed with antibodies raised against rat CYP4A (Amer-sham Biosciences), revealed expressed proteins with an apparent M_r of approx. 52 kDa, similar to that of the purified taurocheno-deoxycholic acid 6 α -hydroxylase [10], which was used as control together with pig kidney microsomes. No immunoreactive pro-

Figure 4 Predicted secondary structure of variable regions in the aligned sequences of CYP4A24 and CYP4A25 between positions 1–120 (A) and 400–504 (B)

The complete sequence of CYP4A24 is shown together with the variable residues in CYP4A25. Lines above the sequences indicate the positions of secondary structures, identified as helices and β -sheets by the secondary structure prediction program (PHD) [18]. The overall nomenclature, used for CYP4A11, is adopted from Chang and Loew [19] with addition of the transmembrane helix.

Figure 5 Western-blot analysis of microsomal proteins from transformed yeast cells with antibodies raised against CYP4A1

Samples were applied as follows: lane 1, 30 μ g of pig kidney microsomes; lane 2, 100 μ g of microsomes from yeast cells transformed with CYP4A24 cDNA; lane 3, 100 μ g of microsomes from yeast cells transformed with CYP4A25 cDNA; lane 4, 100 μ g of microsomes from mock-transformed yeast cells; lane 5, 0.4 μ g of purified pig liver microsomal taurochenodeoxycholic acid 6 α -hydroxylase (CYP4A21).

Table 1 Rates of ω - and (ω –1)-hydroxylation of lauric acid and palmitic acid in microsomal preparations of transformed yeast cells

Data are presented as means $\pm$ S.D. for three separate determinations (pmol/min per mg of microsomal protein). The reaction conditions are given in the Experimental section. Microsomes from mock-transformed cells were devoid of ω - and (ω –1)-hydroxylase activities towards lauric acid. Low intrinsic palmitic acid ω - and (ω –1)-hydroxylase activities (< 10 pmol/min per mg of microsomal protein) were detected using microsomes from mock-transformed yeast cells.

Enzyme	Hydroxylation of ...			
	Lauric acid		Palmitic acid	
	ω	(ω –1)	ω	(ω –1)
CYP4A24	372 $\pm$ 7	174 $\pm$ 10	138 $\pm$ 20	75 $\pm$ 10
CYP4A25	200 $\pm$ 22	77 $\pm$ 16	89 $\pm$ 33	48 $\pm$ 7

Figure 6 HPLC analysis of ω - and (ω –1)-hydroxylated products of lauric acid and palmitic acid formed by microsomes from transformed yeast cells

The radiolabelled substrates and products were detected by Radiomatic detector as described in the Experimental section. Typical chromatograms of CYP4A24 activities (top panels) and CYP4A25 activities (bottom panels) are shown. Lauric acid eluted at 22 min, and ω - and (ω –1)-hydroxylated products eluted at 19 and 22 min respectively. Palmitic acid eluted at 30 min, and ω - and (ω –1)-hydroxylated products eluted at 13 and 12 min respectively.

teins were detected in this region with microsomes from mock-transformed yeast cells (Figure 5).

The activity towards lauric acid and palmitic acid was assayed using 100 μ M substrate. In Table 1 the activities of the recombinant enzymes expressed in yeast cell microsomes are shown. Both sequences clearly encode proteins that exhibit ω - and (ω –1)-hydroxylase activities towards fatty acids. Increasing the fatty acid length decreased the hydroxylation rates for both enzymes. Typical examples of chromatograms from the HPLC analysis are shown in Figure 6. Microsomes from mock-transformed yeast cells were devoid of or had very low ω - and (ω –1)-hydroxylase activities towards lauric acid and palmitic acid.

No hydroxylase activity towards taurochenodeoxycholic acid, the substrate for CYP4A21, could be detected in microsomes from yeast cells transformed with CYP4A24 cDNA, CYP4A25 cDNA or vector without insert.

DISCUSSION

Enzymes with high sequence similarity are difficult to separate and characterize as individual proteins. Likewise, sequences with high sequence similarity can be co-amplified using PCR as shown

in the present study. The presence of two additional sequences, besides the previously reported CYP4A21 [8], in the pig liver 1430 bp PCR product would have remained undetected if it had not been for investigations carried out to verify the aberrant amino acids, found in CYP4A21, within an otherwise conserved CYP4A motif [8,20].

Cloning of sequences with high nucleotide sequence identity by PCR could be hazardous due to premature polymerase drop off and amplification of *in vitro*-produced chimaeras. In the present paper, however, identification of two subpopulations of sequences in PCR products, generated with RNA from both liver and kidney obtained from two individuals, clearly supports the presence of two distinct CYP4A mRNAs, in addition to CYP4A21.

The CYP4A fatty acid hydroxylases are defined by their ability to favour ω -hydroxylation over $(\omega-1)$ -hydroxylation of fatty acids [4]. Lauric acid has been extensively used as a model substrate to characterize CYP4A enzymes. Using a three-dimensional model of human CYP4A11, three residues (Tyr¹²⁰, Glu³¹⁵ and Val³⁸⁶) were predicted to be the major determinants for ω -hydroxylation of lauric acid [19]. These amino acids, corresponding to Tyr¹¹⁴, Glu³⁰⁹ and Val³⁸⁰, are conserved in both CYP4A24 and CYP4A25. The two expressed enzymes show ω - and $(\omega-1)$ -hydroxylase activities towards lauric acid and also towards the longer fatty acid, palmitic acid. The $\omega/(\omega-1)$ ratios for lauric acid are comparable with the previously reported activity of a purified pig kidney ω - and $(\omega-1)$ -hydroxylase [9].

The two CYP4A enzymes reported in the present study differ by only 15 amino acid residues. The extensive sequence identity could indicate that these new CYP4A enzymes are alleles of the same gene. Indeed, the pattern of lauric acid and palmitic acid hydroxylase activities of recombinant expressed proteins is similar. However, using a secondary structure prediction program the majority of the variable amino acids were found to be confined to β -sheets 1 and 4, which are secondary structures with a potential involvement in substrate recognition and binding. All cytochrome P450s are considered to take on a similar fold structure. The β -sheet 1 is among the structurally conserved elements within cytochrome P450 enzymes which, together with β -sheet 2, is located at the entrance of the substrate access channel [21]. From the crystal structure model of CYP2C5 this β -sheet system is suggested to be buried in the hydrophobic core of the microsomal membrane, facilitating entrance of lipophilic substrates from the bilayer membrane [22]. The other β -sheet structure containing residues that differ between the two sequences (β -sheet 4) is, on the other hand, among the more variable structural elements, proposed to be involved in substrate specificity of P450s [21]. The position of the variable amino acids thus indicates a possible difference in substrate specificity or hydroxylase regioselectivity between the two enzymes. The only observed difference so far is that the expressed fatty acid hydroxylase activity of CYP4A24-transformed yeast cell microsomes is higher throughout all experiments compared with that of CYP4A25-transformed yeast cell microsomes.

Similar to the pig CYP4A sequences reported in the present study, rat CYP4A2 and CYP4A3 share a high level of sequence identity (96%) but show a somewhat more pronounced difference in lauric acid ω - to $(\omega-1)$ -hydroxylase ratios (6:1 and 3:1 respectively). Using site-directed mutagenesis, the amino acids involved in determining the ω - to $(\omega-1)$ -hydroxylase regioselectivity in these rat CYP4As have been mapped to the three-residue insert Ser¹¹⁴-Gly¹¹⁵-Ile¹¹⁶ in CYP4A3 and the residue corresponding to position 119 in CYP4A3 [23]. Interestingly, the corresponding amino acids in CYP4A24 and CYP4A25 are those residues encoded for by the nucleotides differentially

cleaved by *EcoRI*. The deduced amino acids are those positioned in β -sheet 1, strand 5 (Figure 4).

The CYP4A subfamily is presumed to have arisen through numerous species-specific gene duplication and conversion events [24,25]. This could well explain the complex picture of species-related differences in regulation and catalytic specificity found among members of the CYP4A subfamily [4]. The CYP4 family is considered to be ancient, having evolved approx. 1.25 billion years ago [4], and inter-species specialized evolution thus seems likely to have occurred. Hydroxylation of bile acids by CYP4A21 could be one example of an inter-species specialized evolution, since the pig is so far the only species known where a CYP4A enzyme plays a major role in bile acid biosynthesis. Whether this could indicate an evolutionary connection between fatty acid and cholesterol metabolism in the pig is an interesting question, which remains to be investigated.

Studies of homologous enzymes within a species are hampered by the high degree of sequence identity. Methods to differentiate between sequences are needed for future studies of these CYP4A enzymes in pig liver and kidney. From the present study, CYP4A21 seems to be the major liver CYP4A sequence captured by the primers, whereas the CYP4A fatty acid hydroxylases are more abundant in kidney.

Taken together, the present study reports on the cloning of two new forms of pig CYP4A enzymes, CYP4A24 and CYP4A25. The two cDNA sequences show exceptionally high sequence identity and encode enzymes with similar catalytic activity towards lauric acid and palmitic acid. The position of the variable amino acids indicates a possible catalytic difference between the two enzymes. Together with the previously cloned CYP4A21, these new pig CYP4A enzymes could contribute to a future understanding of some aspects concerning the structure-function relationships and evolution of the CYP4A subfamily.

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