27-Oxygenation of C₂₇-sterols and 25-hydroxylation of vitamin D₃ in kidney: cloning, structure and expression of pig kidney CYP27A

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This paper describes the molecular cloning of a cytochrome P450 enzyme in pig kidney that catalyses the hydroxylations of vitamin D₃ (cholecalciferol) and C₂₇-sterols. DNA sequence analysis of the cDNA revealed that the enzyme belongs to the CYP27 family. The first 36 amino acids have many hallmarks of a mitochondrial signal sequence. The mature pig kidney CYP27 protein contains 498 amino acids. The *M_r* of the mature protein was calculated to be 56 607. The structure of pig kidney CYP27, as deduced by DNA sequence analysis, shows 77–83 % identity with CYP27A in rat, rabbit and human liver. Transfection of the renal CYP27A cDNA into simian COS cells resulted in the synthesis of an enzyme that catalysed the 25-hydroxylation of vitamin D₃ and the 27-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol, and the further oxidation of the product into the cor-

responding C₂₇-acid 3 α ,7 α ,12 α -trihydroxy-5 β -cholestanoic acid. As part of these studies, the enzymic activities of cultured human embryonic kidney cells were examined using vitamin D₃ and C₂₇-sterols as substrates. The cells were found to express CYP27A mRNA and to convert the respective substrates into the same products as recombinantly expressed CYP27A, i.e. 25-hydroxy-vitamin D₃ and 27-oxygenated C₂₇-sterols. The results of the present study describing the structure and expression of CYP27A in kidney suggest that this enzyme is involved in the renal metabolism of vitamin D₃ and that the kidney plays a role in the metabolism of cholesterol and other C₂₇-sterols.

Key words: bile acids, cholesterol, sterol 27-hydroxylase, vitamin D hydroxylases.

INTRODUCTION

Liver mitochondrial sterol 27-hydroxylase (CYP27A) is a multifunctional cytochrome P450 that is known to catalyse the 27-oxygenation of C₂₇-steroids in the formation of bile acids. A 27-hydroxylation initiates degradation of the C₂₇-steroid side chain. The subsequent oxidation of the 27-hydroxysterol into the corresponding C₂₇-acid can also be catalysed by CYP27A [1–5]. Another important reaction carried out by hepatic CYP27A is the 25-hydroxylation of vitamin D₃ (cholecalciferol), which is the first step in the conversion of the inactive vitamin into the biologically active 1 α ,25-dihydroxyvitamin D₃ [2,6–8]. In addition, experiments with purified and recombinantly expressed liver CYP27A have shown that the enzyme is also able to catalyse the 1 α -hydroxylation step [9,10]. Recent progress in the biochemistry of 1 α ,25-dihydroxyvitamin D₃ has disclosed its physiological importance as a hormone not only in the maintenance of calcium and phosphorus homeostasis, but also in regulation of cellular differentiation [11–13].

It is well established that sterol 27-hydroxylase is obligatory for bile acid biosynthesis, which is considered to take place only in the liver [1,2]. However, the demonstration by Northern blot analysis that CYP27A mRNA is expressed in many tissues indicates a broader biological role for this enzyme [3]. Patients with the rare inherited lipid storage disease cerebrotendinous xanthomatosis have a deficient sterol 27-hydroxylase due to point mutations of the *CYP27A* gene [14–16]. Manifestations of this genetically determined CYP27A deficiency range from accelerated atherosclerosis, extensive osteoporosis and increased risk for bone fractures to progressive neurological impairment [14,17]. These findings indicate that CYP27A also has an important metabolic role in extrahepatic tissues.

Sterol 27-hydroxylase has been reported to produce oxysterols, such as 24-, 25- and 27-hydroxycholesterol, which affect the regulation of several genes [18–22]. A mechanism for the elimination of cholesterol from macrophages and endothelial cells involving the 27-hydroxylation of cholesterol and subsequent oxidation into the C₂₇-acid has been described [23]. It was proposed that this mechanism may also be utilized for elimination of cholesterol from other tissue cells. Although possible roles for CYP27A in various tissues have been suggested, there is no information available on the complete structure or expression of a functional enzyme from an extrahepatic CYP27A cDNA.

The 27-hydroxylation of cholesterol and the 25-hydroxylation of vitamin D₃ in pig kidney have so far only been characterized by enzymic analysis of partially purified enzyme fractions and by immunological methods [24–26]. The availability of a cDNA for renal CYP27A should be a prerequisite for detailed studies on the biological role of this enzyme in the kidney. A cDNA encoding CYP27A in the pig has not previously been isolated or characterized. In the present paper we report the isolation, sequence determination and expression of a cDNA encoding pig kidney CYP27A that is active in the 25-hydroxylation of vitamin D₃ and the 27-oxygenation of C₂₇-sterols.

EXPERIMENTAL

Materials

25-Hydroxy[23,24(n)-³H]vitamin D₃ (100 Ci/mmol), [α -³²P]CTP (3000 Ci/mmol), [α -³⁵S]ATP (1000 Ci/mmol), the Megaprime DNA labelling system and restriction endonucleases were purchased from Amersham. Unlabelled vitamin D₃ was from Sigma, and 25-hydroxyvitamin D₃ was obtained from Solvay (Duphar, The Netherlands). 1 α -Hydroxyvitamin D₃ was generously

Abbreviation used: RACE, rapid amplification of cDNA ends.

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Table 1 Oligonucleotide primers used in the present study

Restriction endonuclease recognition sites used for subcloning of PCR fragments are indicated in *italics* (5'-RACE anchor primer and 5'-RACE UAP, *Sal*I; PK5'-end, *Xba*I).

Name	Sequence
5'-RACE anchor primer	5'-CUACUACUACUAGGCCACGCGTGGACTAGTACGGGIIIGGGIIG-3'
5'-RACE UAP	5'-CUACUACUACUAGGCCACGCGTGGACTAGTAC-3'
PK1 (GSP1)	5'-CTCAGGAGGACTGAGCTGTCCGCTGGT-3'
PK2 (GSP2)	5'-CATGAAGTCATCGACCACCTCATT-3'
PK5'-end	5'-CGCTGC7CTAGAATGGCTGCGCTGGGCTGCGGAGGCTGCGGTGGGCGCTGC-3'

donated by Leo (Copenhagen, Denmark). 5 β -[7 β -³H]Cholestane-3 α ,7 α -diol and 5 β -[7 β -³H]cholestane-3 α ,7 α ,12 α -triol (500 mCi/mmol) were synthesized as described previously [24]. Oligo(dT)-cellulose, T3 and T7 primers and the pSVL expression vector were from Pharmacia. The Librarian⁺ cDNA construction system was from Invitrogen. Gigapack II Gold packaging extracts, λ ZAP II cloning kit and pBluescript II KS⁻ and SK⁻ were from Stratagene, and the Sequenase Version 2.0 DNA sequencing kit was from United States Biochemicals. The 5'-RACE (rapid amplification of cDNA ends) system and medium for cell cultures were obtained from Life Technologies. Nitrocellulose BA 85 disc membranes used in hybridization experiments were obtained from Schleicher & Schuell, and T4 DNA ligase was from New England Biolabs. The adrenodoxin expression vector, pBadX, was generously provided by Professor David Russell (Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). Wizard[®] DNA purification and reverse transcription systems were from Promega, and oligonucleotide primers were purchased from Scandinavian Gene Synthesis. *Taq* polymerase, deoxynucleotides and 10 $\times$ buffer IV containing 15 mM MgCl₂ used in PCR reactions were obtained from Advanced Biotechnologies. Human embryonic kidney 293 cells (ATCC CRL 1573) were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The remaining chemicals were of the highest quality available. Homology searches were performed using the NCBI database and the BLAST network service.

RNA isolation

Total RNA was isolated from 10 g of pig kidney or liver, and poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography according to standard procedures [27].

Library construction and cDNA cloning

Pig kidney cDNA was prepared from kidney poly(A)⁺ RNA according to the manufacturer's instructions (Invitrogen), ligated into λ ZAP II arms and packed to reveal the library. The pig kidney cDNA library was screened with a ³²P-labelled rabbit liver CYP27A cDNA insert (bp 8–1641) [3] by standard methods [27]. Hybridization-positive clones were purified through several rounds of screening. Subcloning into pBluescript SK⁻ was then performed.

DNA sequence analysis

Restriction fragments derived from the hybridization-positive clones were isolated and cloned into pBluescript SK⁻ or pBluescript II KS⁻. The DNA sequence was generated directly from the double-stranded plasmids by the chain-termination method, and compression was resolved using 7-deaza-dGTP and

dITP. Electrophoresis was performed in 6% (w/v) polyacrylamide gels containing 7 M urea.

Isolation of the 5'-end of the CYP27 cDNA

Attempts to isolate the cDNA sequence encoding the mitochondrial leader sequence were performed using the 5'-RACE system according to the protocol supplied by the manufacturer (Life Technologies). The PK1 primer and pig kidney poly(A)⁺ RNA were used for first-strand synthesis, and primer PK2 as a nested primer together with the 5'-RACE anchor or UAP primer for PCR amplification (Table 1). Amplified fragments were subjected to digestion with *Sal*I and *Kpn*I and cloned into the pBluescript KS⁻ vector. PCR amplifications were carried out using an MJ Research minicycler. The reaction mixture contained first-strand cDNA obtained with the 5'-RACE system, 5 μ l of each primer (10 μ M) dissolved in water, 1 μ l of each of dATP, dCTP, dGTP and dTTP (200 μ M), and 4.5 μ l of 10 $\times$ reaction buffer containing 15 mM MgCl₂ in a total volume of 45 μ l. The reaction was overlaid with 2 drops of mineral oil in order to prevent evaporation and was kept at 95 °C for 5 min, followed by 80 °C for 3 min. During the latter period the reaction was initiated by the addition of 1 unit of *Taq* polymerase in 5 μ l of reaction buffer. The first two cycles were carried out at 94 °C for 10 min, 50 °C for 1 min and 72 °C for 2 min, followed by 38 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. The last cycle was followed by a 10 min period at 72 °C, and then the temperature was kept constant at 4 °C. The fragment containing the entire coding region for the mitochondrial signal sequence was amplified using the 5'-RACE fragment as template together with the PK5'-end and PK2 primers (Table 1), as described above. The amplified fragment (fragment 1; see Figure 2) was cloned into the pBluescript KS⁻ vector after digestion with *Xba*I and *Kpn*I.

Northern blot analysis

Analysis of CYP27 transcripts was carried out following electrophoresis of poly(A)⁺ RNA on denaturing 1% agarose gels containing formaldehyde. Following transfer of the RNA to a nitrocellulose filter, hybridization was carried out at 42 °C for 16 h with a ³²P-labelled 1746 bp pig kidney cDNA fragment in hybridization buffer containing 50% formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 1 $\times$ PE (50 mM Tris/HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA and 0.2% BSA), 100 μ g/ml salmon sperm DNA and 100 μ g/ml tRNA. After hybridization the filter was washed in 2 $\times$ SSC containing 0.1% SDS at room temperature, followed by washing at 55 °C for 2 h in 0.1 $\times$ SSC containing 0.1% SDS, and then used to expose Fuji RX film in the presence of an intensifying

screen for 12 h at -70°C . The filter was then stripped and probed with a 1.7 kb *Bam*HI–*Sac*I fragment of human β -actin cDNA. In Northern blot experiments with total RNA from human embryonic kidney 293 cells, a ^{32}P -labelled human liver CYP27A cDNA fragment was used [5].

Southern blot analysis

Pig genomic DNA (Clontech) was digested with *Eco*RI, *Sac*I, *Bgl*II or *Eco*RV, and 10 μg of DNA from each digestion was electrophoresed in agarose, transferred to a nylon membrane, and probed with the ^{32}P -labelled 1746 bp pig kidney CYP27A cDNA [3,6].

Construction of pig kidney CYP27 expression vector

Fragment 1 containing the nucleotide sequence encoding the entire mitochondrial signal sequence in pBluescript KS[−] and clone 1 in pBluescript SK[−] (see Figure 2) were subjected to digestion with *Xba*I/*Kpn*I and *Kpn*I/*Bam*HI respectively. A 472 bp *Xba*I/*Kpn*I fragment and a 1398 bp *Kpn*I/*Bam*HI fragment were isolated and ligated into the pSVL vector digested with *Xba*I and *Bam*HI to generate the expression plasmid pSVL-PK27.

Transfection in COS-M6 cells

COS-M6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics. The cells were transfected with the pSVL expression vector containing pig kidney CYP27 cDNA [9]. Substrates, dissolved in 10 μl of DMSO (0.3% final concentration), were added to 5×10^5 cells in each experiment and incubated for 48 h prior to analysis of products in the cell medium. The substrates were: 10 μg of ^3H -labelled 5 β -cholestane-3 α ,7 α -diol, 10 μg of ^3H -labelled 5 β -cholestane-3 α ,7 α ,12 α -triol, 10 μg of vitamin D₃ and 10 μg of 1 α -hydroxyvitamin D₃. Incubations with 5 β -cholestane-3 α ,7 α -diol and 5 β -cholestane-3 α ,7 α ,12 α -triol were extracted with acidified ether [28], and those containing vitamin D₃ and 1 α -hydroxyvitamin D₃ were extracted with trichloroethane/methanol (2:1, v/v), as described [24,25]. Incubations with 5 β -cholestane-3 α ,7 α -diol and 5 β -cholestane-3 α ,7 α ,12 α -triol were analysed by TLC and radioactivity scanning [28]. Incubations with vitamin D₃ and 1 α -hydroxyvitamin D₃ were analysed by straight-phase and reverse-phase HPLC, as described [24,25].

Cultures of human embryonic kidney cells

Human embryonic kidney cells (293 cells) were seeded at 7.5×10^5 cells per 60 mm tissue culture dish in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics. Enzymic activities in the cells were examined by addition of the respective substrates [vitamin D₃ (10 μg), 1 α -hydroxyvitamin D₃ (10 μg) or ^3H -labelled 5 β -cholestane-3 α ,7 α -diol (20 μg), dissolved in 10 μl of DMSO (0.3% final concentration)] to the medium and incubation for 24 or 72 h. Following incubation with substrate, the medium was collected and extracted with trichloroethane/methanol (2:1, v/v) or acidified ether, and the organic phase was analysed for hydroxylated metabolites.

Other methods

Adrenodoxin and adrenodoxin reductase were prepared and protein determination was performed as described previously [28].

RESULTS

cDNA cloning

Screening of 5×10^5 plaques of a pig kidney λ ZAPII cDNA library with a ^{32}P -labelled rabbit liver CYP27A cDNA insert (bp 8–1641) [3] resulted in five positive clones, which were purified through several rounds of screening. *In vivo* excision to form the pBluescript SK[−] plasmid revealed the plasmid to contain cDNA inserts varying in size between ≈ 1000 bp and 1746 bp. Attempts to isolate the 5'-end of the cDNA were performed using the 5'-RACE system. Pig kidney poly(A)⁺ RNA and the PK1 primer were used for first-strand synthesis. Primer PK2 was then used as a nested primer together with the 5'-RACE anchor or UAP primer (see Table 1) for PCR amplification. The longest fragment isolated (5'-RACE; see Figure 2) was, however, missing the first 22 bp of the mitochondrial leader sequence. The 5'-end of the pig kidney CYP27 cDNA containing the entire coding region of the mitochondrial leader sequence was amplified by

Figure 1 Northern blot analysis of RNA isolated from pig kidney and liver (A) and human embryonic kidney cells (B)

(A) Poly(A)⁺ RNA was isolated from kidney and liver by the guanidine-HCl procedure followed by oligo(dT) chromatography. Portions of 10 μg of kidney mRNA (lane 1) and 5 μg of liver mRNA (lane 2) were electrophoresed on a 1% (w/v) agarose gel, blotted and hybridized with pig kidney CYP27A cDNA by standard methods. After autoradiography for 12 h the filter was stripped and hybridized with human β -actin cDNA. (B) A portion of 30 μg of total RNA isolated from human embryonic kidney 293 cells was subjected to the same procedure as in (A), with the exception that a human CYP27A cDNA [5] was used as the labelled probe.

Figure 2 Restriction map and sequencing strategy for the pig kidney CYP27 cDNA and fragments

Arrows indicate the direction and extent of sequencing.

Figure 3 For legend see facing page.

Figure 4 Southern blot analysis

Pig genomic DNA was digested with *EcoRI*, *SacI*, *BglII* or *EcoRV*, and 10 µg portions of digested DNA were subjected to Southern blot analysis, as described in the Experimental section, using pig kidney CYP27A cDNA as probe.

Figure 3 shows the nucleotide and deduced amino acid sequence of the pig kidney CYP27 cDNA, as determined by analysis of the cDNA inserts in the clones and the 5'-RACE fragment. The first 36 amino acids (residues -36 to -1) of the cDNA sequence have many hallmarks of a mitochondrial signal sequence. The mature pig kidney CYP27 contains 498 amino acids and has an overall hydrophobic nature. The M_r of the mature protein was calculated as 56607, which is slightly greater than that estimated by SDS/PAGE (53000). The haem-liganded cysteine that is conserved in all cytochrome P450 proteins occurs at residue 443. A short 3'-untranslated region of 265 bp was present in both clones and contains a consensus AATAAA sequence for polyadenylation (Figure 3).

The pig kidney CYP27 cDNA shares high similarity with reported sequences of liver CYP27A enzymes. Alignment indicated 79% sequence similarity with that from human [5], 78% similarity with that from rabbit [3] and 71% similarity with that from rat [6,7]. The amino acid sequence displays 83%, 81% and 77% similarity with human, rabbit and rat CYP27A respectively [3,5–7]. The cloned human CYP27A gene contains nine exons and eight introns [16]. The junctions between the exons and introns in the human gene have all been identified in the kidney cDNA, as well as the conserved regions around the haem-liganded cysteine (residues 435–456) and residues 337–365, which may form part of the ferredoxin binding domain [29].

Genomic Southern blot analysis

It has been reported previously that CYP27A appears to be encoded by a single gene in rabbit [3] and rat [6]. To estimate the number of CYP27A genes in the pig genome, we carried out Southern blotting experiments. Pig genomic DNA was digested separately with four restriction enzymes and electrophoresed, transferred to nylon membranes and hybridized with the probe employed in Northern blotting experiments, as described in the Experimental section. As demonstrated in Figure 4, a small number of hybridizing bands was visualized in each lane of the gel. The restriction enzymes *EcoRI* and *SacI*, which do not cut

Table 2 Hydroxylase activities in COS-M6 cells transfected with pig kidney CYP27A cDNA

Transfection experiments were performed as described in the Experimental section. As negative controls, COS-M6 cells were transfected with the pSVL vector containing no insert. Unlabelled vitamin D₃ or 1α-hydroxyvitamin D₃, or ³H-labelled 5β-cholestane-3α,7α-diol or 5β-cholestane-3α,7α,12α-triol (8 µM of each substrate), was added to the culture medium containing approx. 5 × 10⁵ cells in each experiment. On day 2 after substrate addition, the medium was removed for assay of product formation and the cells were harvested for protein determination. Product formation is expressed as pmol/mg of protein. The results from four experiments are given as means ± S.D. The amount of substrate converted into product during the incubation was approx. 0.5% for vitamin D₃, 1.5% for 1α-hydroxyvitamin D₃, 9% for 5β-cholestane-3α,7α-diol and 90% for 5β-cholestane-3α,7α,12α-triol.

Reaction measured	Product formation (pmol/mg of protein)	
	pSVL	pSVL-PK27
Vitamin D ₃ 25-hydroxylation	< 2	32 ± 8
1α-Hydroxyvitamin D ₃ 25-hydroxylation	< 2	105 ± 11
5β-Cholestane-3α,7α-diol 27-hydroxylation	< 10	700 ± 81
5β-Cholestane-3α,7α,12α-triol 27-Hydroxylation	< 10	5705 ± 419
Oxidation to acid	< 10	1925 ± 888

Table 3 Endogenous hydroxylase activities in human embryonic kidney cells

Human embryonic kidney 293 cells were cultured and enzyme activities were assayed as described in the Experimental section. The amount of substrate added was 8 µM (vitamin D₃ and 1α-hydroxyvitamin D₃) or 16 µM (5β-cholestane-3α,7α-diol). Product formation is expressed as pmol/mg of protein. The results from three experiments are given as means ± S.D. The amount of substrate converted into product during the incubation was approx. 2% for vitamin D₃, 3% (24 h) and 10% (72 h) for 1α-hydroxyvitamin D₃, and 16% (24 h) and 70% (72 h) for 5β-cholestane-3α,7α-diol. ND, not determined.

Reaction measured	Product formation (pmol/mg of protein)	
	24 h	72 h
Vitamin D ₃ 25-hydroxylation	220 ± 39	ND
1α-Hydroxyvitamin D ₃ 25-hydroxylation	350 ± 130	1000 ± 325
5β-Cholestane-3α,7α-diol 27-hydroxylation*	2000 ± 644	8600 ± 850

* Analysis of 72 h incubations by TLC also revealed another polar product with an R_f value corresponding to that of 3α,7α-dihydroxy-5β-cholestanoic acid.

the cDNA, yielded only single bands. *BglII* and *EcoRV*, which do cut the cDNA, yielded two to three bands. These results suggest the presence of a single gene for CYP27A in the pig.

PCR experiments with RNA from pig kidney and liver

Since the structure of pig liver CYP27A cDNA is not known, we carried out PCR experiments with RNA from kidney and liver in order to compare sequences of CYP27A in the two tissues. First-strand cDNA was synthesized using the random primer method (Reverse Transcription System; Promega). Primers hybridizing to nucleotides 614–633 (forward primer) and 1427–1447 (reverse primer) in the pig kidney CYP27A cDNA (cf. Figure 3) were then used in the PCR experiments. A DNA fragment of the same size, 833 bp, was amplified from the cDNA of both liver and kidney. The nucleotide sequence of the liver and kidney fragments was the same. Taken together, the results from the Southern blotting and PCR experiments strongly indicate that CYP27A in kidney and liver is encoded by a single gene in the pig.

Expression of the pig kidney CYP27A cDNA in COS-M6 cells

To confirm that the sequence shown in Figure 3 encodes a functional enzyme, we expressed the cDNA in simian COS-M6 cells. A DNA fragment corresponding to nucleotides 22–1848 (see Figure 2) was ligated into the eukaryotic expression vector pSVL to generate the pSVL-PK27 vector. The expression vector was transfected, together with the pBadX plasmid encoding bovine adrenodoxin, into COS cells by the DEAE-dextran method [9]. As control, COS cells transfected with vector containing no insert were used. Table 2 shows that COS cells transfected with pSVL-PK27 catalysed the 25-hydroxylation of vitamin D₃ and 1 α -hydroxyvitamin D₃ and the 27-hydroxylation of 5 β -cholestane-3 α ,7 α -diol and 5 β -cholestane-3 α ,7 α ,12 α -triol, whereas the mock-transfected cells did not. The transfected cells also catalysed the further oxidation of 5 β -cholestane-3 α ,7 α ,12 α ,27-tetrol into its corresponding C₂₇-acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholestanoic acid.

27-Oxygenation of C₂₇-sterols and 25-hydroxylation of vitamin D₃ by human embryonic kidney cells

In order to study the catalytic activities towards vitamin D₃ and C₂₇-sterols in a kidney cell line, we examined the 27-hydroxylase and 25-hydroxylase activities in human embryonic kidney cells (293 cells). The cultured cells converted vitamin D₃, 1 α -hydroxyvitamin D₃ and 5 β -cholestane-3 α ,7 α -diol into the same products as the recombinantly expressed kidney CYP27A, i.e. 25-hydroxyvitamin D₃, 1 α ,25-dihydroxyvitamin D₃ and 27-oxygenated C₂₇-sterol respectively (Table 3).

Northern blot experiments showed that the cells expressed CYP27A mRNA (Figure 1B).

DISCUSSION

The results of the present study, describing the molecular cloning, structure and functional expression of pig kidney CYP27A, imply that CYP27A has a biological role in kidney in the metabolism of vitamin D₃ and C₂₇-sterols.

Renal metabolism of vitamin D₃

The recombinantly expressed kidney CYP27A catalysed the 25-hydroxylation of vitamin D₃, suggesting that the enzyme is involved in the formation of 25-hydroxyvitamin D₃ in kidney. Although the liver is regarded as the most significant source of 25-hydroxyvitamin D₃, several previous reports indicate that the liver is not the sole site for this 25-hydroxylation and that substantial activity can be shown to occur in other tissues, such as kidney [25,30–34]. Measurement of the concentrations of vitamin D₃ in various tissues has shown that vitamin D₃ is present in kidney in almost the same concentration as that found in liver [35]. The enzyme(s) responsible for the formation of 25-hydroxyvitamin D₃ in kidney has, however, not been characterized. Mitochondrial CYP27A is known to be a vitamin D₃ 25-hydroxylase in mammalian liver [2]. The present study, demonstrating the presence in kidney of CYP27A that has the capacity to convert vitamin D₃ into 25-hydroxyvitamin D₃, supports a role for the kidney in the production of 25-hydroxyvitamin D₃. The finding that human embryonic kidney cells express CYP27A mRNA and endogenous activity resulting in the formation of 25-hydroxyvitamin D₃ lends further support to the contention that this metabolite is formed in kidney. Since kidney mitochondria contain both the 25-hydroxylating CYP27A and 1 α -hydroxylases [36–40] that convert 25-hydroxyvitamin D₃ into 1 α ,25-dihydroxyvitamin D₃, the kidney would then be

capable of the complete conversion of vitamin D₃ into its hormonal form.

The role of hepatic CYP27A as a physiological vitamin D₃ 25-hydroxylase has been discussed [14,17,41,42]. Berginer et al. [17] found that extensive osteoporosis and increased risk of bone fractures occurred in some patients with cerebrotendinous xanthomatosis, caused by an anomaly of the *CYP27A* gene. The serum levels of some vitamin D₃ metabolites, such as 25-hydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃, were lower than normal in these patients [17]. Okuda et al. [42] have suggested that osteoporosis in these patients may be explained by low or null activity of the mitochondrial vitamin D₃ 25-hydroxylase, owing to the abnormal enzyme formed from the defective *CYP27A* gene. Other authors have questioned the role of CYP27A as a vitamin D₃ 25-hydroxylase because of the finding that patients with cerebrotendinous xanthomatosis, lacking the CYP27A enzyme, and mice with a disrupted *CYP27A* gene do not have markedly reduced circulating 25-hydroxyvitamin D₃ levels [14,41]. Although these findings argue against an important role for CYP27A in vitamin D₃ metabolism, these data do not rule out the possible function of CYP27A as a 25-hydroxylase. The lack of effect on the levels of circulating vitamin D₃ metabolites may, for example, be due to a compensatory increased activity of the microsomal vitamin D₃ 25-hydroxylase [43] under these pathological and unphysiological conditions. The results of a recent study on vitamin D₃ hydroxylation in piglets suffering from rickets suggest that CYP27A is important for 25-hydroxyvitamin D₃ production, at least in the pig [44].

The regulated 1 α -hydroxylation step is apparently catalysed by several cytochrome P450 enzymes in the kidney [36–40]. Studies with purified and recombinantly expressed liver CYP27A have shown that it catalyses 1 α -hydroxylation [9,10], and experiments with partially purified pig kidney CYP27A have indicated that this enzyme plays a role as a renal mitochondrial 1 α -hydroxylase [45]. In the present study, the rate of 1 α -hydroxylation catalysed by the recombinantly expressed pig kidney CYP27A was below the limit of detection with the methods used. However, considering the similarities between kidney CYP27A and liver CYP27A in structure and other catalytic properties examined, it appears possible that the kidney CYP27A has the capacity to catalyse 1 α -hydroxylation.

Renal metabolism of C₂₇-sterols

The recombinantly expressed kidney CYP27A enzyme also carried out the 27-hydroxylation of C₂₇-sterols and the further oxidation into C₂₇-acids. Cultured human embryonic kidney cells were found to express CYP27A mRNA and endogenous enzyme activity resulting in the formation of the same metabolites as those produced by the recombinantly expressed renal CYP27A. These findings, together with previous work describing partially purified 27-hydroxylase from kidney [24,26], indicate that the kidney may play a hitherto unrecognized role in the metabolism of C₂₇-sterols.

A possible physiological role for CYP27A in sterol metabolism in the kidney could be the formation of oxysterols, such as 27-hydroxycholesterol, which appears to regulate cholesterol homeostasis in several ways [18,19,23,46,47]. Liver mitochondrial CYP27A is known to carry out the 27-oxygenation of cholesterol and other C₂₇-sterols, such as 5 β -cholestane-3 α ,7 α ,12 α -triol. The rate of 27-oxygenation is about 50 times lower with cholesterol than with 5 β -cholestane-3 α ,7 α ,12 α -triol [10,28]. The recombinantly expressed kidney CYP27A used in the present study catalysed the 27-oxygenation of 5 β -cholestane-3 α ,7 α ,12 α -triol into 5 β -cholestane-3 α ,7 α ,12 α ,27-tetrol and 3 α ,7 α ,

12 α -trihydroxy-5 β -cholestanic acid. Although the rate of 27-hydroxylation of cholesterol was below the limit of detection, it seems likely that CYP27A in kidney is capable of the 27-oxygenation of cholesterol, and may be involved in the elimination of cholesterol [23].

The formation of bile acids is considered to take place only in the liver. It is surprising that, in spite of this contention, several enzymes involved in the biosynthesis and metabolism of bile acids are expressed in kidney. In a study on oxysterol 7 α -hydroxylase, Schwartz et al. [48] found that 25-hydroxycholesterol was 7 α -hydroxylated by human embryonic kidney cells. Work in our laboratory has shown that 27-hydroxycholesterol can also be 7 α -hydroxylated by this cell line (M. Norlin and K. Wikvall, unpublished work). Pedersen et al. [49] reported the molecular cloning of a 3 α ,7 α ,12 α -trihydroxy-5 β -cholestanoyl-CoA oxidase from rabbit liver peroxisomes. This enzyme catalyses the further oxidative cleavage of the steroid side chain after the formation of the C₂₇-acid. The enzyme was also found to be expressed in kidney. The authors discussed the possibility that small amounts of cholesterol hydroxylated in the 27-position in other organs may eventually be further oxidized and chain-shortened in the kidney. The results of the present study show that CYP27A in kidney can produce the substrate for 3 α ,7 α ,12 α -trihydroxy-5 β -cholestanoyl-CoA oxidase locally. Bile acid-CoA synthetase and bile acid-CoA:amino acid *N*-acyltransferase are two enzymes that catalyse the conjugation of bile acids with either taurine or glycine in the liver. Kwakye et al. [50,51] demonstrated the presence of these enzyme activities in rat kidney. The authors suggested that the kidney has the potential for bile acid metabolism, and may have a role in bile acid physiology. Taken together, these findings indicate a role for the kidneys in cholesterol metabolism. Since enzymes catalysing the 27-oxygenation, 7 α -hydroxylation and side-chain oxidation of cholesterol and cholesterol metabolites are expressed in kidney, the results in fact show that the kidney has the capacity for the formation and conjugation of bile acids.

In conclusion, the results of the characterization of pig kidney CYP27A described in the present study strongly indicate that CYP27A is involved in the renal metabolism of vitamin D₃, and that the kidney plays a role in the metabolism of cholesterol and other C₂₇-sterols.

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