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Molecular cloning and expression of guinea pig cytochrome P450c21 cDNA (steroid 21-hydroxylase) isolated from the adrenals☆

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Abstract

In mammals, the P450c21 enzyme mediates 21-hydroxylase activity by transforming progesterone and 17-hydroxyprogesterone into deoxycorticosterone (DOC) and 11-deoxycortisol (11-DOC), respectively. Previous studies have shown that among the adrenal steroid hydroxylase enzymes involved in C19 steroid and glucocorticoid syntheses, P450c21 plays an important role, because it is localized at the key branch between glucocorticoids and C19 steroid production. Its implication in congenital adrenal hyperplasia is also of great clinical interest. In this study, in addition to describing the isolation of the P450c21 cDNA from guinea pig (GP) adrenal and comparing it to those from other species, we report on its tissue-distribution and on the activity of the recombinant protein towards progesterone and 17-hydroxyprogesterone. The guinea pig P450c21 includes the full-length coding region (1464 nucleotide) that is translated to a protein of 488 amino acids. The clone shares highly conserved regions with other species. The guinea pig P450c21 cDNA hybridized with a major transcript of 2.1 kb and with two minor related transcripts of 1.8 and 1.5 kb and was found to be adrenal-specific among the various tissues analyzed. Characterization of the enzymatic activity by transient transfection of the guinea pig P450c21 cDNA in human embryonic kidney 293 cells indicated a net preference for the 21-hydroxylation of 17-hydroxyprogesterone in comparison to the progesterone substrate. Assays showed a maximum conversion rate of 12.5% for the conversion of progesterone into deoxycorticosterone (mineralocorticoid pathway), whereas the guinea pig P450c21 demonstrated a higher activity with 17α-hydroxyprogesterone, with 55% of 11-deoxycortisol formation (glucocorticoid pathway) after 48 h. Adrenocorticotropin and an analogue of the second messenger cyclic adenosine monophosphate specifically increased the abundance of P450c21 mRNA levels in guinea pig adrenal cells.

Keywords: Guinea pig; 21-Hydroxylase; P450c21; Adrenals; Progesterone; 17α-Hydroxyprogesterone; Glucocorticoids

1. Introduction

Steroid synthesis is a complex process involving cholesterol as a parent compound from which all different steroid classes are produced. Steroid transformations are mediated by enzymes from the cytochrome P450 family, which are membrane-bound, heme-containing proteins serving as terminal oxidases in electron-transfer chains originating with NADPH [1], or the hydroxysteroid dehydrogenase superfamily. In several mammalian species including human, primate, bovine, porcine and guinea pig (GP), the predominant glucocorticoid cortisol and mineralocorticoid aldosterone are synthetized by the adrenal cortex and are characterized by the presence of a hydroxyl group at position C21 of the steroid ring structure. Hydroxylation at position C21 of progesterone and of 17-hydroxyprogesterone, which gives rise to deoxycorticosterone (DOC) and to 11-deoxycortisol (11-DOC), respectively, is catalyzed by the microsomal steroid 21-hydroxylase cytochrome P450 enzyme (P450c21, steroid monooxygenase, EC 1.14.99.10).

Partial or complete 21-hydroxylase deficiency activity in human is of great clinical interest and is generally called congenital adrenal hyperplasia (CAH), which brings minor to severe symptoms associated with a low cortisol production, and endogenous stimulation of ACTH secretion. This condition is also associated with an increased adrenal C19 androgen synthesis [2].

Two linked genes for P450c21 are present in rodent [3] and human [4] genomes and lie in humans within an approximately 100 kb duplication of the Class III HLA major histocompatibility complex on chromosome 6p21.1 [5]. The

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two genes in human (CYP 21A1P, a pseudogene and CYP 21A1) are generally referred to as 21A and 21B and are duplicated in tandem with the fourth component of serum complement gene C4 [6], and with the extracellular matrix protein gene tenascin-X [7]. All this region is duplicated and the array in human is 5′-C4A/21A-XA/C4B/21B-XB-3′ [8]. In mice, the 21A gene encodes the functional enzyme and begins at 3′ of the nonhemolytic homologue of C4, termed sex-limited protein (slp). The 3′ end of this region contains the *21B* gene (pseudogene). The array in mice is 5′-C4(slp)/21A/C4/21B-3′ [3]. These genes have a 5′ to 3′ transcriptional orientation except for the human tenascin-X gene, for which transcription occurs in the opposite direction [5,7]. According to genomic analysis, Northern studies in mice and humans indicate the presence in the adrenal of a unique 2 kb P450c21 mRNA species. Although, in the bovine genome, only one of the *CYP21* gene is functional, analysis of the bovine adrenocortical polyadenylated RNA reveals two bands of about 2.2 and 2.4 kb in length, which suggests that additional splicing events are responsible for these mRNAs [9–11]. The various mutations responsible for congenital adrenal hyperplasia occur through several mechanisms involving gene conversion, unequal cross-over and recombination between the active *21B* gene and portions of the 21A pseudogene [12–14].

Adrenocortical cells in culture have served as a useful model to study the mechanisms underlying the actions of ACTH on adrenal steroidogenesis. In the guinea pig, we showed the presence of enzymes producing cortisol and C19 steroids [15] and identified androstenedione and its 11β-hydroxylated derivative as the main C19 steroids produced by the adrenal in response to ACTH [16,17]. Because the guinea pig is a good animal model for in vivo and in vitro investigation of the mechanisms by which ACTH regulate 21-hydroxylase activity, we have isolated from guinea pig adrenals a full-length cDNA encoding P450c21, which consists of an open reading frame of 488 amino acids. In this study, in addition to describing the isolated P450c21 cDNA and comparing it to those form other species, we report on its tissue-distribution and on the activity of the recombinant protein towards progesterone and 17-hydroxyprogesterone. We also describe the regulation of the guinea pig P450c21 enzyme by ACTH and cAMP.

2. Materials and methods

2.1. cDNA synthesis and library construction

A cDNA library from guinea pig (GP) adrenals was constructed with 5 μg of purified poly(A)$^+$ RNA using the superscript λ directional cDNA synthesis (BRL Life Technologies, Gaithersburg, MD). The first cDNA strand was synthesized using a Mo-MLV-RT enzyme that no longer has RNase H activity in the presence of a Not I-Oligo (dT_{15}) primer-adapter. The second strand was synthesized by *E. coli* DNA polymerase I in the presence of *E. coli* RNase H and *E. coli* DNA ligase. Afterward, the cDNAs were blunt-ended, ligated to Sal I adapter and digested with Not I. Molecules longer than 1 kb were size-selected by chromatography on Sepharyl S500 HR columns and, finally cDNAs were ligated into Sal I–Not I cloning sites of the phage λgt22. The recombinant phage DNA was packaged and propagated in *E. coli* Y1090(r−). The library contained 4.75×10^6 pfu/ml of primary recombinants and was amplified to 2×10^8 pfu/ml for screening. The average length of cDNA recombinants determined by Sal I–Not I digestion of λ DNA was up to 1.5 kb. The second library was constructed with a 3′-specific 18 nucleotides Xba I/Not I primer adapter (5′-gtt-cta-gag-cgg-ccg-ccc-ggg-ata-atc-act-gtg-tcc-ttg-ggg-3′) localized 800 bp downstream from the 5′ beginning GP272-1 clone.

2.2. Screening, analysis and sequencing of cDNA clones

Amplified recombinant phages were plated at 2.5×10^4 pfu/15 cm plate. Plaque screening was first done using purified 1.2 kb fragment of human P450c21 cDNA [18]. Hybridization was performed at 42 °C in 50% formamide, 5× Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin), 5× Standard Saline Citrate (SSC IX; NaCl 0.15 M, sodium citrate 0.015 M), 0.1% sodium dodecyl sulfate, 200 μg/ml sonicated salmon sperm DNA. Filters were washed at 65 °C in 0.1× SSC and 0.1% SDS. DNA from plaque-purified phage was mapped by restriction endonuclease cleavage with Sal I–Not I, analyzed on ethidium bromide-stained agarose gel for sizing cDNA inserts, and analyzed by Southern blot using hP450c21 cDNA as probe. Inserts from putative λ clones were purified on agarose gel and subdoned into Sal I/Not I digested pSV-SPORT-1 (BRL Life Technologies, Gaithersburg, MD) for sequencing with the T7 Sequencing Kit (Pharmacia Biotech, Montreal, QC). Screening the first cDNA library with the human probe yielded only partial clones. The longest clone (GP272-1) missed about 330 nucleotides in its 5′ coding sequence. The missing segment was obtained by rescreening these libraries with the GP272-1 3′ partial clone. This second screening yielded about 300 clones. Hybridization of these clones with a 445 bp fragment that fits with the most 5′-coding part of the hP450c21 cDNA permitted to isolate 15 overlapping putative positives. The 1F 5′-partial clone was used for the fusion with the GP272-1 3′-partial clone to obtain the full-length guinea pig P450c21 cDNA.

2.3. Messenger RNA preparation and analysis

Total RNA from guinea pig tissues and adrenal cells were isolated by homogenization in 4 M guanidium thiocyanate, centrifuged through a 5.7 M CsCl cushion [19] and fractionated by electrophoresis [20]. Northern blots were performed with 20 μg of total RNA, and hybridization was done under high-stringency conditions [21]. Northern blots were probed with purified GP P450c21/*Sma* I cDNA

coding sequence purified with QIAEX (Qiagen, Chatsworth, CA). A rat 18S ribosomal oligodeoxyribonucleotide probe (5′-ccg-cat-gta-tta-gct-cta-gaa-tta-cca-cag-3′) [22] was used as an internal control as described previously [23].

2.4. Preparation of dispersed GP fasciculata/glomerulosa (FG) cells

Primary cultures of FG cells were prepared as reported [24]. Cells were plated at 1.25×10^6 cells/ml in MEM supplemented with 12% (v/v) dextran-coated charcoal-treated FCS, 10^5 U/l of penicillin, 50 mg/ml of streptomycin and 2.2 g/l of $NaHCO_3$ (MEMS) for measuring RNA levels. Twenty-four hours after plating, the medium was changed and 10 nM ACTH (synthetic tetracosapeptide $ACTH_{1–24}$ cortrosyn) was added for 24 h. At the end, cells were resuspended in guanidium thiocyanate and total RNA isolated and analyzed by Northern blots. Autoradiograms from Northern blots were densitometrically scanned, and the abundance of P450c21 mRNA relative to control value arbitrarily fixed at 1.0 was reported.

2.5. Transient expression of GP P450c21 in HEK293 cells

The guinea pig P450c21 cDNA was subcloned in pcDNA3 expression vector (Invitrogen, Carlsbad, CA) by ligating the entire cDNA fragrnent with Sal I–Not I restriction endonucleases to produce the recombinant plasmid pcDNA3-gpCYP21 transiently expressed in human embryonic kidney cells (HEK293) obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% dextran-coated, charcoal-treated foetal calf serum. HEK293 cells were plated at an initial density of 2×10^5 cells in 6-well plates and no 21-hydroxylase activity in intact cells was detected. Transient transfections were performed in triplicate using Exgen 500 protocol (Euromedex, France). Each assay received 2 μg of plasmid expressing guinea pig P450c21 and 1 or 2 μg of Green Fluorescent Protein (GE-GFP-C1) (Clontech, Palo Alto, CA) used as a control of transfection efficiency. Catalytic activities of the recombinant P450c21 enzyme were carried out by incubating intact HEK293 cells with tritiated HPLC-purified [1,2,6,7-^{3}H]progesterone (1 μCi per assay, 94.0 Ci/mmol) or 17α-hydroxy [1,2,6,7-^{3}H]progesterone (1 μCi per assay, 77.0 Ci/mmol) (Amersham, Oakville, Canada). For the determination of enzymatic activities, tritiated steroids were incubated in intact cells for 24 and 48 h. The enzymatic reaction was stopped by adding 0.05 ml of 0.5 N CH_3COOH and unconjugated steroids were extracted from medium with diethyl ether, applied on silica gel-coated thin-layer chromatography (TLC) and developed with hexane:ethyl acetate 4:7 (v/v). Radioactivity was quantified with a Berthold digital autoradiograph (Weldbrod, Germany) coupled with a Dar Signal Analyzer Imaging System (San Diego, CA). The substrates and newly produced steroids were determined by comigration on each TLC with HPLC authentic purified labelled steroids.

3. Results

3.1. Cloning, sequencing and analysis of cDNA

The comparison of amino acid sequence of guinea pig P450c21 with that of human [4], canine [25], bovine [26], porcine [27], ovine [28] and murine [3] is shown in Fig. 1. The guinea pig P450c21 shares all the consensus sequence described, the NH_2-signal anchor (residues 1–15) and the heme binding site (FXXGXXXCXG). The guinea pig amino acid sequence also shares protein kinase C phosphorylation sites (ST-x-RK: residues 94–96; 364–366), a cAMP-dependant protein kinase phosphorylation site (residues 118–121) and a N-glycosylation site (residues 343–346). Two additional putative protein kinase C sites are found in the guinea pig amino acid sequence at residues 14–16 and 25–27. As for all other P450 enzymes, the guinea pig heme-binding site is also presumed to be centred on a cysteine residue at position 223. The percentage of identity of guinea pig P450c21 based on amino acid sequence (see Fig. 2), calculated in Table 1, is greatest with human, followed by the canine, bovine, porcine, ovine and murine species P450c21.

We denoted five highly conserved regions among the amino acid sequence among different species. The region comprised between amino acids 104 and 128 (region II; 25 residues) is conserved in an average of 92.7% and includes the cAMP phosphorylation site consensus sequence. The consensus for this region is D[L/I]SLGDYSLXWKA-HKKL[T/s]RSAL[L/M]LG. The NH_2-signal anchor sequence is conserved in an average of 68.6% and the heme-binding region in an average of 84.3%. The highly conserved region IV includes the N-glycosylation site, which we found an exclusive sequence for the guinea pig between amino acids 304–308. The guinea pig sequence is CCTI, whereas the other species share the sequence LLHH at these positions.

3.2. Tissue-distribution of the guinea pig P450c21

We examined the presence of gpP450c21 mRNA in several tissues by Northern blot hybridization using the gpP450c21/*Sma* I cDNA fragment as a probe. Analysis of the guinea pig adrenals (Fig. 3), reveals a major band of 2.1 kb, and two additional related minor bands at 1.8 and 1.5 kb. To ensure whether such P450c21 mRNAs were also present in other tissues, total RNA was extracted from adult male guinea pigs from heart, lung, liver, testis, brain, spleen and kidney and were also analyzed. Northern results (Fig. 3) shows that only the adrenals express the P450c21 in the guinea pig. Hybridization of the same blot with the rat 18S ribosomal oligodeoxyribonucleotide probe did not reveal any significant variation in loading RNA (data not shown).

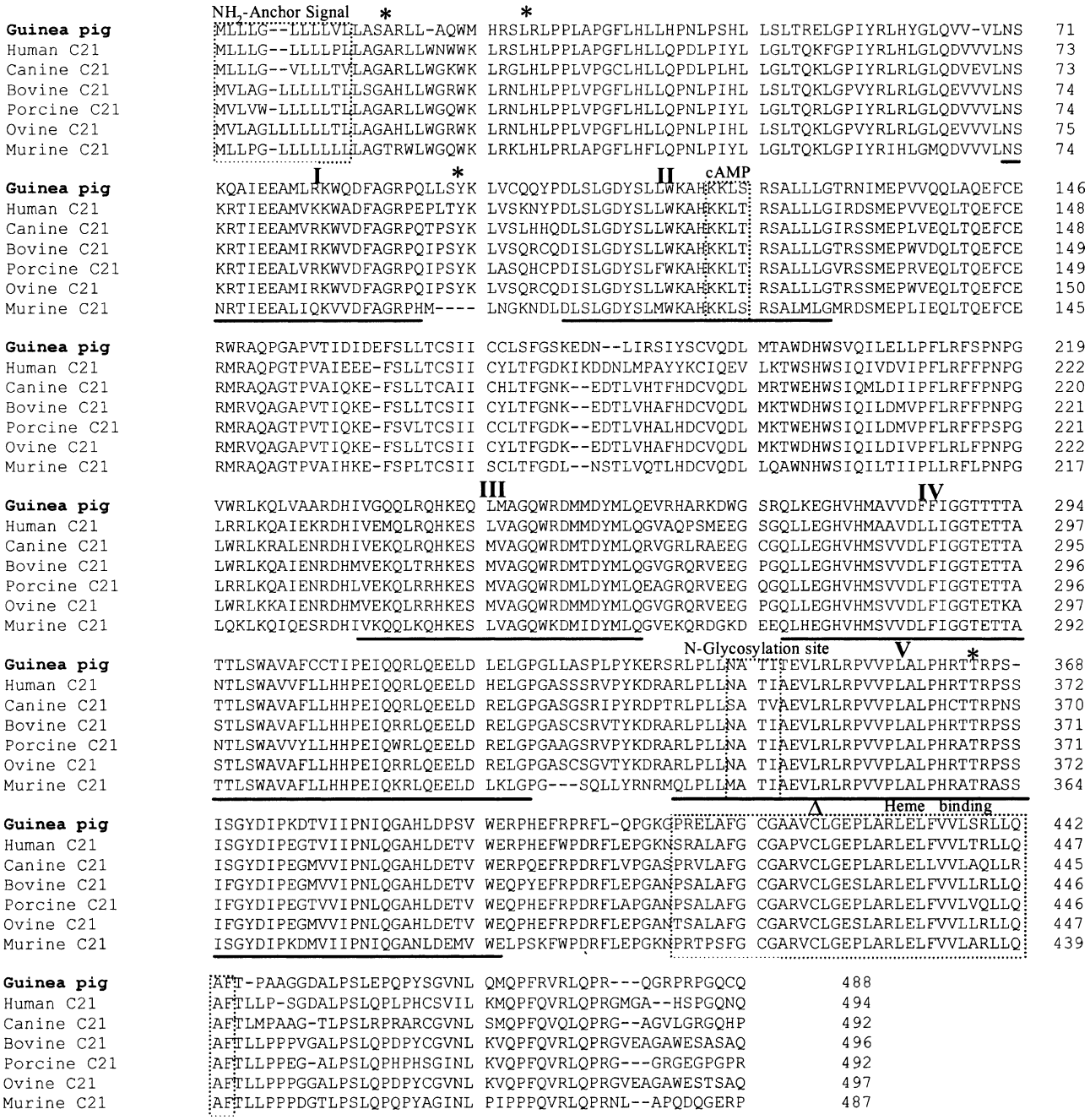

Fig. 1. Comparison of the amino acid sequence deduced from the nucleotide sequence of the guinea pig P450c21 with that from other species. Amino acid sequences of P450c21 from murine [3], porcine [27], bovine [26], ovine [28], canine [25] and human [4] species are designated by the universal single-letter code and residues are numbered relative to the first NH_2-terminal methionine. Gaps indicate sites where comparable amino acids are not present. Highly conserved regions are underlined. The triangle indicates the cysteine residue presumed to provide the ligand for the heme iron. The star indicates a protein kinase C phosphorylation site, the doted boxes indicate the cAMP-dependant protein kinase phosphorylation and the N-glycosylation sites. Highly conserved regions between all the species are underlined.

3.3. Progesterone and 17-hydroxyprogesterone metabolism by 293 cells transfected with pcDNA6-gpP450c21

The expression of P450-dependent enzymes in non-steroidogenic HEK293 cells allowed for the investigation of steroid 21-hydroxylase activity without interference of other P450s steroidogenic enzymes. Monkey kidney COS-1 cells have also been used, and similar data were obtained in both cell lines (data not shown). Time course assays for both progesterone and 17α-hydroxyprogesterone conversion during 0, 2, 5, 12, 24 and 48 h showed that enzyme activity was relatively linear after 48 h in the presence of substrate. Fig. 4A shows that the guinea pig P450c21 catalyzes the conversion of progesterone to deoxycorticosterone (miner-

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ATGCTGCTCCTTGGG CTGCTGCTGCTGGTA CTGCTGGCCAGTGCC CGCCTGCTCGCGCAG TGGATGCATAGGAGC CTGCGCCTTCCACCT   90
MetLeuLeuLeuGly LeuLeuLeuLeuVal LeuLeuAlaSerAla ArgLeuLeuAlaGln TrpMetHisArgSer LeuArgLeuProPro

CTGGCCCCAGGCTTC CTGCACCTCCTGCAC CCCAACCTCCCCAGT CACCTGCTCAGCCTC ACCCGGGAGCTTGGG CCCATCTATAGGCTG  180
LeuAlaProGlyPhe LeuHisLeuLeuHis ProAsnLeuProSer HisLeuLeuSerLeu ThrArgGluLeuGly ProIleTyrArgLeu

CACTATGGGCTTCAA GTAGTGGTGCTGAAC TCCAAGCAGGCCATC GAAGAGGCCATGCTC AGGAAGTGGCAAGAC TTCGCCGGCAGACCC  270
HisTyrGlyLeuGln ValValValLeuAsn SerLysGlnAlaIle GluGluAlaMetLeu ArgLysTrpGlnAsp PheAlaGlyArgPro

CAGCTGCTGTCCTAC AAGCTGGTGTGCCAG CAGTACCCGGACCTG TCGCTGGGGGACTAC TCCCTGCTCTGGAAG GCCCACAAGAAGCTC  360
GlnLeuLeuSerTyr LysLeuValCysGln GlnTyrProAspLeu SerLeuGlyAspTyr SerLeuLeuTrpLys AlaHisLysLysLeu

TCGCGCTCGGCCCTC CTGCTGGGCACCCGC AATATCATGGAGCCG GTGGTGCAGCAGTTA GCCCAGGAGTTCTGC GAGCGCTGGAGAGCC  450
SerArgSerAlaLeu LeuLeuGlyThrArg AsnIleMetGluPro ValValGlnGlnLeu AlaGlnGluPheCys GluArgTrpArgAla

CAGCCTGGTGCCCCC GTGACCATTGACATT GACGAGTTCTCCTTG CTCACCTGCAGCATC ATCTGCTGCCTCTCC TTTGGAAGCAAGGAG  540
GlnProGlyAlaPro ValThrIleAspIle AspGluPheSerLeu LeuThrCysSerIle IleCysCysLeuSer PheGlySerLysGlu

GACAACCTGATTCGG AGCATTTACAGCTGT GTACAGGACTTGATG ACCGCCTGGGACCAC TGGTCTGTCCAAATT CTGGAGCTACTTCCC  630
AspAsnLeuIleArg SerIleTyrSerCys ValGlnAspLeuMet ThrAlaTrpAspHis TrpSerValGlnIle LeuGluLeuLeuPro

TTTCTCAGGTTCTCC CCCAACCCAGGCGTC TGGAGGCTGAAGCAG TTGGTGGCGGCCAGG GATCACATTGTAGGG CAGCAGCTGAGGCAG  720
PheLeuArgPheSer ProAsnProGlyVal TrpArgLeuLysGln LeuValAlaAlaArg AspHisIleValGly GlnGlnLeuArgGln

CACAAGGAGCAGCTG ATGGCCGGGCAGTGG AGGGACATGATGGAC TACATGCTCCAGGAG GTGCGGCACGCCAGG AAGGACTGGGGCTCC  810
HisLysGluGlnLeu MetAlaGlyGlnTrp ArgAspMetMetAsp TyrMetLeuGlnGlu ValArgHisAlaArg LysAspTrpGlySer

AGACAGCTCAAGGAG GGCCACGTGCACATG GCTGTGGTGGACTTC TTCATCGGTGGCACC ACGACCACAGCCACC ACTCTCTCCTGGGCC  900
ArgGlnLeuLysGlu GlyHisValHisMet AlaValValAspPhe PheIleGlyGlyThr ThrThrThrAlaThr ThrLeuSerTrpAla

GTGGCTTTCTGCTGC ACCATCCCAGAGATC CAGCAACGACTCCAA GAAGAGCTGGACCTC GAGCTGGGCCCTGGT CTTCTTGCCTCCCCA  990
ValAlaPheCysCys ThrIleProGluIle GlnGlnArgLeuGln GluGluLeuAspLeu GluLeuGlyProGly LeuLeuAlaSerPro

CTTCCATACAAGGAG CGCTCTCGGCTGCCC CTGCTCAATGCCACC ATCACCGAGGTGCTG CGCCTACGGCCTGTG GTGCCCCTGGCCCTG 1080
LeuProTyrLysGlu ArgSerArgLeuPro LeuLeuAsnAlaThr IleThrGluValLeu ArgLeuArgProVal ValProLeuAlaLeu

CCCCATCGTACCACT CGACCCAGCATCTCC GGCTACGACATCCCC AAGGACACAGTGATT ATCCCCAACATCCAA GGTGCCCACCTGGAC 1170
ProHisArgThrThr ArgProSerIleSer GlyTyrAspIlePro LysAspThrValIle IleProAsnIleGln GlyAlaHisLeuAsp

CCGTCGGTGTGGGAG CGGCCCCACGAGTTC CGGCCACGCTTCCTG CAGCCGGGCAAGGGC CCCCGAGAGCTGGCC TTCGGCTGCGGGGCC 1260
ProSerValTrpGlu ArgProHisGluPhe ArgProArgPheLeu GlnProGlyLysGly ProArgGluLeuAla PheGlyCysGlyAla

GCGGTGTGCCTGGGG GAGCCCCTGGCGCGG CTGGAACTCTTCGTG GTGCTGAGCCGCCTT CTCCAGGCCTTCACG CCTGCCGCTGGAGGG 1350
AlaValCysLeuGly GluProLeuAlaArg LeuGluLeuPheVal ValLeuSerArgLeu LeuGlnAlaPheThr ProAlaAlaGlyGly

GACGCCCTGCCCTCC CTAGAACCCCAGCCC TACAGCGGCGTCAAC CTCCAAATGCAGCCT TTCCGGGTGCGGCTG CAGCCCCGGCAAGGG 1440
AspAlaLeuProSer LeuGluProGlnPro TyrSerGlyValAsn LeuGlnMetGlnPro PheArgValArgLeu GlnProArgGlnGly

CGTCCGCGCCCGGGC CAGTGCCAGTGATGC GCAGGACCGGAGCCA GTCGCGTGCCTCAGT TTCTCCTTTATTGCC CCCATACGAACCCCT 1530
ArgProArgProGly GlnCysGln...Cys AlaGlyProGluPro ValAlaCysLeuSer PheSerPheIleAla ProIleArgThrPro

CTCCTCCCCCCTGTA AACATGGTGCTGTGG GGTCGCGAGCAGAGA GGACTTCGGGAGCTT CTCTGGGCGCGACTT CTCAGTGCTCGGCAG 1620
LeuLeuProProVal AsnMetValLeuTrp GlyArgGluGlnArg GlyLeuArgGluLeu LeuTrpAlaArgLeu LeuSerAlaArgGln

TCCTCCCGGGCGCAG GCGCGGCGGCTCAGC CCCGCCGGGCGCCCG GTAACTCCTCGGTCT CAGCTTCATTTCCGT GAAGGGCACGGAGAA 1710
SerSerArgAlaGln AlaArgArgLeuSer ProAlaGlyArgPro ValThrProArgSer GlnLeuHisPheArg GluGlyHisGlyGlu

GTCGAAGCCCTTCCA GTGGTACCAGCTCAC CCCCTGGGCAAGGAG GCCGAGCATGGCAGG CCACAGCCCCAACCC GGACACCCACCCGCT 1800
ValGluAlaLeuPro ValValProAlaHis ProLeuGlyLysGlu AlaGluHisGlyArg ProGlnProGlnPro GlyHisProProAla

CAGCCCGGTAGCCCC AGCCCCGGTCCACCG CGCGGAACCCCAAGC CGCCCGTCCGCCCAA TAAACCGCTTCCGAG CCGCCAAAAAAAAAAA 1890
GlnProGlySerPro SerProGlyProPro ArgGlyThrProSer ArgProSerAlaGln ...ThrAlaSerGlu ProProLysLysLys

AAAAAAAAAA                                                                                    1900
LysLysLys
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Fig. 2. Nucleotide and predicted amino acid sequences of the guinea pig P450c21 cDNA. The numbers on the right indicate the nucleotide position. Residue >1 is the putative methionine initiator. The 3′ uncoding nucleotides are uncapitalized. The single open reading frame beginning at the ATG is in capital letters and shown below the nucleotide sequence. The putative polyadenylation signal is underlined. The cDNA was sequenced in both orientations.

Table 1
Percent Identity of Guinea Pig P450c21 amino acid sequence with other species P450c21

Parameter	Human	Canine	Bovine	Porcine	Ovine	Murine
Overall similarity (1–488) (%)	70.5	68.1	68.1	68.0	67.9	64.2
NH_2-signal Anchor (1–17) (%)	88.3	76.5	58.8	64.7	58.8	64.7
Heme binding (41–1444) 34 nts (%)	88.2	82.3	85.3	85.3	82.3	82.3
Highly conserved region I (68–91) 23 nts (%)	73.9	82.6	82.6	78.3	82.6	60.9
Highly conserved region II (104–128) 25 nts (%)	**96.0**	**96.0**	**92.0**	**88.0**	**92.0**	**92.0**
Highly conserved region III (234–259) 25 nts (%)	84.6	76.9	73.1	73.1	76.9	76.9
Highly conserved region IV (271–325) 53 nts (%)	77.4	84.9	81.1	75.5	77.4	83.0
Highly conserved region V (338–395) 57 nts (%)	87.7	80.7	86.0	84.2	84.2	82.5

Numbers in parentheses refer to the guinea pig amino acid sequence.

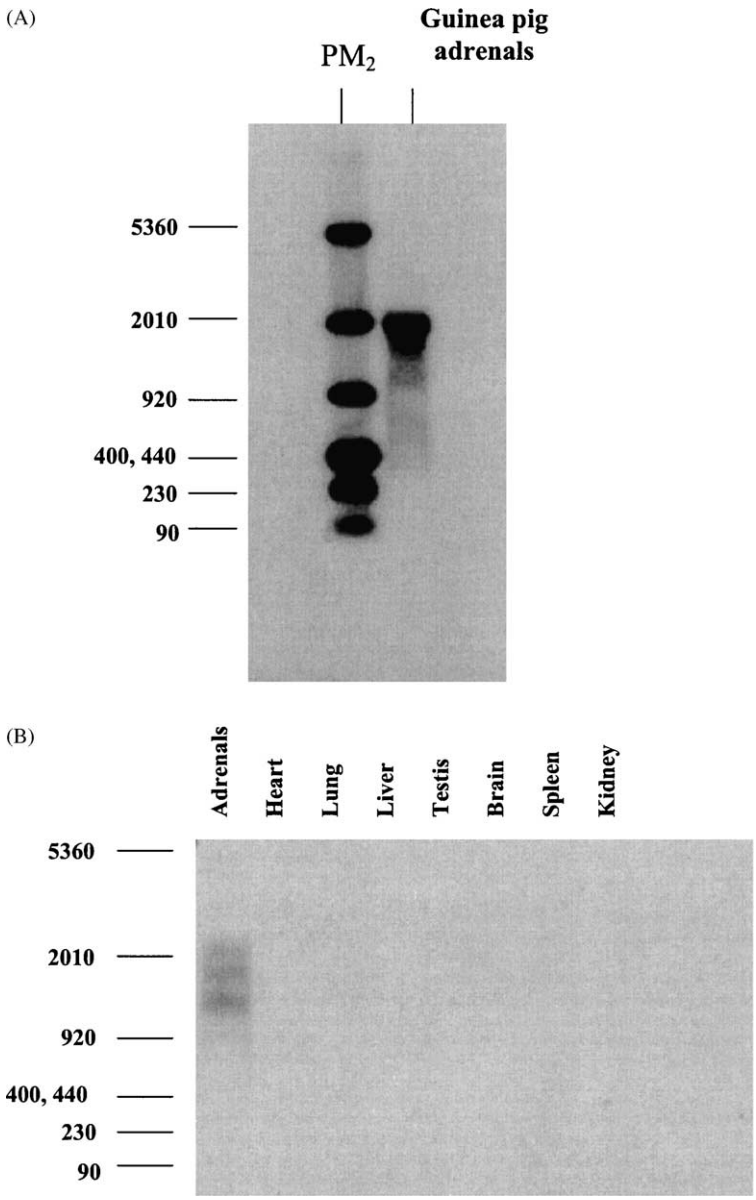

Fig. 3. Northern blot analysis and tissue-distribution of the guinea pig P450c21 mRNA. (A) Total RNA from adult guinea pig adrenals was probed with ^{32}P-labeled guinea pig P450c21/*Sma* I fragment corresponding to the coding sequence. The blot was exposed to X-ray film for 24 h. (B) Total RNA from adult guinea pig adrenals, heart, lung, liver, testis, brain, spleen and kidney were analyzed for the presence of gpP450c21 mRNA.

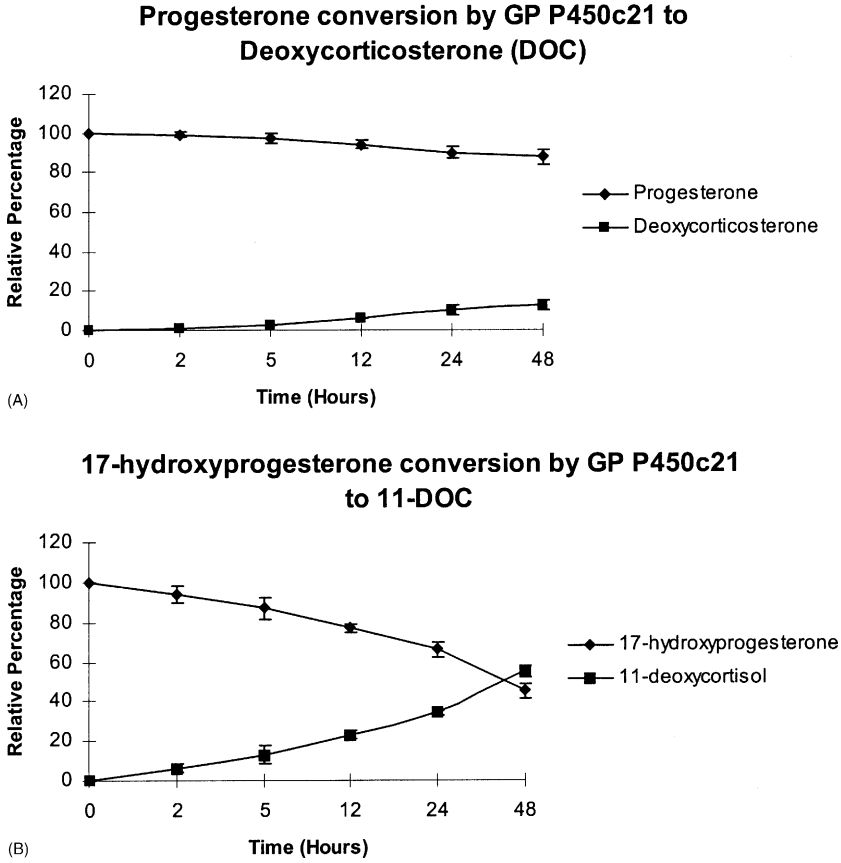

Fig. 4. Enzymatic activity of the expressed guinea pig P450c21 protein in HEK293 cells. Time course of conversion of progesterone (panel A) and 17α-hydroxyprogesterone (panel B) were performed with intact cells for various durations. At the indicated times, the steroids were extracted and resolved by TLC and scanned as described in Section 2. The radioactivity of each spot was quantified relatively to the same steroids incubated only with the vector pcDNA3 transfected cells, and results were reported as normalized percentage of radioactivity of steroids present in the medium. Points are means of closely agreeing triplicate experiments. Prog: progesterone, 17α-OHProg: 17α-hydroxyprogesterone, DOC: deoxycorticosterone, 11-DOC:11-deoxycortisol.

alocorticoid pathway) at a lower range. Repeated assays in triplicate showed a maximum conversion rate of 12.5% after 48 h. In Fig. 4B, the guinea pig P450c21 demonstrated an higher activity with 17α-hydroxyprogesterone, with 55% of 11-deoxycortisol formation (glucocorticoid pathway) after 48 h. In the conditions used for these assays, the guinea pig 21-hydroxylase transcript showed a net preference for the formation of glucocorticoid precursors.

3.4. Regulation of P450c21 mRNA in guinea pig fasciculata cells

Fasciculata cells were isolated and incubated with 10 nM ACTH or 1.5 mM 8CPTcAMP, a non-metabolizable cAMP analogue, for 24 h. Northern blot analysis of the P450c21 mRNA showed that both ACTH and cAMP equally increased to above basal levels the abundance of the guinea pig P450c21 mRNA. The similarity in the maguitude of stimulation indicates that the inductins are cAMP-dependent. No siguificant effect of these treatments on 18S ribosomal RNA was observed (Fig. 5).

4. Discussion

The guinea pig P450c21 is closely related structurally to the P450c21 from other species, especially human, with which, in its nucleotide and amino acid sequences, the guinea pig shares the highest percentage of homology. By comparison of gpP450c21 with other P450c21 cDNAs, we noted several highly conserved regions, including the heme-binding region which is an important feature of the cytochrome P450 family. We denoted a 25 amino acid region between positions 104–128 that is conserved in over 90% for all the species, with the consensus sequence D[L/I]SLGDY-SLXWKAHKKL[T/S]RSAL[L/M]LG. All the species also share a great homology in their sequence containing the cAMP phosphorylation site and the *N*-glycosylation site. We also observed that the guinea pig amino acid sequence contains two additional putative protein kinase C phosphorylation sites in comparison with other species so far characterized.

The prominent guinea pig transcript hybridizing with P450c21 cDNA clone of 2.1 kb long was detected only

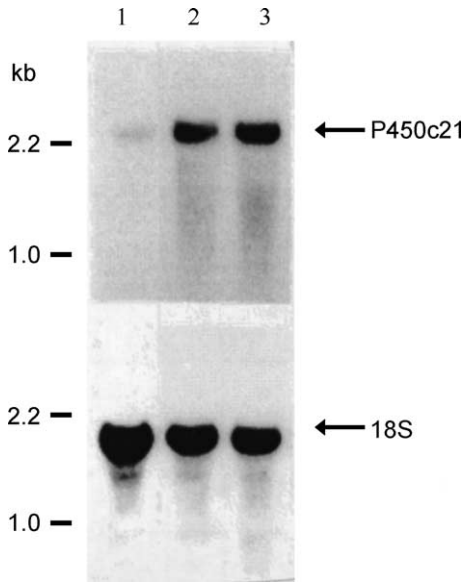

Fig. 5. Effects of ACTH (10 nM) and 8CPTcAMP (1.5 mM) on guinea pig P450c21 mRNA in primary cultures of fasciculata cells. Experiments were carried out as described in materials and methods. Upper panel, blot probed with P450c21. Lower panel, same blot probed with 18S rat ribosomal oligodeoxyribonucleotide probe. Lane 1: control; Lane 2: ACTH; Lane 3: 8CPTcAMP.

in the adrenals. All other steroidogenic (testis) and non-steroidogenic tissues (heart, lung, liver, brain, spleen and kidney) were negative. However, it has been reported that 21-hydroxylase activity was found in other tissues in human, such as in lymphocytes B and leukocytes [29]. Extra-adrenal 21-hydroxylase activity has been proposed to compensate for the loss of the activity in adrenals in patients with CAH [6]. This hydroxylation activity has also been detected in the mouse and rat kidney [30]. An 21-hydroxylase activity is also found in rat hepatocytes and is provided by cytochrome P4502C6 and has been shown to be regulated by estrogens [31]. Another cytochrome, P4502C2 demonstrated 21-hydroxylase activity when the valine residue at position 473 was mutated with a serine [32].

In contrast with the human species, in which a single 2 kb mRNA was identified [1], the present study shows that two additional shorter P450c21-hybridizing bands of 1.8 and 1.45 kb are also present in the guinea pig. Similar observations have been made with the cytochrome P450c17 isolated from the guinea pig adrenals [20].

Expression of pcDNA3-gpP450c21 in HEK293 cells by transient transfection allowed the analysis of the enzymatic activity with its specific steroid substrates, progesterone and 17α-hydroxyprogesterone. In the conditions used for this study, we showed that this guinea pig cytochrome enzyme has a five-fold higher activity with 17α-hydroxyprogesterone than with progesterone. Similar results were reported before [33] and showed that different types of cytochrome P450c21-containing preparations from bovine adrenocortical microsomes have exhibited different steroid specificities for 21-hydroxylation. The ratio of 21-hydroxylation for 17α-hydroxyprogesterone/progesterone ranged between 2.6 and 0.86. These variations indicated that the purified P450 mixture contained either higher affinity for 17α-hydroxyprogesterone or progesterone P450 isoforms. They showed that these isoforms were associated with variations in their net charge and steroid preference for 21-hydroxylation and binding. The presence in our studies of multiple mRNA transcripts could be involved in this substrate specificity. Furthermore, studies with the guinea pig adrenal P450c17, which regulation is of primary importance because it is localized at the key branch between glucocorticoid and C19 steroid synthesis [20], showed that progesterone was rapidly converted into 17α-hydroxyprogesterone (17-hydroxylase activity), and then into androstenedione (17,20-lyase activity). It is possible that the guinea pig P450c21 has a lower affinity for progesterone, so that 17α-hydroxyprogesterone can be used rapidly to produce glucocorticoids and androstenedione, which leads to the production of C19 steroids.

Previous studies of the adrenal steroidogenic enzymes in guinea pigs [15] demonstrated that in both reticularis and fasciculata-glomerulosa zones, the concentration of progesterone was between 4.5 and 8.8 higher then 17α-hydroxyprogesterone, and DOC was between 3.6 and 6.8 lower than the 11-deoxycortisol metabolite (11-DOC), which is in accordance with the activity measured with the guinea pig P450c21 in intact HEK293 cells.

ACTH and cAMP have been shown to modulate the expression of 21-hydroxylase transcripts [16,17,34–38]. Treatment with ACTH greatly increased the amount of cortisol produced in the fasciculata-glomerulosa zone, which favours for a preference of the 21-hydroxylase activity with 17α-hydroxyprogesterone, which leads to the production of glucocorticoids. Although transfection of the mouse P450c21 cDNA has indicated ACTH as a major trophic hormone that regulates *CYP21* gene expression [39], other regulators at the level of the 21-hydroxylase activity have been identified. Among those, androstenedione inhibits 21-hydroxylase activity by a mechanism that does not involve the formation of oxygen-derived radicals [17]. Using the RU486, a non-metabolizable synthetic C18 steroid having a 4-ene-3-ketosteroid with an aryl group at position 11, it was shown that the inhibition of 21-hydroxlase activity in RU-treated cells, required a new synthesis of protein that can be reinduced by ACTH [35]. Other modulators have also been studied, such as NGFI-B, an analog of the factor SF-1, binds to the P450c21 promoter and stimulates the expression a regulated gene in mice [40]. The compound 1-aminobenzotriazole inhibits 21-hydroxylase activity in vivo in the guinea pig [41]. Inhibition of 21-hydroxylase activity has also been observed with exogenous compounds such as Acrolor1254 in membrane-depleted assays [42]. Hydroxylation activity of P450c21 is also modulated by the androgenic activity of C19 steroids [43].

The guinea pig has been shown to be a good in vivo and in vitro model for the study of the human steroidogenesis. A

better knowledge of the enzymatic activities specific to this model may be useful to study their implication in disorders of the mineralocorticoid and glucocorticoid pathways.

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