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THE HYBRID RAT CYTOCHROME P450 CONTAINING THE FIRST 5 EXONS OF THE CYP11B1 AND LAST 4 EXONS FROM THE CYP11B2 ENZYME RETAINS 11ß-HYDROXYLASE ACTIVITY, BUT THE ALTERNATIVE HYBRID IS INACTIVE

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Summary: Human, mouse and rats have 2 different cytochrome P-450 11ß-hydroxylases in the adrenal cortex. The classical rat 11ß-hydroxylase or CYP11B1 enzyme hydroxylates deoxycorticosterone to corticosterone and 18-hydroxydeoxycorticosterone and is located throughout the adrenal. The second aldosterone synthase or CYP11B2 enzyme is located in the zona glomerulosa and converts deoxycorticosterone to corticosterone, 18-hydroxycorticosterone and aldosterone. In rat the coding nucleotide sequence and the deduced amino acid sequences of the CYP11B1 and CYP11B2 genes are homologous by 88% and 83%,respectively. We have constructed two different hybrid cDNAs by exchanging two fragments of the rat CYP11B1 and CYP11B2 at the junction of the 5/6 exon and expressed them in COS 7 cells. The hybrid CYPH11B1 construct containing the first 5 exons of the CYP11B1 when expressed, retains 11ß-hydroxylase activity, but cannot process corticosterone to 18-hydroxycorticosterone or aldosterone. The hybrid CYPH11B2 construct containing the first 5 exons of the CYP11B2 enzyme when expressed is inactive.

Mineralocorticoids and glucocorticoids are steroids produced in the adrenal cortex from cholesterol by the action of a series of enzymes known as cytochrome P450s (1). Glucocorticoids are mainly formed in the zona fasciculata, while aldosterone, the main mineralocorticoid, is synthesized only in the zona glomerulosa (1). At least two cytochrome P450 11ß-hydroxylase enzymes that are involved in the final steps of the biosynthesis of mineralocorticoids and glucocorticoids have been found and their genes have been cloned in the rat, mouse and human (2-4). The cytochrome P-450 11ß-hydroxylase (CYP11B1) is distributed throughout the adrenal and mainly catalyzes the conversion of deoxycorticosterone(DOC) to corticosterone (B), 18-hydroxydeoxycorticosterone (18-OH-DOC) and 19-hydroxydeoxycorticosterone (19-OH-DOC) (5). A second cytochrome P-450 11ß-hydroxylase, aldosterone synthase (CYP11B2), has also been isolated and

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ABBREVIATIONS: deoxycorticosterone (DOC), 21-hydroxy-preg-4-ene-3,20-dione; corticosterone (B) 11ß,21-dihydroxy-preg-4-ene-3,20-dione; 18-hydroxydeoxycorticosterone (18-OH-DOC), 18,21-dihydroxy-preg-4-ene-3,20-dione; 18-hydroxycorticosterone (18-OH-B), 11ß,18, 21-trihydroxy-preg-4-ene-3,20-dione; aldosterone, 11ß,21-dihydroxy-preg-4-ene-3,18,20-trione.

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cloned and is capable of converting DOC to B, 18-hydroxycorticosterone (18-OH-B) and aldosterone (ALDO). CYP11B2 is located only in the zona glomerulosa (5-7).

The coding sequences and the deduced amino acid sequences of the human CYP11B1 and CYP11B2 are 93% identical (2,8-10). In rat the coding nucleotide sequence and the deduced amino acid sequences of the CYP11B1 and CYP11B2 genes are homologous by 88% and 83% respectively (11-13). Most of the differences in nucleotides between the rat CYP11B1 and CYP11B2 cDNAs occur in exons 3, 4 and 5, with exon 5 having the lowest homology among the 9 exons (11-13). A similar case occurs in mice (4). The substitutions in the CYP11B2 responsible for the different enzymatic specificity from the CYP11B1 is still unkown. In the human, two point mutations in the CYP11B2 has been found to be responsible for the patients with corticosterone methyl oxidase II (CMO II) deficiency in whom DOC is converted to B and 18-OH-B, but its conversion to aldosterone is decreased very significantly (14,15). In patients with the disease known as Glucocorticoid-suppressible aldosteronism there is gene duplication produced by the unequal crossover recombination of the early exons and promoter region of the CYP11B1 with the late exons of the CYPB2 which result in the expression of the new enzyme in the zona fasciculata of the adrenal. This new enzyme has the capacity to produce aldosterone in an area where it is not normally produced (16-19). These examples demonstrate that the structure-function relationship of these two enzymes is important for the explanation of the pathological mechanisms. In this study we report the construction of two rat hybrid cytochrome P450 genes and their enzymetic activities after expression in COS-7 cell lines.

MATERIALS AND METHODS

Restriction enzymes were purchased from Promega (Madison, WI) or United States Biochemical Corp (Cleveland, OH). Plasmids pcDNAII and pcDNAI/neo were from Invitrogen Corp (San Diego, CA). pBAdx-4, an expression plasmid encoding bovine adrenodoxin, was a kind gift from Dr. M. Waterman from the University of Texas, Southwestern Medical Center. Steroids and other chemicals were from Sigma (St. Louis, MO).

Construction of the Hybrid Gene Expression Plasmids and Nucleotide Sequence Analysis--The plasmids pCYP11B1/neo and pCYP11B2/neo, which contain the coding cDNA of rat 11ß-hydroxylase and aldosterone synthase respectively (20), were digested with restriction enzymes Bam HI and Xba I and the inserts were purified by electrophoresis and cloned into plasmid pcDNAII at the same restriction sites to construct pcDNAII/CYP11B1 and pcDNAII/CYP11B2 respectively. The plasmids were digested with Bam HI and Hinc II. Two sets of fragments were purified and recovered from low-melting agarose gel. The first set was denoted as AS-948 and 11ß-945, containing the 1-948bp fragment of aldosterone synthase cDNA coding region and 1-945bp fragment of 11ß-hydroxylase cDNA coding region respectively. The second set, AS-555 and 11ß-555, contained the 555bp 3'-fragments of the Hinc II restriction site of the above cDNA coding regions respectively, along with the attached plasmid (Fig.1). The fragments AS-948 and 11ß-555 or 11ß-945 and AS-555 were ligated at 4 C overnight by T4 DNA ligase to construct the hybrid gene plasmids pHAS or pH11ß respectively (Fig.1). The resulting clones were checked by restriction mapping and sequencing. pHAS or pH11ß were digested with Bam HI and Xba I and the inserts were purified by gel electrophoresis and cloned into plasmid pcDNAI/neo at Bam HI and Xba I sites to construct two eukaryotic expression plasmids. pCYPH11B2/neo contained AS-948 and 11ß-555 fragments and pCYPH11B1/neo contained 11ß-945 and AS-555 fragments respectively. The positive clones were checked by restriction mapping and sequencing. DNA sequencing was done using the Taq Dyedeoxy Terminator Cycle Sequencing Kit and the automatic DNA sequencing instrument Model 373A (Applied Biosystems).

Transient Expression of the Plasmids in COS-7 Cells and the Assay of the Converted Steroids--Plasmid DNA was transfected into COS-7 cells using Lipofectin reagent (Gibco-BRL) in triplicate. Cells in 6-well plates at 60-80% confluence were incubated in 1 ml of

serum-free Dulbecco's MEM medium pre-mixed with 2 μg of cytochrome P450 cDNA plasmids (or pcDNAI/neo as control) , 1 μg of pBAdx-4 and 10 μl of Lipofectin. In the case of mock control transfection, no other DNA except pBAdx-4 was added. After 4-5 h, 1 ml of medium containing 20% of bovine calf serum (BCS) was added and incubated for another 16-20 h, then the medium was replaced with 10% BCS-DMEM medium containing 10 μM of deoxycorticosterone. The medium was collected after 48 h incubation and frozen until assayed . The conversion products aldosterone, 18-hydroxycorticosterone and 18-hydroxydeoxycorticosterone were measured by ELISA and corticosterone was assayed by radioimmunoassay(RIA) as described before (21).

RESULTS AND DISCUSSION

The hybrid cDNA construction took advantage of the presence of the unique Hinc II restriction site in both the CYP11B1 and CYP11B2 cDNAs. First the CYP11B1 and CYP11B2 cDNA were subcloned into pcDNAII plasmid, which does not contain the Hinc II restriction site. Digestion with BamHI and Hinc II only formed two fragments. The hybrid plasmids were constructed by ligation of the two-fragments (Fig 1). Restriction maps and automatic DNA sequencing confirmed the correct DNA sequences of the constructed hybrid plasmids. There were no changes in the DNA reading frames of the hybrid genes.

The hybrid cDNA and the original constructs were transfected into COS-7 cells separately and the cells were incubated with 10 μM of DOC. The steroid hydroxylase activities are summarized in table 1. As expected, the expressed CYP11B2 gene product (aldosterone synthase) converted DOC to B, 18-OH-B and aldosterone, while the CYP11B1 gene product (11ß-hydroxylase) converted DOC to B, but no 18-OH-B or aldosterone were detected. Both the CYP11B1 and CYP11B2 formed 18-OH-DOC from DOC, but the former produced a much greater quantity. Similar to CYP11B1, the hybrid CYPH11B1 transfected cells efficiently converted DOC to B, but aldosterone and 18-OH-B were not detectable. COS-7 cells transfected with the hybrid CYPH11B2 gene failed to convert DOC to either B, 18-OH-B, 18-OH-DOC or aldosterone. In separate transfection experiments, the incubated media containing DOC was extracted with methylene chloride and the extracts were analyzed by high performance liquid chromatography (HPLC). No conversion products were detected from CYPH11B2, while B and other products were identified by HPLC in the case of CYP11B1,CYP11B2 and H11B1 transfects.

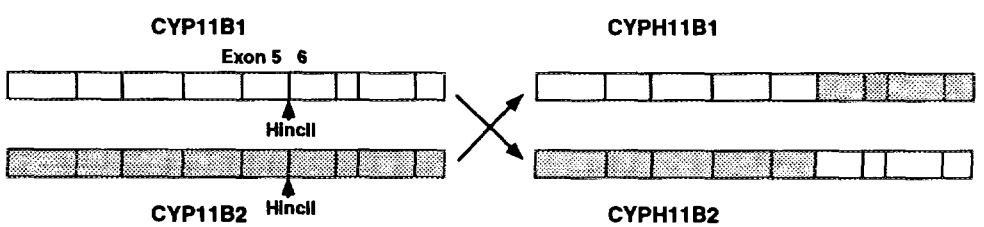

Figure 1. Top. Transposition of the first 5 exons of the CYP11B1 or CYP11B2 with the last 4 exons of the CYP11B2 or CYP11B1 cDNAs is shown.
Bottom. Sequence of the junction where the transposition was performed is shown. Top sequence corresponds to the CYP11B1 and bottom to the CYP11B2. Identical nucleotides shown by dots.

Table 1. Steroid Hydroxylase Activity of COS-7 Cells Transfected with Plasmids Encoding Cytochrome P450s

Product	Hydroxylase Activity (ng/ml)			
	CYP11B1	CYP11B2	H11B2	H11B1
Corticosterone	530	394	UD	553
18-OH-B	UD	122	UD	UD
Aldosterone	UD	37.9	UD	UD
18-OH-DOC	221	30.0	UD	37.4

UD = undetectable. Cells were incubated with 10 μM of 11-deoxycorticosterone for 48 hr.

The functional role of the CYP11B1 (11ß-hydroxylase) is the 11ß-hydroxylation of steroids, while the CYP11B2 (aldosterone synthase) processes the 11ß-hydroxylated product further with a double hydroxylation at the 18 position followed by a spontaneous dehydration to yield aldosterone. The selectivity and efficiency of steroid hydroxylation by the cytochrome P450s is different with the various steroid substrates and may depend on the interaction between the enzymes and the specific stereo-structure and conformation of steroid substrates (5). In the rat, the CYP11B1 enzyme also hydroxylates DOC to 18-OH-DOC efficiently, but is significantly less active in the hydroxylation of B to 18-OH-B (5). DOC is a better substrate than B for the CYP11B2 for the production of 18-OH-B and aldosterone (5).

Hybrid genes of the CYP11B1 and CYP11B2 enzymes causes an autosomal dominant form of familial hypertension known as glucocorticoid-suppressible hyperaldosteronism.(16-19). The extra gene in these patients is created by an uneven crossover recombination of the inital exons and promoter region of the CYP11B1 with the later exons of the CYP11B2 (16-19). A series of hybrid cDNAs of human CYP11B1 and CYP11B2 in which the various length of the 3'-coding region of the CYP11B1 were replaced by the corresponding region of the CYP11B2 have been constructed and expressed in COS cells (18). Only those hybrid cDNAs containing sequences of the CYP11B2 starting from exons 2, 3 or 4 to exon9 confered aldosterone synthase acitivity, but all of the hybrid constructs have 11ß-hydroxylation activity although the activities were lower than the parent CYP11B1 (18).

There are 83 amino acid residue substitutions between rat CYP11B1 and CYP11B2. Of the 83 amino acid residues, 63 appear at the region encoded by exons 3, 4 and 5 and only 4 at exons 1 and 2. The two hybrid cDNAs CYPH11B1 and CYPH11B2 exchanged the 3'-coding regions at the conjunction site of exon 5/6 (Fig.1), resulting in 16 amino acid substitutions as listed in table 2. In comparison to CYP11B1, CYPH11B1 produced slightly higher amount of corticosterone from DOC, but the amount of 18-OH-DOC produced was greatly decreased to a level equivalent to CYP11B2. These results show that the 16 amino acid replacement of CYP11B1 sequence by the corresponding amino acids of CYP11B2 have little effect on the 11ß-hydroxylation activity of CYP11B1, but the 18-hydroxylation activity toward DOC was greatly reduced. CYPH11B2 failed to convert DOC to B, 18-OH-B, 18-OH-DOC or aldosterone. In an separate experiment when corticosterone was used as substrate instead of DOC, still no conversion products were

Table 2. Differences in deduced amino acid sequences encoded by the CYP11B1, CYP11B2 and hybrid enzymes

Position	CYP11B1	CYPH11B1	Position	CYP11B2	CYPH11B2
321	Ser	Pro	322	Pro	Ser
343	Ser	Thr	344	Thr	Ser
351	Val	Ala	352	Ala	Val
379	Ser	Gly	380	Gly	Ser
381	Val	Leu	382	Leu	Val
385	Val	Leu	386	Leu	Val
386	His	Asn	387	Asn	His
401	Phe	Leu	402	Leu	Phe
403	Ile	Leu	404	Leu	Ile
404	Ile	Leu	405	Leu	Ile
477	Met	Val	478	Val	Met
480	Val	Ala	481	Ala	Val
481	Phe	Tyr	482	Tyr	Phe
484	Leu	Val	485	Val	Leu
492	Phe	Val	493	Val	Phe
498	Val	Ile	499	Ile	Val

detected by CYPH11B2. The 16 amino acid residue substitution of CYP11B2 by the counterparts of CYP11B1 inactivate enzymatic activity. Site-directed mutations will be needed to find out which of the 16 amino acid residues are necessary for the CYP11B2 enzyme activity.

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