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CLONING AND EXPRESSION OF A RAT CYTOCHROME P-450 11ß-HYDROXYLASE/ALDOSTERONE SYNTHASE (CYP11B2) cDNA VARIANT

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Summary: A variant cytochrome P450 11ß-hydroxylase/aldosterone synthase cDNA (CYP11B2) clone, pALDO-4, was isolated and sequenced from a rat zona glomerulosa cDNA library. This clone contains a 1521 bp open reading frame coding for a 506 amino acid residue protein which has a 30 amino acid extension peptide which is 6 residues longer than that reported by others. Seven nucleotide mutations in the coding region of the mature protein were found in comparison to the reported cDNAs. These mutations resulted in three amino acid changes in the mature protein which were identical to those present in the cytochrome P-450 11ß-hydroxylase (CYP11B1). Two nucleotide substitutions and two nucleotide insertions were also identified in the 3'-noncoding region. The expression product of this cDNA transfected into COS7 cells converted DOC to aldosterone, corticosterone and 18-hydroxycorticosterone less efficiently than the plasmid containing the cDNA with normal 24 aminoacid extension peptide. © 1993 Academic Press, Inc.

The adrenal synthesis of glucocorticoids and mineralocorticoids is zone specific. Glucocorticoids are formed mainly in the zona fasciculata, while aldosterone is synthesized in the zona glomerulosa from deoxycorticosterone (DOC) under the action of two different cytochrome P-450 11ß-hydroxylases (1,2). The 2 cytochrome P-450 11ß-hydroxylase cDNAs (CYP11B1 and CYP11B2) have been isolated and sequenced from rat, mouse and human adrenal cDNA libraries (2-7). The CYP11B1 gene is responsible for 11ß- and 18-hydroxylation of DOC to corticosterone and 18-hydroxy-deoxycorticosterone and is distributed throughout the adrenal (1). The CYP11B2 gene can hydroxylate succesively DOC to corticosterone, 18-hydroxycorticosterone and aldosterone and is distributed in the zona glomerulosa (1). In the Sprague Dawley rat, 2 different CYP11B2 cDNA clones have been isolated which differ in the size of the leading peptide and in one aminoacid (4,5).

Two more rat CYP11B genes have been recently described (8,9). The CYP11B3 gene is highly homologous to the CYP11B1 gene, but no expression was found using RT-PCR from adrenal gland mRNA (8). The fourth gene, CYP11B-4, is probably a pseudogene (9). A genetic polymorphism in the CYP11B1 and CYP11B2 genes has been detected in 2 Sprague Dawley-derived inbred strains which exhibit salt-sensitive

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hypertension (Dahl SS/jr) and salt-resistance (SR/jr rats). The SR/jr rat has 6 mutations of the CYP11B1 gene (10,11) and show a different RFLP of the CYP11B2 gene, the nature of which has not been elucidated (11). We isolated cDNAs for the CYP11B2 from an adrenal library from Sprague-Dawley rats and are reporting their sequence which is at variance with the published ones.

MATERIALS AND METHODS

Materials: [α-^{32}P]dCTP and [α-^{35}S]dATP were obtained from DUPONT NEN (Boston, MA). pcDNAIneo, a eukaryotic expression vector, was purchased from Invitrogen Corp (San Diego, CA). An expression plasmid pBAdx-4 encoding bovine adrenodoxin was a kind gift of Dr. M. Waterman from the University of Texas. 11ß-deoxycorticosterone was from Sigma. DNA primers used for PCR and sequencing were purchased from the DNA Synthesis Lab of the University of Florida in Gainesville.

Cloning and Sequencing of Rat CYP11B2 Gene: Sprague-Dawley rats (Harlan Sprague-Dawley, Madison WI) were fed a low sodium diet for 7 days. The adrenal zona glomerulosa was frozen in liquid nitrogen after it was separated from the core by making a small cut in the gland and extruding the zonas fasciculata and reticularis along with the medulla. mRNA was prepared using a Micro-FastTrack kit from Invitrogen Corp (San Diego, CA). A cDNA library, in which synthesized cDNA was unidirectionally inserted into pcDNAI at the Not I/BstX I site, was constructed using the Unidirectional Librarian I cDNA library construction system from Invitrogen Corp. In the initial studies, a cDNA for CYP11B2-coding region was prepared by polymerase chain reaction (PCR) using a GeneAmp kit from Perkin Elmer Cetus using a linearized library cDNA as template together with a sense primer 5 '-TTT GGA TCC GCA ATG GCT CTC AGG GTG ACA G-3', in which a BamH I restriction site has been introduced, and an antisense primer 5'-GAT GTA AGG TGA CTA GCT GAT GG-3'. The PCR was carried out for 30 cycles with the hot-start technique at the following conditions: 94C, 60s; 55C, 60s; 72C, 120s. The amplified product was digested with BamH I and inserted into pcDNAI/neo at BamH I/EcoRV site. The DNA sequence was determined using Sequenase (version 2.0) DNA sequencing kit from United States Biochemical (Cleveland, OH). The cDNA library was screened using a µWAVE colony screening kit (Invitrogen Corp) with a 345bp probe corresponding to the area of the reported CYP11B2 cDNA where the most differences occur between the CYP11B2 and CYP11B1. The probe was obtained by PCR amplification and labelled using a random primed DNA labeling kit (USB). The inserts of the positive clones were first digested with Hind III to differentiate between CYP11B2 and CYP11B1, and the longest clones sequenced.

Expression of CYP11B2 Gene in COS-7 Cells and the Assay of Converted Steroids: plasmid DNA was transfected into COS-7 cells using Lipofectin reagent (Gibco-BRL). Cells in 6-well plates at about 80% confluence were incubated in 1 ml of serum-free Iscove medium premixed with 2µg of aimed cDNA, 1µg of pBAdx-4 and 7.5µl of Lipofectin. After 5 h, 1ml of medium containing 20% fetal calf serum(FCS) was added and incubated for another 20 h, then the medium was replaced with 10% FCS-Iscove medium containing 10µM of deoxycorticosterone. The medium was collected for assay after 48 h incubation. Aldosterone,18-hydroxycorticosterone and corticosterone were assayed by ELISA or radioimmunoassay(RIA) as described before (12).

PCR: The presence of the modified extended peptide sequenced above was also determined by using RT-PCR. mRNA was prepared as above and first strand was prepared using reverse transcriptase Superscript (GIBCO) and primed using the antisense sequence 5'-AGG TGT AGG TTC TCT TGG CCC-3'. PCR was then done using the reagents described above and the sense primer 5'-AAT GAA TAA AGC ACC TGC CAA GGC-3' with the antisense described above resulting in the amplification of the expected 216 bp fragment.

RESULTS AND DISCUSSION

During the initial studies, the coding region of CYP11B2 and CYP11B1 cDNA was amplified by PCR and cloned into pcDNAI/neo, the constructed plasmids were denoted pCYP11B2/neo and pCYP11B1/neo, respectively. The sequencing of several independent clones showed that 6 mutations occured in CYP11B2 compared with reported results, while the CYP11B1 sequence was unchanged. The 6 mutations resulted in 2 amino acid changes in deduced protein sequence. In order to discard the possible PCR errors during amplification, the cDNA library was screened. Six clones with various insert lengths were obtained. Hind III digestion demonstrated all 6 clones contained CYP11B2 insert, no CYP11B1 insert was found. This was consistent with prediction because of the use of the CYP11B2 specific probe which hybridizes with CYP11B1 poorly. The clone with the longest insert, refered to as pALDO-4 (the fourth CYP11B2 cDNA found), was completely sequenced. As shown in Fig.1 the whole sequence contains an open reading frame of 1521 bp, a 291 bp 5'-noncoding region and a 1188 bp 3'-noncoding sequence plus a polyA tail. It deduces a protein of 506 amino acid residues. This protein was 6 residues longer in the extention peptide than the normal CYP11B2 and 4 residues shorter than a second cDNA reported (4,5). Seven mutations were found in the coding region instead of 6 mutations found by PCR. The 7th mutation

Fig.1. The complete cDNA sequence of the pALDO-4 and deduced amino acid sequence is shown.

appeared 6 nucleotides ahead of the stop codon and was not present in pCYP11B2/neo because we designed the pair of PCR primers according to the reported sequence and one of them was located in the stop codon area containing this mutation, so that the PCR product amplified by this pair of primers does not have the 7th mutation. Seven mutations result in 3 amino acid changes, i.e., positions136-Glu, 251-Gln and 499-Ile changed to 136-Asp, 251-Arg and 499-Val respectively. In the 3'-noncoding region two nucleotide substitutions and two nucleotide insertions were identified (Fig.2) Five of the 7 mutations in the coding area resulted in nucleotides which were the same as those of the corresponding area of the CYP11B1. The three mutated amino acids were also the same as those of the corresponding area of the CYP11B1. In a recently found genomic CYP11B3 gene, four nucleotides of the 5 corresponding positions were also identical to the ones found in the CYP11B1 sequence (8,9). Our findings support that CYP11B2 evolve from the same CYP11B1 origin. Three positive clones sequenced contained all of the 7 mutations in the coding area. We also found and sequenced an incomplete cDNA clone containing most of the coding area which did not have the corresponding

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                                        MetAsnLysAlaProAlaLysAlaLeu            Gly
pALDO-4            (I)          -18  ATGAATAAAGCACCTGCCAAGGCTCTC 9     (II)  GGT 75
                         MetGlyAlaCysAsp   AspPheIleGluLeuHisSer
pcP-45011B,aldo-46   -30 ATGGGAGCGTGTGAC---G-CTTCAT--AGCTTCACAG-               --C
                                                        Met
pALD23                                                  -T-------              --C
                                                        Met
pcP-45011B-62                                           -T-------              --C
                                                        Met
CYP11B3                                                 -T-------              --C


                           AlaAsp              ThrArg                Thr
pALDO-4            (III)   GCAGAC 408    (IV)  ACTCGG 753      (V)   ACC 978
                              Glu                 Gln
pcP-45011B,aldo-46         --T--A              --C-A-                --A
                              Glu                 Gln
pALD23                     --T--A              --C-A-                --A
pcP-45011B-62              ------              --C---                ---
                                                  Gln
CYP11B3                    ------              --C-A-                ---


                         Val
pALDO-4            (VI)  GTC 1497 (VII) TGGGGGGGGGGGA 1602  (VIII) TAGATAGATG 1992
                         Ile
pcP-45011B,aldo-46       A--            ----------**-              ----------
                         Ile
pALD23                   A--            ----------**-              -********-
pcP-45011B-62            --G            -T-T***---**-              ----------
CYP11B3                  --G


pALDO-4                 (IX)  ACT 2168   (X) GAT 2207    (XI)  G*******A  2692
pcP-45011B,aldo-46            -G-            -G-               ---------
pALD23                        -G-            -G-               -AAGTCCC-
pcP-45011B-62                 ---            -G-               ---------
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Fig.2. Comparison of the nucleotide and deduced amino acid sequences of CYP11B2 cDNA clones pALDO-4, pALD23 (4) and pcP-450B,aldo-46 (5) is shown. The numbering start at the initial ATG of pALD23, which contains the normal 24 amino acid signal peptide. Only those regions where the sequences which are different between the Aldo cDNAs are compared with the corresponding regions of pcP450-11B-62 and CYP11B3. In the nucleotide sequences, hyphens indicate identity with pALDO-4 and asterisks indicate deleted nucleotides. The amino acids are shown above its nucleotide sequence.

Table 1. Conversion of Deoxycorticosterone by Transfected COS7 Cells (DOC=10 μM [ng/ml])

	pcDNAI/neo	pcDNAI/neo-11B1	pcDNAI/neo-11B2	pcDNAI- ALDO4
B	1.78 ±.0.95	133±13.0	29±22.0	3.97±2.00
18OH-B	0.841±0.046	0.998±0.039	104±5.49	2.29±0.251
ALDO	0.113±0.001	0.111±0.017	15.5±0.769	0.525±0.074

mutations, indicating the existence of normal CYP11B2 cDNA in the library. The cDNA library was prepared from the adrenal gland capsules of 4 outbred Sprague-Dawley rats and these cDNAs may result from variations between individual rats or different alleles.

The pCYP11B2/neo (PCR product) and pALDO-4 (from cDNA library) were transiently transfected with lipofectin into COS7 cells, a nonsteroidogenic cell line, and the transfected cells were incubated with 10μM of deoxycorticosterone. The conversion products were assayed by ELISA or RIA. As shown in table 1, both pCYP11B2/neo and pALDO-4 can convert DOC to aldosterone, 18-hydroxycorticosterone and corticosterone, although conversion by pALDO-4 was much less, while pCYP11B1 transfected cells could only produce conticosterone. The lower conversion rate of the pALDO-4 might be due to the long 5' untranslated region.

The cloning of 2 different CYP11B2 cDNAs has been reported (4,5). One of the clones had an extension peptide which was the same as that of the CYP11B1 (24 residues) ($P\text{-}450_{11\beta,aldo\text{-}2}$) and contained Lys at the 320th position in comparison to the CYP11B2 (($P\text{-}450_{11\beta,aldo\text{-}1}$) which contained a 34 residue extension peptide and Glu at the 320th position. The cDNA with the long extension peptide impaired the ability of the clone to be expressed in COS7 cells and only small amount of corticosterone was formed from DOC, but no aldosterone was detected (5). When the long extension peptide region was substituted with the 24 residue extension peptide, the transfected cDNA was expressed well. The cDNA containing Lys ($P\text{-}450_{11\beta,aldo\text{-}2}$) at the 320th position failed to show steroid converting activity. This clone could have be created by a PCR misincorporation of a nucleotide (5). RT-PCR using primers which incorporated the nucleotides coding for the 6 extra aminoacids shown in the isolated cDNA indicated that the long extended peptide was expressed in adrenal glands from Sprague-Dawley rats.

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