BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 86–89 (1996)
ARTICLE NO. 1761

Full-Length cDNA Sequence of a Progesterone Membrane-Binding Protein from Porcine Vascular Smooth Muscle Cells

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Received October 10, 1996

A full-length cDNA clone for a progesterone membrane binding protein from porcine vascular smooth muscle cells was isolated and the complete nucleotide sequence determined. The cDNA encodes a protein of 194 amino acids with a transmembrane segment. This protein is likely to represent the first steroid membrane receptor or a part of it for which sequence information is available.

The common assumption that steroid hormones penetrate into cells by diffusion, bind to intracellular receptors resulting in transcription of specific gene sequences (1) represents the classical, genomic theory of steroid action. A superfamily of intracellular steroid receptors has been defined (2,3) and extensively studied by techniques of molecular biology (4). However, during the past two decades, specific binding sites have been described in membranes for various steroids exposing pharmacological properties distinct from those of the intracellular receptors. These sites are discussed in the context of rapid non-genomic effects of steroids, for example the rapid stimulation of the Na^+/H^+ exchanger in human mononuclear leukocytes (HML) by aldosterone (5-7) or the rapid stimulation of ion fluxes in human sperm induced by progesterone (8-10). Moreover, in hepatocytes a rapid, progesterone induced increase of cytosolic Ca^{2+} has been described resulting from Ca^{2+} influx (11).

Recently, a progesterone specific binding protein from porcine liver microsomes has been identified, purified and partially sequenced (12). This protein is likely to represent the first putative steroid membrane receptor or a part of it for which sequence information is available. Here we describe a full-length cDNA clone encoding for this progesterone binding protein from porcine vascular smooth muscle cells.

MATERIALS AND METHODS

Reverse transcriptase-PCR and cDNA cloning. From known amino acid sequence (12) two degenerate oligonucleotide primers were synthesized: Oligo Chr. 1: 23mer, sense primer based on AEDVAATG (N-terminus) 5′-GCI GA(A/G) GA(T/C) GTI GCN GCN ACN GG-3′, Oligo Chr. 3: 26mer, anti-sense primer based on AINGKVFDV (CNBr-fragment) 5′-AC (A/G)TC (A/G)AA IAC (T/C)TT ICC (A/G)TT (A/G/T)AT NGC-3′. 2 μg of total RNA derived from porcine liver was reverse-transcribed using random primer and SuperscriptII reverse transcriptase (Gibco BRL, Eggenheim, FRG). PCR amplification was carried out in a total volume of 100 μl containing 20 μl of cDNA pool, 60 pmol of each degenerate oligonucleotide primer, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μm each dATP, dGTP, dCTP, dTTP, and 2.5 units of *Thermus aquaticus* DNA polymerase (Biometra). For PCR reaction the following conditions were used: 45 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. The amplified DNAs with a length of 290 bp were

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The sequence data reported in this paper have the accession number X99714 in the EMBL Data Library.

0006-291X/96 $18.00

subcloned into pUC 18 (Pharmacia) and sequenced by the dideoxy-chain termination method with a Sequenase Version 2.0 DNA sequencing kit (USB, Bad Homburg, FRG).

Screening of Uni-ZAP phage library. A porcine vascular smooth muscle cell Uni-ZAP XR cDNA library (Stratagene, Heidelberg, FRG) was screened. Labeled fragments used as hybridization probes were produced in PCR reactions with DIG labeled nucleotides (Boehringer Mannheim, FRG). Plaque hybridization and washing of the filter were performed following the Boehringer protocol. *In vivo* excision of positive clones was performed as described in the Stratagene protocol.

Northern blot analysis. For Northern hybridization analysis total RNA was isolated from various tissues using TRI-Reagent and poly(A)$^+$RNA was isolated using Oligo(dT)-Cellulose following the manufactures protocol (SIGMA-Aldrich Chemie, Deisenhofen, FRG). Poly(A)$^+$RNAs were separated by electrophoresis in a 1.2 % agarose formaldehyde, MOPS denaturing gel and transferred to a Nylon-membrane (Boehringer Mannheim, FRG). RNA blots were probed with a DIG-labeled cDNA-fragment of the isolated clone. The blot was prehybridized in hybridization solution (Boehringer Mannheim, FRG) and 50% formamide at 42°C for 4 h, then hybridized with the DIG-labeled probe in the same solution at 42°C overnight. The membrane was washed in 5×SSC/50% formamide at 68°C for 30 min. The washed blot was developed using chemiluminescence substrate Lumi-Phos 530 according to the manufactures protocol (Boehringer Mannheim, FRG). RNA molecular weight marker II (Boehringer Mannheim, FRG) was used as a reference.

RESULTS AND DISCUSSION

Screening of approximately 1×10^6 plaques yielded in four positive clones. The nucleotide sequence of the positive cDNA clone PgMEF6.1 and the corresponding amino acid translation of the coding part are shown in Fig. 1. The cDNA consists of a 5′ untranslated region of 41 bp and a 3′ untranslated region of 1228 bp followed by a poly(A) tail of 39 bp. The neighbouring sequence of the potential AUG initiation codon at base pair 42 is consistent with the consensus sequence proposed by Kozak (13). PgMEF6.1 cDNA contains an open reading frame coding for a protein of 194 amino acids with a calculated mass of 21.67 kDa. The amino acids downstream the first methionine are identical to the N-terminus of the partially sequenced progesterone membrane binding protein isolated recently (12). The amino acids of the CNBr-Fragment derived from that protein also are found in PgMEF6.1 beginning at position 91 of the deduced amino acid sequence.

Hydropathy analysis using the method of Kyte and Doolittle (14) as shown in figure 2 revealed a single putative 28 residue membrane spanning region between the amino acids 14 and 42. No potential N-glycosylation sites (N-X-S/T) have been found.

In a data base search (SwissProt, SWALL, EMBL/GenBank) no significantly identical protein was found except for a gene encoding a 25 kDa protein from rat liver. Latter yet unpublished protein, which became available by gene accession number (U63315) only while preparing this manuscript, shows 76% identity and is claimed to be 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible similar to the cytokine/growth factor/prolactin receptor superfamily.

The highly conserved cysteine rich central domain of the classical steroid receptors (15) is not detectable in PgMEF6.1. Also, the consensus polypeptide sequence of the steroid binding pocket (16) found in a number of steroidogenic enzymes, in sex steroid binding proteins, and in classical steroid receptors could not be found. However, this does not exclude the possibility of steroid binding as the same holds true for the corticosteroid binding protein which also lacks the consensus sequence for steroid binding (17).

In Northern blot analyses on mRNAs from a variety of different porcine tissues progesterone binding protein mRNA was found at 1.9 kb consistent with the length of the 1852 bp cDNA clone PgMEF6.1. The hierarchy of expression levels is liver $>$ kidney $>$ lung $>$ cerebellum $>$ spleen $\cong$ heart (Fig. 3). These data correspond to Western blots using polyclonal antibodies against the N-terminus for the detection of the protein and ^{3}H progesterone maximum binding capacities in microsomal membrane preparations of the same porcine tissues (18). Optical densities and maximum binding capacities (liver: 3.8; kidney: 1.13; lung: 0.73; cerebellum: 0.4; heart: 0.55; spleen: 0.58 pmol/mg protein) are closely correlated by linear regression ($r = 0.85$, $p < 0.05$).

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                                                    GAAACGAGTTCCGGAGCCCTCCAACCTTTGCTCCAGCGATC    -1
ATGGCTGCCGAGGATGTGGCGGCTACCGGCGCCGACCCGAGCGAGCTAGAGGGCGGCGGGCTGCTGCATGAGATT    75
 M  A  A  E  D  V  A  A  T  G  A  D  P  S  E  L  E  G  G  G  L  L  H  E  I     25

TTCACGTCGCCGCTCAACCTGCTGCTGCTCGGCCTCTGCATCTTCCTGCTCTACAAGATCGTGCGCGGGGACCAG   150
 F  T  S  P  L  N  L  L  L  L  G  L  C  I  F  L  L  Y  K  I  V  R  G  D  Q     50

CCGGCGGCCAGCGATAGCGACGACGACGAGCCGCCCCCGCTGCCCCGCCTTAAGCGGCGCGACTTCACCCCTGCC   225
 P  A  A  S  D  S  D  D  D  E  P  P  P  L  P  R  L  K  R  R  D  F  T  P  A     75

GAGCTGCGTCGCTTCGACGGCGTCCAGGACCCGCGTATACTCATGGCCATCAACGGCAAGGTGTTCGACGTGACC   300
 E  L  R  R  F  D  G  V  Q  D  P  R  I  L  M  A  I  N  G  K  V  F  D  V  T    100

AAAGGCCGCAAGTTCTACGGGCCCGAGGGGCCGTACGGGGTCTTTGCTGGAAGAGACGCATCCAGGGGCCTGGCC   375
 K  G  R  K  F  Y  G  P  E  G  P  Y  G  V  F  A  G  R  D  A  S  R  G  L  A    125

ACGTTTTGCCTGGATAAGGAAGCCCTGAAGGACGAGTATGATGACCTTTCTGACCTCACTCCTGCCCAGCAGGAG   450
 T  F  C  L  D  K  E  A  L  K  D  E  Y  D  D  L  S  D  L  T  P  A  Q  Q  E    150

ACCCTGAATGACTGGGACTCTCAGTTCACTTTCAAGTACCATCACGTGGGCAAACTGCTGAAGGAGGGGGAGGAG   525
 T  L  N  D  W  D  S  Q  F  T  F  K  Y  H  H  V  G  K  L  L  K  E  G  E  E    175

CCCACCGTGTACTCAGATGAGGAAGAGCCCAAAGATGAGAGCGCTCGGAAAAATGATTAAAGCGTTCGGTGGAAG   600
 P  T  V  Y  S  D  E  E  E  P  K  D  E  S  A  R  K  N  D  *                   194

CATATCTATTTTTGTATTTTGCAGAATCATTTGTAACATTCCAGTCTGTCTCTAAAACATGGTGATTTCAATATT   675
TAGAAGAGTTTTGAACTTGCTGTACATTTTTTTAATTAACATCACTAGTGACAGTGATAAAGCTAACCTCTGTCT   750
TAGACTGCATGATGTGTTTGTGTGTCACAAATCCAGAACGTGAACTGCAGTGCTGTAATACAGACGTTATTACTG   825
TTTTTTCTTCTATCCATAGTTAGTACAGGCTGAATTTGGATTCGTTTTTTGCTTGAGAGACAGGTAAGACGTGGG   900
TATTTCCCCCCAAACAGGTAAAAATGTTAAACGTGCAAAGTCCTTCAAGCACCAAGGAACAGGGGATCAACTTTT   975
AGTCGTGAGGTTCTCTAAATGAGACAGCAAATCCCTTATTTCTCAATTGACCCAACTGCATGATTTCTGTTTGAT  1050
CTACCTCTAAAGCAAATCTGCAGTGTTCCAAAGGCTTTGGTATCGATTAGAGAGCTCTCCAGAAACAAAATGAAA  1125
TCTCAAAGCTGGGGCTCTGTCGGAAGCAGTTTCCTTGCCGTATTTTCATACTGCATCGCTGTCCTTGTCCACCCT  1200
GGGACACTCAGAGGCCCTCACCAACGTAGTCATCCACGGTGAGGACTAAAGCGGGACCTGAGCACCCAGTGGCGA  1275
GCCCTGGGGACAAAATCAGCCGGCTTGCCTAGAAACCGAAATATACGAGGAAGATTGAAGTCCTATCTTCCCTTT  1350
CCCCGTGTTCACGGCGGGGCAGTGGTTCTTTCACACGATTGGACAGAGAGAGGGGCCCCGCAGAGTAGGACCGGG  1425
CGGTGGCTCTCCTAAGAGCCTCCCCCCACACCCCCAAAGCTCAAGGGAACAGTACGCATTACCAGCCAGGCGGTT  1500
TTTAAAGTAAAGCGTATTCATAAAGTGAACAGTTACTCTTCTGTTACAAAACTTGTAGCCACTGCAAGAGTAGTA  1575
AGCGTCTTGGTCTTTGATATTGGTCGTCTTTTGGTTAACAATAAACTTAGCTGAAGTAGACTGTACAGTTGTTTG  1650
AATTGTAAAAGTATTATATGAACAACTAGTGAGGTTTCAAACTCTTGTAATTGTAGTTGAATAGTCATTGTATTT  1725
TTCTTGTGAACCGTGTTTAATGGTTTTACCTCAAATCAGAAAACAAAATGAAGTGCTTTGGTCAGTTAATAAAAT  1800
GGTTTTACCCGGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                          1852
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the cDNA clone PgMEF6.1 coding for the putative progesterone membrane binding protein. Start and stop codons are highlighted by bold letters. The transmembrane domain is indicated by a single underline.

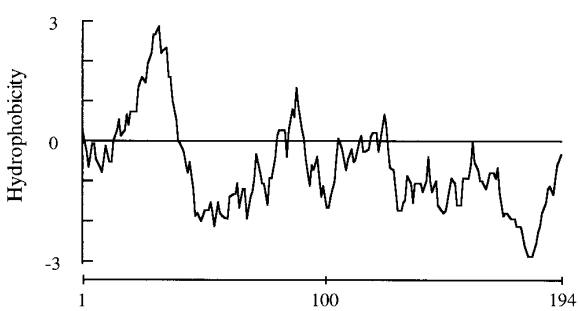

FIG. 2. Hydrophobicity analysis of the deduced amino acid sequence of the cDNA clone PgMEF6.1 according to the method of Kyte and Doolittle (15).

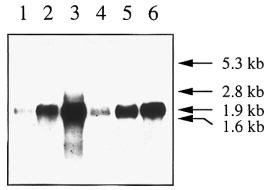

FIG. 3. Northern blot analysis of the progesterone binding protein mRNAs in preparations from different porcine tissues. Lane 1: spleen; lane 2: cerebellum; lane 3: liver; lane 4: heart, lane 5: lung; lane 6: kidney. 5 μg of mRNA were immobilized on nylon-membrane and hybridized with a DIG-labeled PgMEF6.1 cDNA-probe. The sizes of the marker bands are indicated.

In conclusion a cDNA encoding a progesterone membrane binding protein was isolated from a porcine vascular smooth muscle cell cDNA library. Its primary structure and further analysis will be essential to understand rapid nongenomic steroid action through putative membrane receptors.

ACKNOWLEDGMENTS

We thank K. Sippel and K. Pasentsis for expert technical assistance. This study was supported by the *Deutsche Forschungsgemeinschaft* (We 1184/4-2, Sc 4/9-4).

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