Human kidney amiloride-binding protein: cDNA structure and functional expression

(Primary sequence/epithelium/Na⁺ transport)

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ABSTRACT Phenamil, an analog of amiloride, is a potent blocker of the epithelial Na⁺ channel. It has been used to purify the porcine kidney amiloride-binding protein. Synthetic oligonucleotides derived from partial sequences have been used to screen a human kidney cDNA library and to isolate the cDNA encoding the human amiloride-binding protein. The primary structure was deduced from the DNA sequence analysis. The protein is 713 residues long, with a 19-amino acid signal peptide. The mRNA was expressed in 293-S and NIH 3T3 cells, yielding a glycoprotein (i) that binds amiloride and amiloride analogs with affinities similar to the amiloride receptor associated with the apical Na⁺ channel in pig kidney membranes and (ii) that is immunoprecipitated with monoclonal antibodies raised against pig kidney amiloride-binding protein.

High-resistance epithelia actively transport Na⁺ from the luminal side to the blood. Regulation of the Na⁺ transport activity is an important component of salt and fluid homeostasis. Na⁺ transport is controlled by hormones and blocked by amiloride (1). Amiloride is an important diuretic drug that has a dual action on Na⁺ transport by the kidney. It blocks a Na⁺/H⁺ exchange system (2) and a Na⁺-selective ionic channel (3) that are located in the proximal and distal parts of the tubule, respectively. Amiloride derivatives such as phenamil and benzamil are potent blockers of apical Na⁺ channels at concentrations at which they have very little (if any) activity on the Na⁺/H⁺ exchanger (4, 5). [¹H]Phenamil and [¹H]romemamil have been used to purify and affinity label the amiloride-binding protein (ABP) associated with the epithelial Na⁺ channel (6, 7). It is composed of two apparently identical and disulfide-bonded subunits of about 100 kDa.

This paper describes (i) the isolation of the human kidney ABP cDNA and the primary structure of the corresponding protein and (ii) the successful expression of this cDNA in transfected mammalian cells.¶

MATERIALS AND METHODS

Protein Purification and Amino Acid Sequence Analysis. ABP was isolated from frozen pig kidneys as described (7); 1.5 nmol of the purified peptide was used for preparative sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) on an isocratic 7.5% running gel (8). The receptor was electroeluted according to ref. 9 and then concentrated by partial lyophilization. After removal of SDS (10), ABP (35 μg) was redissolved in 50 μl of 0.5 M urea. The protein sample was then digested for 30 hr with 5 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin in two steps at 37°C under argon.

Tryptic peptides were separated by HPLC using an analytical RP300 5-Å column of 1-mm diameter (Brownlee) equilibrated in water containing 0.1% triethylamine, 0.05% trifluoroacetic acid, and 0.001% 2-mercaptoethanol. Elution was performed in 6 hr with a linear gradient of acetonitrile from 0 to 90% at a flow rate of 80 μl/min. Fractions were collected in Pico-Tag glass tubes following the absorbance at 214 nm. Edman degradation was performed with an Applied Biosystems 470A microsequencer, and phenylthiohydantoin-amino acids were identified using an on-line analyzer (Applied Biosystems 120A).

For determination of the N-terminal sequence of ABP, 100–150 pmol of the receptor was submitted to SDS/PAGE analysis. The protein was then transferred onto Polybrene-coated glass fiber sheet by electroblotting (11) and submitted to Edman degradation.

Cloning and Sequencing of the Receptor. Agt10 cDNA libraries were constructed using human adult kidney mRNA as described (12). Initial cDNA clones were identified with a synthetic oligonucleotide based on the sequence of tryptic fragment 3 (Fig. 1) and codon frequency statistics (13). The oligonucleotide sequence was 5'-ACCACAGCTCCTCTTGGCGATGAGCATGAGCAGGAGCTGACCTTCACA-GATGTCGCCCGG-3'. The cDNA insert [500 base pairs (bp)] of the first isolated ABP clone was used in subsequent screening.

Single-stranded DNAs were sequenced by the dideoxy chain-termination method (14). Sequence data were aligned, decoded, and examined using BISANCE sequence analysis programs (C.I.T.I.2 Paris), including programs for hydropathy and secondary structure analysis (15–17) and searches in the National Biomedical Research Foundation (release 22) and GenBank (release 61) data bases.

Transfection of the cDNA into Eukaryotic Cell Lines. The complete 2.4-kilobase (kb) cDNA insert was ligated into the cloning site of the mammalian expression vector pRK5, cotransfected with a plasmid containing the neomycin resistance gene into human 293-S and NIH 3T3 cells using the calcium phosphate technique (18), and then selected for resistance to G418 at 500 μg/ml. Neomycin-resistant cells were analyzed by Western blotting with appropriate antibodies and by [¹H]phenamil binding (4).

Biochemical Characterization of the Transfected Receptor. Western blotting. A polyclonal serum that recognizes the

Abbreviation: ABP, amiloride-binding protein.

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porcine protein was obtained after immunization of a rabbit by injection of 30 μg of highly purified porcine ABP in the popliteal lymph node (19). The different fractions were run onto SDS/PAGE, transferred for 2 hr on a Hybond C membrane, and analyzed according to ref. 20.

**Immunoprecipitation with the monoclonal antibody 452B.** A monoclonal antibody that precipitates the [³H]phenamil-binding protein from solubilized pig kidney membranes (not shown) was used to identify the transfected protein after in vivo labeling of the cells. The cells were pulse-labeled (10 min) in Dulbecco-modified Eagle medium (DMEM) without methionine supplemented with 40 μCi (1 Ci = 37 GBq) of [³⁵S]methionine per 35-mm dish and then “chased” during various incubation times. At the end of the labeling time, cells were washed several times with unlabeled phosphate-buffered saline and solubilized by 500 μl of a buffer containing 1% Nikkol, 200 mM NaCl, 5 mM MgCl₂, 0.5 mM glutathione, 10 μg of aprotinin per ml, 10 μg of trypsin/chymotrypsin inhibitor per ml, and 40 μg of phenylmethylsulfonyl fluoride per ml. The mixture was centrifuged for 45 min at 100,000 × g, incubated with 200 μl of standardized Pansorbin, and centrifuged for 5 min at 4°C. The antibody was added to the supernatant at the appropriate dilution and incubated at 4°C. After 1 hr, 300 μl of a 10% suspension of protein A-Sepharose was added for 15 min, and at least seven washings with the same buffer were performed. Samples were heated in the denaturation buffer at 95°C for 5 min and electrophoresed. After Coomassie staining, gels were soaked for 30 min in 1 M sodium salicylate, dried, and autoradiographed at −70°C with Kodak X-Omat AR film.

**RESULTS AND DISCUSSION**

**Partial Amino Acid Sequence and Cloning.** The phenamil-binding protein is a single 105-kDa peptide (Fig. 1A, lane a). After an electroelution step, the receptor (Fig. 1A, lane b) was digested with trypsin. Fig. 1B shows a typical separation of tryptic peptides. Several well-separated peaks (from two independent experiments) yielded unambiguous sequence information shown in Fig. 1. Synthetic oligodeoxyribonucleotides were prepared on the basis of these partial sequences (13). Screening of a 5 × 10⁶-codon cDNA library from human adult kidney in Agt10 with a 200-base-long probe derived from the tryptic peptide 3 resulted in four identical 500-bp clones that contained the expected ABP sequence. The isolated cDNA fragment was subsequently used for screening 1.5 million clones of the second cDNA library. Twelve recombinant phage of about 50 hybridizing positives were analyzed and the two largest cDNA inserts (about 2.4 kbp) were subjected to nucleotide sequence analysis. The two clones (named ABP1 and ABP2) were essentially identical but the ABP2 clone was 29 bp longer on the 5’ end.

**ABP cDNA Coding Sequence.** Fig. 2 shows the 2396-nucleotide sequence of the cDNA encoding the phenamil receptor from human kidney. The initiation site was assigned to the methionine codon composed of nucleotide residues 1–3 because it is the first ATG triplet that flanks functional initiation codons (21). There is an open reading frame of 713 codons. The different peptides characterized by microsequencing of porcine ABP were identified within the deduced human ABP amino acid sequence. The few sequence differences are probably due to species differences (Fig. 1).

The N-terminal sequence of porcine ABP identified by Edman degradation is Glu-Pro-Ser-Pro. This sequence appears at residue 20 (Fig. 2). It is situated after a hydrophobic 19-amino acid stretch that shows all of the typical features of a signal sequence (22). It seems therefore that the isolated cDNA encodes the complete sequence of the protein that we have purified. This was confirmed by RNA blots of human kidney showing that the ABP mRNA has a size of 2.4 kb (Fig. 3), in good agreement with the length of the insert that has been characterized. From the cDNA sequence, one can then conclude that human ABP is composed of 694 amino acid residues once the signal peptide has been released. The calculated molecular mass of the mature peptide is 78,886 daltons. There are three potential N-glycosylation sites at residues 110, 168, and 536.

RNA blot patterns presented in Fig. 3 indicate that ABP mRNA from mouse and pig kidneys have sizes that are substantially larger (4.4 kb and 3.3 kb, respectively) than those of human ABP mRNA (2.4 kb).

**ABP Expression by Transfection in Eukaryotic Cells.** A eukaryotic expression vector was constructed with the DNA coding for the complete sequence. This plasmid was used to transfect 293-S and NIH 3T3 cells. After selection, the clones
were recovered with the help of cloning disks. The different clones were expanded and tested for the presence of the phenamil receptor by Western blotting with a polyclonal antibody raised against the pig kidney protein. All of the different clones selected with Novobiocin expressed a protein that appeared in SDS/PAGE as a 97-kDa peptide (in reducing conditions) (see Fig. 5, lane b). The different clones were then tested for the presence of [3H]phenamil-binding sites. Typical experimental results are shown in Fig. 4. Equilibrium binding experiments (Fig. 4A) demonstrated the presence in membranes of transfected cells of a high-affinity binding site characterized by a dissociation constant (Kd) of 2.5 nM and a maximum number of binding sites of 11.3 pmol/mg of protein. The 97-kDa peptide was not detected by Western blotting (Fig. 5, lane a) in nontransfected cells. No specific [3H]phenamil binding was found in control cells. All selected clones expressed a receptor with the same characteristics—i.e., a Kd value for [3H]phenamil binding of about 2.5 nM and a molecular mass of 97 kDa. Phenamil binding was reversible and rates of association (Fig. 4B) and dissociation (Fig. 4C) have been measured. Their values (Kd = 4.1 × 10^6 M^-1 s^-1, k2 = 2.9 × 10^10 s^-1) are in good agreement with values previously found with pig kidney membranes (Kd = 6.4 × 10^6 M^-1 s^-1, k2 = 2.45 × 10^10 s^-1 (24)).

Labeling with [35S]methionine and immunoprecipitation with a monoclonal antibody raised against pig kidney ABP was used to confirm the presence of the phenamil receptor in membranes from transfected cells. The pharmacological profile obtained with different ligands of the amiloride family is presented in Fig. 4D. The true dissociation constants (calculated according to ref. 4) are Kd(phenamil) = 2.5 nM, Kd(benzamil) = 10 nM, Kd(ethylpropylamiloride) = 50 nM, and Kd(amiloride) = 100 nM. Corresponding values previously found in intact pig kidney membranes (24) are Kd(phenamil) = 1 nM, Kd(benzamil) = 50 nM, and Kd(amiloride) = 100 nM. Thus, it is clear that this pharmacological profile of the amiloride receptor in cells transfected with human kidney ABP cDNA is similar to that found in original pig kidney membranes.

Labeling with [35S]methionine and immunoprecipitation with a monoclonal antibody raised against pig kidney ABP...
revealed a single peptide at 97 kDa (Fig. 5, lanes c and e). Under nonreduced conditions, the protein migrated at about 180 kDa (not shown). These properties are identical to those of the purified ABP from pig kidney. In 293-S cells, the half-life of ABP biosynthesis was about 30 min.

ABP is a glycoprotein (7). The extent of glycosylation was investigated by treating cells for 16 hr with 20 μg of tunicamycin per ml, an inhibitor of the N-glycosylation process. This treatment led to the disappearance of the 97-kDa peptide and to its replacement by a 70-kDa species (Fig. 5, lanes d and f).

**Protein Structure.** The primary structure of the ABP has no sequence homology with any known protein, including the previously cloned receptors or ion channels. The protein is rich in prolines (64 prolines in 713 residues—i.e., 9% of the total amino acids), especially in two regions. A first region, between Cys-267 and Pro-326, is composed of 22% prolines (13/60) and contains 7% cysteines. Negative residues (Asp and Glu) are underrepresented in this part of the sequence, which has a large positive charge at physiological pH. This domain is largely hydrophilic. A high density of prolines is also observed (12/72 = 16%) in the C-terminal domain (from Pro641 to Leu713), in which basic residues (Lys, Arg, and His) are rare and which has a large negative charge at physiological pH. The hydropathicity profile is not similar to that of membrane-spanning proteins, which usually display numerous hydrophobic helices (not shown).

Although transfection experiments have clearly shown the existence of a well-characterized amiloride/phenamil-binding protein in the cell membrane fraction, no amiloride-sensitive Na+ channel activity was observed using patch-clamp techniques in transfected cells (not shown). Moreover, although George et al. (25) have recently described the expression of an amiloride-sensitive Na+ channel in Xenopus oocytes microinjected with total mRNA, we also failed to observe a Na+ channel signal after injecting ABP cRNA into oocytes. These results suggest that although the cloned protein has amiloride-binding sites with pharmacological features of the kidney Na+ channel, it may not code for the channel structure. The amiloride-sensitive Na+ channel may require other distinct protein subunits for functional expression. Benos et al. (26) have suggested that the apical Na+ channel present in a Xenopus kidney cell line (A6) is a multisubunit assembly of 315-, 149-, 95-, 71-, 55-, and 35-kDa peptides. The amiloride-binding component and the channel subunit might be distinct. Since it has been previously shown (7) that reconstitution of purified ABP displays 22Na+ transport activity, such an interpretation would suggest that other subunits present in the purified material were not detected on gels but are important in the Na+ transport activity.

Another interpretation for the absence of channel activity in transfected cells would be that alternative splicing has removed a short but important transmembrane protein segment that would be essential for Na+ channel function. A similar situation has been recently observed for the human interleukin 7 receptor (27).

Numerous subtypes of voltage-dependent ion channels and of ion channels coupled to neurotransmitter receptors have
been identified (28). Similarly, there is a family of subtypes of amiloride-sensitive Na\(^+\) channels that slightly differ by their conductance, their ionic selectivity, and their affinities for amiloride and its analogs. Amiloride-sensitive Na\(^+\) channels are important for kidney function but they are also present in frog skin (29), in urinary bladder (30), in thyroid cells (31), in blood–brain barrier endothelial cells (32), and in colonic enterocytes (33). They have a role in sensory perception. Amiloride-blockable Na\(^+\) channels seem to provide the transducer mechanism for the "salty" taste (34) and for the response to odorants of receptor cells in the nasal mucosa (35).

Amiloride-sensitive Na\(^+\) channels are present in airway epithelia, where they play an important role in fluid secretion (36). Amiloride inhibits the excessive absorption of Na\(^+\) and liquid that takes place in airway epithelia of patients with cystic fibrosis (CF) (37), and amiloride aerosol therapy is being tried for the treatment of lung disease in CF (38). It turns out that we have recently observed that the structural gene for ABP is situated on human chromosome 7 in a region that flanks the CF locus (39).

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