Identification of a Novel Ankyrin Isoform (AnkG190) in Kidney and Lung That Associates with the Plasma Membrane and Binds α-Na,K-ATPase*

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Ankyrins are a family of adapter molecules that mediate linkages between integral membrane and cytoskeletal proteins. Such interactions are crucial to the polarized distribution of membrane proteins in transporting epithelia. We have cloned and characterized a novel 190-kDa member of this family from a rat kidney cDNA library, which we term AnkG190 based on the predicted size and homology with the larger neuronal AnkG isoform. AnkG190 displays a unique 31-residue amino terminus, a repeats domain consisting of 24 repetitive 33-residue motifs, a spectrin binding domain, and a truncated regulatory domain. Probases derived from the unique amino terminus hybridize to an 8-kilobase message exclusively in kidney and lung and specifically to the kidney outer medullary collecting ducts by in situ hybridization. Transfections of Madin-Darby canine kidney and COS-7 epithelial cell lines with a full-length hybridization. Transfections of Madin-Darby canine kidney and COS-7 epithelial cell lines with a full-length hybridization. Transfections of Madin-Darby canine kidney and COS-7 epithelial cell lines with a full-length hybridization. Transfections of Madin-Darby canine kidney and COS-7 epithelial cell lines with a full-length hybridization.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF086925.

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‡ The abbreviations used are: kb, kilobase pair(s); MDCK, Madin-Darby canine kidney; PCR, polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; DEPC, diethylpyrocarbonate; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid.
the unique amino terminus hybridize to an 8-kb message exclusively in kidney and lung, and cRNA probes derived from the same unique sequences hybridize specifically to kidney outer medullary collecting duct cells by in situ hybridization. Transfections of MDCK (Madin-Darby canine kidney) and COS-7 epithelial cell lines with full-length AnkG190 constructs result in (a) expression at the lateral membrane, (b) functional assembly with the cytoskeleton, and (c) interaction with at least one integral membrane protein, namely Na,K-ATPase. These findings significantly extend the known diversity of ankyrin isoforms, directly confirm the interaction of AnkG190 with Na,K-ATPase, and suggest that ankyrins may interact with integral membrane proteins in a complex, pleiotropic manner that requires complex tertiary structural determinants not easily deducible from the primary structure.

**EXPERIMENTAL PROCEDURES**

**Isolation of the AnkG190 cDNA—**All molecular biologic procedures were carried out using standard methods (22). Oligonucleotides bracketing a conserved 525-bp region extending from repeat 11 to 16 of human erythrocyte and brain ankyrins (7, 9, 10) were used in a high stringency PCR reaction (23), with rat kidney RNA reverse-transcribed with random hexamer priming and avian myeloblastosis virus reverse-transcriptase (Boehringer Mannheim) as template. The sense primer was GGCTTTACCCCCATACATGCTGCTAAAAGAACAG and the antisense primer was CTGTTGCGCGCACTGAGGTTATCATC CTTCTTGTG. This yielded a single 525-bp product which was subcloned into the TA vector (Invitrogen) and sequenced in both directions. Three full-length clones were isolated, which were identical in sequence and differed only in the length of the 5'-untranslated region; the largest clone was completely sequenced in both directions.

**Northern Blot Analysis—**Oligonucleotides targeted to the unique amino terminus of AnkG190 (Fig. 1) were used in standard PCR reactions, with the full-length clone as template. The sense primer was GGAGACCGCGACCGCGAGAG (from within the 5'-untranslated region) and the antisense primer was TCGTGAATACAGGATCTTTCTTG (corresponding to residues 19–28). The 146-bp fragment was subcloned into the TA vector (Invitrogen) and sequenced (26). Immunodetection of transferred proteins was by enhanced chemiluminescence (Amersham Pharmacia Biotech). Antibody to non-erythroid spectrin binding domain of ankyrins (13). Finally, the blot was probed with actin as a control for RNA loading.

**In Situ Hybridization—**In situ hybridizations were performed as described previously (34). The 146-bp PCR product containing sequences unique to AnkG190 was subcloned into the TA vector (Invitrogen) in both directions, to yield sense and antisense constructs. 35S-labeled antisense and sense cRNA probes were generated by in vitro transcription with T7 RNA polymerase (Promega, Madison, WI) in the presence of [35S]s-thio-UTP (NEN Life Science Products, 1100 Ci/mol).

Rats were sacrificed by decapitation, and the kidneys were snap-frozen in O.C.T. compound (Sakura Finetechical, Tokyo). Sections (15 μm) were cut in a cryostat, mounted onto slides, air-dried for 15 min, and stored at –80 °C. For prehybridization, slides were warmed to room temperature and fixed in fresh 4% paraformaldehyde, pH 7, in PBS treated with 0.1% diethylpyrocarbonate (PBS-DEPC). Slides were rinsed in PBS-DEPC (2 × 5 min), deproteinized in 0.2% n HCl for 20 min, rinsed in PBS-DEPC (1 × 5 min), and acetylated by immersion in 0.1 M triethanolamine in DEPC/water (pH 8.0, 2 min), followed by addition of acetic anhydride (0.25% final concentration) for 20 min. Slides were then rinsed in PBS-DEPC (1 × 5 min), dehydrated through ascending alcohols, air-dried, and used immediately. For hybridization, sections were covered with 40 μl of hybridization buffer containing 1.5 × 10⁷ cpm/ml. The hybridization buffer contained 50% formamide, 10% w/v dextran sulfate, 2% Denhardt’s solution (0.04% each polyvinylpyrrolidone, bovine serum albumin, Ficoll), 0.9 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, 0.1% SDS, 100 μM dithiothreitol, 500 μg/ml herring sperm DNA, 500 μg/ml yeast total RNA. Sections were then covered with glass coverslips and placed in humidified chambers overnight at 53 °C. The following morning, the coverslips were removed in 2 × SSC, and the slides were washed in 2 × SSC (30 min, room temperature), incubated in RNase A (10 μg/ml in 0.5 M NaCl, 10 mM Tris-HCl, pH 8) for 30 min at 37 °C, and washed in 2 × SSC (30 min, room temperature), 0.1× SSC (twice for 30 min at 53 °C), and 0.1× SSC (twice for 30 min at room temperature). Slides were then dehydrated through ascending alcohols containing 0.3 M ammonium acetate and air-dried. Film autoradiograms were generated by apposing slides to Kodak SB-5 x-ray film for 7 days.

**Transfection of AnkG190 Constructs—**PCR techniques were utilized to create a series of AnkG190 expression constructs, each containing the eight-residue FLAG tag (IBI Biosystems) added to the carboxyl terminus. Primes were targeted to AnkG190 residues as shown in Fig. 7A; the FLAG sequence and a stop codon were added to the 3′ end of each antisense primer. PCR products were verified by sequencing, subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen), and transfected into MDCK and COS-7 cells either transiently or stably with G418 (400 μg/ml) selection (Life Technologies, Inc.). Subconfluent cells were exposed to the DNA-LipofectAMINE complex in Opti-MEM (Life Technologies, Inc.) for 6 h, and the transiently transfected cells were analyzed upon reaching confluence, after an additional 48 h incubation in high serum growth medium.

**Extractions, Immunofluorescence, and Immunoprecipitations—**Both MDCK and COS-7 cells, transiently transfected with FLAG-tagged full-length AnkG190 in pcDNA3, were grown to confluence in 150-cm² culture flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and sequentially extracted in situ as described (13, 19). Briefly, cells were first extracted for 10 min at 4 °C in buffer 1 (yielding fraction 1) containing 10 mM Pipes, pH 6.8, 100 mM NaCl, 500 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, and protease inhibitors. The second extraction (yielding fraction II) was for 20 min at 4 °C in buffer 2 containing 250 mM ammonium sulfate instead of NaCl and 1% Triton X-100. Equal amounts of total proteins in each extract were analyzed by SDS-PAGE and Western blotting with various antibodies, to determine their distribution in the soluble (fraction 1) or cytoskeletal (fraction 2) fractions.

MDCK cells stably transfected with the full-length AnkG190-FLAG fusion construct were grown to confluence on coverslips and processed for indirect immunofluorescence microscopy as described (19, 24). All incubations were at room temperature. Cells were washed with PBS, fixed, and permeabilized with 100% acetone for 20 min, blocked with normal goat serum for 60 min, and incubated first with the M2 monoclonal antibody against FLAG (IBI Biosystems) for 1 h and then with rhodamine-conjugated goat anti-mouse secondary antibody for 30 min (Amersham Pharmacia Biotech). The coverslips were mounted on slides with Crystalmount (Biomeda) and viewed with a microscope equipped for epiillumination (IX70, Olympus).

COS-7 cells transiently transfected with each of the five AnkG190 constructs were grown to confluence in six-well plates and processed for immunoprecipitations as described (24). Cells were lysed for 20 min at 4 °C in 2 ml of IP buffer (10 mM Tris-HCl, pH 7, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Pefablock), the lysate was centrifuged for 1 min at 10,000 × g, and precleared by a 60-min incubation at 4 °C with 25 μl of nonimmune serum and 200 μl of a 50% protein A-Sepharose solution (Amersham Pharmacia Biotech). The cleared supernatant was incubated for 4 h at 4 °C with 10 μg of the M2 monoclonal antibody against FLAG and for an additional 2 h at 4 °C with 200 μl of a 50% protein A-Sepharose solution. The lysate was centrifuged for 1 min at 10,000 × g, washed three times with IP buffer, and the pellet subjected to SDS-PAGE and Western analysis with a monoclonal antibody against a-ankyrin (a-ATPase [Upstate Biotechnology]).

**RESULTS**

**Isolation and Characterization of a Novel Ankyrin (AnkG190) from Rat Kidney—**A comparison of cDNA sequences encoding human erythrocyte (7, 27) and brain (10) ankyrins revealed a
FIG. 1. Nucleotide and deduced amino acid sequence of Ank_{G190}. The start of each repeat is marked. The spectrin binding domain (starting with residues EDAIT) is in italics. The regulatory domain (starting with ALRK) follows the spectrin binding domain. The putative polyadenylation site is underlined.
AnkG190 Is Expressed Exclusively in Kidney and Lung—Plasmatic membrane ankyrin isoform (AnkG190) is expressed exclusively in kidney and lung. This region of high conservation extending from repeat 11–16. Oligonucleotides bracketing this region were used in a PCR reaction with reverse-transcribed rat kidney RNA as template, yielding a 525-bp PCR product whose amino acid sequence was only 55% homologous to red cell or brain ankyrin. Using the PCR product as a probe, three full-length clones were isolated from a rat kidney cDNA library, which were identical in sequence and differed only in the length of the 5'-untranslated region. The largest clone provided a 6134-bp contiguous cDNA sequence for AnkG190 (Fig. 1, GenBank™ accession number AF069525), with a putative polyadenylation signal (AATAAA) approximately equal loading of message in all lanes (not shown).

Collectively, these data suggest that AnkG190 is an alternatively spliced, tissue-specific isoform of the Ank3 family.

AnkG190 Is Expressed by the Outer Medullary Collecting Duct Cells of the Rat Kidney—In situ hybridization of rat kidney tissue sections with an antisense cRNA probe generated from the unique amino-terminal sequences of AnkG190 showed that it is distributed primarily in the outer medullary region, indicating that it is expressed by the outer medullary collecting duct cells (Fig. 4). The sense cRNA probes did not hybridize at all to the same region (not shown). These results are comparable to those recently obtained for mouse epithelial Ank3, the expression of which was found to be predominantly in the outer medullary collecting ducts (12).

AnkG190 Assembles with the Cytoskeletal Fraction, at the Lateral Membrane of Transfected Cells—Since antibodies that specifically recognize AnkG190 were not available, we used transfected MDCK (dog kidney collecting duct cell line) and COS-7 (monkey kidney) cells to examine the biochemical and cellular properties of an AnkG190-FLAG fusion construct. In situ extraction (13, 19) followed by Western blot analysis with FLAG antibody revealed that in both cell types, the transiently transfected AnkG190 assembled predominantly in the cytoskeletal fraction II, or the Triton-inextractable, cytoskeletal fraction of the rat kidney plasma membrane in these cells, as expected (not shown) (13, 16).

The association of AnkG190 with the plasma membrane by extractions and immunofluorescence microscopy. In confluent, polarized MDCK cells stably transfected with the AnkG190-FLAG fusion construct, the antibody against AnkG190-FLAG was found to be predominantly localized to the lateral membrane in these cells, as expected (not shown) (13, 16). The association of AnkG190 with the plasma membrane-associated spectrin was confirmed by immunofluorescence microscopy. In confluent, polarized MDCK cells stably transfected with the AnkG190-FLAG fusion construct, the antibody against AnkG190-FLAG was found to be predominantly localized to the lateral membrane in these cells, as expected (not shown) (13, 16).
AnkG190 displays a unique amino terminus, a highly conserved repeats domain and spectrin binding domain, and a conserved repeats domain. The initial cloning and characterization of red cell ankyrin, and the elucidation of its characteristic repeats domain of 24 tandemly arrayed 33-residue motifs (7, 27), lead to the postulate that different repeats, singly or in combination, constitute the binding sites for integral membrane proteins. Recent evidence suggests that this membrane binding domain consists of four independently folded subdomains of six repeats each (28). Thus, in in vitro assays, both the voltage-dependent sodium channel (29) and the anion exchanger (30–32) bind red cell ankyrin predominately in the outer medullary region, consistent with the distribution of collecting ducts.

**DISCUSSION**

Ankyrins are critical adapter molecules that are thought to maintain the polarized distribution of integral membrane proteins by mediating their linkage with the underlying spectrin-based cytoskeleton (4–6). Renal epithelial cells express a prominent 190-kDa ankyrin polypeptide (13, 15–18); data with cross-reacting antibodies have suggested that it co-distributes with spectrin and Na,K-ATPase along the basolateral membrane (3, 4, 15–18, 20, 21). The present study identifies the primary structure of this novel ankyrin which is expressed exclusively in kidney and lung, termed AnkG190 based on its predicted size and homology to the larger neuronal AnkG isoform (10). AnkG190 displays a unique amino terminal sequences of AnkG190, and Na,K-ATPase. Indeed, immunoprecipitations with FLAG antibody of COS-7 cells transiently transfected with the full-length AnkG190-FLAG fusion construct (construct I, Fig. 7A), followed by Western analysis with antibody against α-Na,K-ATPase, revealed a functional complex between these proteins. Furthermore, a similar analysis using a series of AnkG190 constructs showed that both the distal 12 repeats (construct III) and the spectrin binding domain (construct IV) were capable of interacting with α-Na,K-ATPase, whereas the proximal 12 repeats and the regulatory domain were devoid of Na,K-ATPase binding activity.

**FIG. 3.** *AnkG190 mRNA expression is restricted to kidney and lung.* A rat multiple tissue Northern blot (CLONTECH) was hybridized to probes derived from AnkG190-specific sequences (A) and from a region within the spectrin binding domain that is highly conserved among ankyrins (B). The AnkG190-specific probe yielded an 8-kb message exclusively in kidney and lung. The spectrin binding domain probe recognized several additional transcripts, presumably representing other alternatively spliced AnkG/Ank3 isoforms (9–13). Hybridization with actin showed equal loading of all lanes (not shown).

**FIG. 4.** *AnkG190 is expressed by outer medullary collecting duct cells.* Rat kidney sections were hybridized in situ with an antisense cRNA probe directed to the unique amino-terminal sequences of AnkG190. Specific hybridization was detected in the outer medullary region, consistent with the distribution of collecting ducts.

**FIG. 5.** *AnkG190 assembles with the cytoskeletal fraction in transfected MDCK and COS-7 cells.* Confluent cells transiently transfected with AnkG190-FLAG fusion constructs were extracted in situ to yield soluble (fraction I) and cytoskeletal (fraction II) fractions (13, 19), followed by Western blotting with antibodies as indicated. Note that AnkG190 cocolocalizes with spectrin and α-Na,K-ATPase predominantly in fraction II, whereas the Golgi-associated AnkG190 is found exclusively in fraction I (13). Molecular masses in kDa are shown. Similar results were obtained in cells stably transfected with AnkG190 (not shown).
to interact independently with both repeats 7–12 as well as repeats 13–24 (32). However, previous in vitro competition assays have shown that the spectrin binding domain of red cell ankyrin can also associate with liposomes containing Na,K-ATPase (31). Our studies demonstrate for the first time that AnkG_{190}, the isoform of ankyrin associated with the plasma membrane of polarized kidney epithelial cells, forms a functional immunoprecipitable complex with α-Na,K-ATPase in vivo. Furthermore, we show that two distinct ankyrin domains, namely the distal 12 ankyrin repeats as well as the spectrin binding domain, are capable of this interaction in vivo, whereas the proximal 12 repeats and the regulatory domains are devoid of Na,K-ATPase binding ability. These results are compatible with previous findings that both red cell and kidney ankyrins bind to two distinct cytoplasmic domains of α-Na,K-ATPase (19, 33). Furthermore, our results provide a structural basis for the ability of even significantly truncated ankyrin isoforms, such as the Golgi-associated AnkG_{119} (which contains only the distal 12 ankyrin repeats and a diminutive regulatory domain), to not only bind Na,K-ATPase but indeed to mediate its trafficking from endoplasmic reticulum to Golgi apparatus (13, 24).

In summary, ankyrins interact with integral membrane proteins such as Na,K-ATPase in a complex, pleiotropic manner that requires recognition of multiple binding sites on both ligands. Such combinatorial complexity presumably allows not only for the acquisition of the high binding affinities that characterize ankyrin-Na,K-ATPase interactions (16, 19, 31) but also for the ability of ankyrins to accommodate an increasing number of integral membrane proteins and sequester them into specialized domains of polarized epithelial cells. With the kidney AnkG_{190} clone in hand, it will be important in future studies to determine the minimal Na,K-ATPase-binding residues on ankyrin, the relative contribution of the two independent binding sites, and the consequences of interference with this binding on the generation and maintenance of polarized distribution of Na,K-ATPase in epithelial cells.

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