Ank3 (Epithelial Ankyrin), a Widely Distributed New Member of the Ankyrin Gene Family and the Major Ankyrin in Kidney, Is Expressed in Alternatively Spliced Forms, Including Forms That Lack the Repeat Domain

Luanne L. Peters,* Kathryn M. John,* Frederick M. Lu,* Eva M. Eicher,† Ann Higgins,‡ Maria Yialamas,* L. Christine Turtzo,* Anthony J. Otsuka,~ and Samuel E. Lux*

*The Division of Hematology/Oncology, Children's Hospital and the Dana Farber Cancer Institute, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115;†The Jackson Laboratory, Bar Harbor, Maine 04609; and~Department of Biological Sciences, Illinois State University, Normal, Illinois 61790

Abstract. We cloned a novel ankyrin, Ank3, from mouse kidney cDNA. The full-length transcript is predicted to encode a 214-kD protein containing an 89 kD, NH₂ terminal “repeat” domain; a 65 kD, central “spectrin-binding” domain; and a 56 kD, COOH-terminal “regulatory” domain. The Ank3 gene maps to mouse Chromosome 10, ~36 cM from the centromere, a locus distinct from Ankl and Ank2.

Ank3 is the major kidney ankyrin. Multiple transcripts of approximately 7.5, 6.9, 6.3, 5.7, 5.1, and 4.6 kb are highly expressed in kidney where Ankl and Ank2 mRNAs are barely detectable. The smaller mRNAs (~<6.3 kb) lack the entire repeat domain. These transcripts have a unique 5’ untranslated region and NH₂-terminal sequence and encode a predicted protein of 121 kD. Two small sequences of 21 and 18 amino acids are alternatively spliced at the junction of the repeat and spectrin-binding domains in the larger (~>6.9 kb) RNAs. Alternative splicing of a 588 bp sequence (corresponding to a 21.5-kD acidic amino acid sequence) within the regulatory domain also occurs.

Ank3 is much more widely expressed than previously described ankyrins. By Northern hybridization or immunocytochemistry, it is present in most epithelial cells, in neuronal axons, in muscle cells, and in megakaryocytes/platelets, macrophages, and the interstitial cells of Leydig (testis). On immunoblots, an antibody raised to a unique region of the regulatory domain detects multiple Ank3 isoforms in the kidney (215, 200, 170, 120, 105 kD) and in other tissues. The 215/200 kD and 120/105-kD kidney proteins are close to the sizes predicted for the 7.5/6.9- and 6.3/5.7-kb RNAs (with/without the 588-bp acidic insert). Interestingly, it appears that Ank3 exhibits a polarized distribution only in tissues that express the ~7.0-kb isoforms, the only isoforms in the kidney that contain the repeat domain. In tissues where smaller transcripts (~<6.3 kb) are expressed, Ank3 is diffusely distributed in some or all cells and may be associated with cytoplasmic structures.

We conclude that Ank3 is a broadly distributed epithelial ankyrin and is the major ankyrin in the kidney and other tissues, where it plays an important role in the polarized distribution of many integral membrane proteins.

The ankyrins are a family of conserved proteins that link integral membrane proteins to the spectrin-based membrane skeleton (reviewed in reference 53). Ankyrins are present in a wide variety of vertebrate cells, and ankyrin-like proteins have been described in primitive organisms such as fruit flies (Drosophila melanogaster) and worms (Caenorhabditis elegans) (Otsuka, A. J., R. Franco, B. Yang, K-H. Shim, L. Z. Tang, and A. Jeyaprakash. 1991. J Cell Biol. 115:465a).

Two vertebrate ankyrin genes have been cloned and sequenced. The first, erythrocyte ankyrin (human gene symbol ANK1/mouse gene symbol Ankl) is located on chromosome 8 in both humans and mice (41, 45, 46, 64, 65). Ankl links the β subunit of spectrin to the cytoplasmic domain of the transmembrane anion exchanger, band 3 (41). Nomenclature: In this paper ankyrin genes are indicated by their assigned genetic symbols: ANK1, ANK2, etc. (human genes) and Ankl, Ank2, etc. (mouse genes) (53). The corresponding proteins are Ankl, Ank2, etc. Ank1 is also called erythrocyte ankyrin or ankyring, and Ank2 is called brain ankyrin or ankyrinb (12). Ankyrinα, a novel ankyrin localized to axons (37) is an isofrom of Ank3.

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(AE1), in the red blood cell (RBC)\(^2\) (7). This interaction is critical to the stability of the RBC membrane; genetic defects in Ankl can result in severe hemolytic anemia (44, 46, 65). Ankl is highly expressed not only in erythroid tissues, but also in Purkinje cells and granule cells of the cerebellum, in motor neurons of the spinal column, and in a small subset of neurons in the hippocampus (35, 54). However, it is the second member of the ankyrin gene family, brain ankyrin or Ank2, that is the major form of ankyrin in the nervous system (52). Ank2 is alternatively spliced to form several species, including 220- and 440-kD isoforms (12). In rats, the 220-kD isoform predominates in adults, while the 440-kD isoform is most highly expressed during the neonatal period (12). The 220-kD isoform is broadly distributed in the brain, being present in neuron cell bodies and dendrites and in glia (12, 36). In contrast, the 440-kD isoform appears to be targeted specifically to unmyelinated axons and dendrites. Its expression decreases coincident with myelination (12). Ank2 is encoded by a gene on human chromosome 4q (62) and mouse Chromosome 3 (54).

Structurally, both Ankl and Ank2 consist of three domains: an NH\(_2\)-terminal “repeat” domain that binds band 3 (AE1) (16), AE3 (48), tubulin (16), and other proteins; a central “spectrin-binding” domain that binds spectrin and possibly vimentin (18, 28, 55); and a COOH-terminal “regulatory” domain, so-called because it can modulate the binding affinities of the first two domains (19).

The wide tissue distribution of ankyrin immunoreactive proteins and the limited expression of Ankl and Ank2 transcripts suggest that additional ankyrin genes exist. Here, we report the isolation, cDNA sequence, genetic localization and protein/mRNA distribution of a novel ankyrin, Ank3, in the mouse. Ank3 is the most widely distributed ankyrin. It is the major form of ankyrin in mouse kidney and other epithelial tissues, and it is also expressed in macrophages, megakaryocytes, and Leydig cells; in cardiac, smooth, and skeletal muscle; in the initial segments

| Table I. Oligonucleotide Primers |
|----------------------------------|
| **Name** | **Sequence** | **Location in Ank3** (bp, 5'-3') |
| Degenerate oligonucleotides* | | |
| A | 5'-GCA | CTAG | TGG | GAA | GAT | ATT | ACI | AGG | AC1 | AC1 | AC-3' |
|  | A | "Q" | W | E | D | I | T | G | T | T | 3822-3850 |
| B | 3'-ACA | GAC | TGG | GAG | GCT | AAT | ACC | ACC | ACC | ACC-5' |
|  | 3'-ATC | TG1 | CT1 | CT1 | CTT | CAG | GCG | TTT | TTT | TTT-5' |
|  | 3'-GTA | AAT | TAC | CG1 | TTT | AA-5' |
|  | 3'-AAA | GAT | TGG | G | G | N | V | S | A | "R" |
|  | 5'-CTT | ACC | ACC | ACC | ACC | CTI | CTI | CAT | TGG | GC-3' |
|  | 5'-CGT | ACC | ACC | ACC | ACC | CTI | CTI | CAT | TGG | GC-3' |
| E2a | 5'-CAA | GGT | ACC | ACC | ACC | ACC | ACC | ACC | ACC | ACC | ACC | ACC-3' |
|  | 5'-CAA | GGT | ACC | ACC | ACC | ACC | ACC | ACC | ACC | ACC | ACC | ACC-3' |
| Other oligonucleotides | | |
| 4B | 3'-GTTCCTCGCTAGCCACACCAGGGTCC-5' | 3600-3574 |
| 19D | 5'-CAGAAGAGTGAAGTACGTCACTCGAT-3' | 2515-2541 |
| A3REGRI | 3'-TACTAGTTCTACCTCACTCACCCAGTCGAC-5' | 6053-6021 |
| A3REGRII | 3'-CAGTGTCGGTCTAGCCGAGAGAGATAGTCAG-5' | 5985-5953 |
| A3REGF1 | 5'-CAGCATAAAAGGCTGAGAAAGGCAGACACAGCC-3' | 4372-4404 |

*Degenerate oligonucleotides were constructed to match the amino acid sequences shown, with the exception that the 5' end of some oligonucleotides were modified to include a restriction site. Sense oligonucleotides are shown with the 5' end on the left; antisense oligonucleotides have the 5' end on the right. The nucleotides that match sequences in Ank-3 are underlined. The locations of the matching sequences is shown at the right (numbered from the 5' end of Ank3-7kb, Fig. 3).

1, deoxynosine triphosphate.
of axons; and in the nodes of Ranvier (37). Ank3 is the third distinct member of the ankyrin gene family to be described.

**Materials and Methods**

**Preparation of RNA and Northern Blots**

Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (13). Polyadenylated RNA (polyA(+)-RNA, also termed mRNA) was isolated by oligo(dT) chromatography (57). For northern blots total RNA or polyA(+)-RNA was separated by denaturing gel electrophoresis, transferred to nylon filters (Zetabind; AMF Cuno, Meriden, CT), and hybridized with 32P-labeled probes using standard techniques (57). Hybridizations and post-hybridization washes were performed at 42°C and 65°C, respectively, as described (54).

**Primer Sequences and Hybridization Probes**

The primer sequences used in PCR and reverse transcription PCR (RT-PCR) experiments described in this study are listed in Table I. The locations of the hybridization probes used to screen cDNA libraries, map Ank3 by Southern hybridization, and analyze Ank3 transcripts by northern hybridization are illustrated in Fig. 3.

**Cloning of Ank3 by RT-PCR**

**PCR1.** This RT-PCR product was the initial clone of Ank3 and was obtained using degenerate oligonucleotide primers A, B, and C (Table I). The cDNA synthesis reaction included (20 μl total volume): 1 μg mouse polyA(+)-RNA (kidney, brain, thymus), 4 μl 5× cDNA synthesis buffer (GIBCO BRL Life Technologies, Gaithersburg, MD), 1 mM DTT, 20 U RNasin (Promega Biotech, Madison, WI), 10 pmol of primer B, and 200 U SuperscriptⅢ™ RNase H- M-MLV reverse transcriptase (GIBCO BRL). The mixture was incubated at 37°C for 60 min and the cDNA-mRNA complex was purified by phenol extraction and ethanol precipitation. The first-strand cDNA was amplified by PCR in a reaction mixture containing (100 μl total volume): 10 μl 10× PCR buffer (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 200 μM of each deoxynucleotide triphosphate (dNTP), 100 pmol of degenerate oligonucleotide primers A and B, 5 μM (25%) of the first-strand cDNA reaction, and 2.5 U Taq DNA polymerase (Boehringer-Mannheim Biochemicals). The PCR reaction was conducted for 30 cycles (95°C, 1 min; 65°C, 1.5 min; 72°C, 1.5 min) with a 15-min incubation at 72°C following the last cycle. The RT-PCR reaction yielded a 260-bp product from brain and kidney mRNA that hybridized with a 280-bp fragment of the cDNA fragment (PCR1). This product was purified from agarose using a Spin-X centrifuge filter (Costar Corp., Cambridge, MA), and subcloned (57) into the BlueScript KSII (+) vector (Stratagene, La Jolla, CA) for sequencing.

**PCR2.** This RT-PCR clone bridged the junction between the repeat and spectrin-binding domains. It was obtained as described above for PCR1. Reverse transcription was primed with primer B and the cDNA was amplified using primer D (representing a conserved sequence in the spectrin-binding domain) and a 1:1 mixture of primers E2a and E2b (which encode several versions of the highly conserved -G-TPLH-A sequence found at the beginning of many ankyrin repeats). The product (1,925 bp) was cut at two internal PstI sites (bps 2,257 and 3,429) and the major fragment (PCR2, ~1.1 kb) was subcloned into Bluescript KSII (+) and sequenced.

**PCR3, PCR4, and PCR5.** These products were obtained by RT-PCR using a single tube assay (63) under the following reaction conditions (25 μl total volume): 0.5–1.0 μg mRNA, 20 pmol each of oligonucleotides 4B and 4D, and 100 μM of each of dNTP, 2 mM MgCl₂, 67 mM TrisCl, pH 8.8, 17 mM (NH₄)₂SO₄, 6 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.02% gelatin, 2 μg yeast tRNA, 20 U RNasin (Promega Biotech), 33 U reverse transcriptase (AMV Super RT; Molecular Genetic Resources, Tampa, FL), and 2.5 U Taq I DNA Polymerase (Boehringer-Mannheim Biochemicals). The cDNA synthesis reaction was carried out for 60 rain at 42°C following by 5 min at 94°C. Subsequent amplification was performed for 30–40 cycles (94°C, 1 min; 60°C, 2 min; 72°C, 2 min) with a 7-min incubation at 72°C following the last cycle. The products (1.0–1.1 kb) were subcloned into the PCRⅢ™ vector using the TA cloning system (Invitrogen, San Diego, CA) and sequenced.

**Ank3 Regulatory Domain (Including PCR6 and PCR7).** The regulatory domain of Ank3 was amplified by RT-PCR, using a method designed to amplify large fragments (2), to test for alternatively spliced products, such as those characteristic of the Ank1 regulatory domain (41, 45). Reverse transcription was performed in a final volume of 20 μl containing: 1.4 μg total RNA, 500 μM each dNTP, 10 mM DTT, 40 U RNasin, 50 mM TrisCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 400 U M-MLV reverse transcriptase (GIBCO BRL). cDNA synthesis was primed with 1 μg oligo-dT (d(T)₂₀₋₂₅) (Collaborative Biomedical Products, Lexington, MA) or with A3REG1 (Table I). The mixture was incubated for 120 min at 42°C. Amplification of the first-strand cDNA was performed using a mixture of KlenTag I and Pfu DNA polymerases (2). The reaction mixture (50 μl) included: 5 μl (25%) of the RT reaction mixture, 20 mM TrisCl, pH 8.75, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 100 μg/ml BSA, 250 μM of each dNTP, 125 ng of the appropriate forward and reverse primers (A3REG2 and A3REG8, Table I), 11.7 U KlenTag I (AB Peptides, St. Louis, MO), and 0.078 U Pfu DNA polymerase (Stratagene). The amplification was performed for 30 cycles (94°C, 15 s; 68°C, 24 min) with a 7-min incubation at 72°C following the last cycle. PCR6 and PCR7 were obtained in this manner and subcloned into the TA vector (Invitrogen) and sequenced.

**cDNA Library Construction and Screening**

Oligo(dT) and random primed kidney cDNA was prepared from C57BL/6J mice using the Timesaver cDNA synthesis kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. Following size selection (~400 bp) and ligation of NotI/EcoRI adapters, the cDNAs were cloned into the lambda ZAP II vector (Stratagene). The unamplified ligates of each contained 460 bp plaques and were used to screen the number of fractions. The libraries were initially screened by Southern hybridization (57) of Dur- alon UV nylon filters (Stratagene) using PCR1 (described in the previous section). Subsequent screenings using PCR2 and an Ndel restriction enzyme fragment of clone pA3-19 (probe 2) were also performed to obtain a complete cDNA sequence. Hybridizations and post-hybridization washes were performed at 65°C, as described (54). Positive lambda Zap II clones were excised in vivo with helper virus according to the manufacturer’s directions, which generated subclones in the BlueScript SK(+)+ plasmid.

**DNA Sequencing**

Plasmid DNA was prepared by the cetyltrimethylammonium bromide (CTAB) method (3) and sequenced on both strands by the dyeoxyxynucle- otide chain termination method, using Sequenase Version 2.0 (United States Biochemicals Corp., Cleveland, OH) according to the manufacturer’s directions. 7-Deaza-dGTP was used (Deaza Sequenase Kit; U.S. Biochemicals Corp.) and formamide was included in the gels as needed to alleviate compressions. Synthetic oligonucleotide primers were used to extend all sequences. The data were analyzed using the Genetics Computer Group (GCG) programs (21).

**Genetic Mapping**

An Mspl restriction fragment length polymorphism (RFLP) was detected between the parental strains of an interspecific backcross ([BALB/cJ x CAST/Ei]F₁ X BALB/cJ) set. The segregation of this RFLP was followed in 77 progeny by Southern blot analysis (57). In addition, to map Ank3 relative to SSLP (simple sequence length polymorphism) markers, a Taq I RFLP was typed by Southern blot analysis in 94 progeny from an interspe- cific backcross involving (C57BL/6J X Mus spretus);F₁ females mated to M. spretus males (56). In both cases the hybridization conditions were identical to those detailed above. The hybridization probe was the Ank3 PCR1 product.

**Antibody Preparation**

**Regulatory Domain Antibodies.** The pGEX-2TK vector (32) was used to generate a 45-kD fusion protein containing the NH₂-terminal portion of glutathione-S-transferase (GST) and amino acids 1550–1587 and 1784–1905 of the regulatory domain of Ank3 (amino acids 1588 to 1783 are alternatively spliced and were excluded). This segment of Ank3 was chosen because it has minimal similarity to Ank1 and Ank2, and no significant homology to other proteins in the GenEMBL, PIR, and SWISSPROT databases. The Ank3 cDNA inserted into pGEX-2TK was generated by the polymerase chain reaction (see section entitled PCR3, PCR4, and PCR5

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Affinity Purification of Ank3-R1 Antiserum

The Ank3-R1 antiserum was purified by batch adsorption to the GST/Ank3 fusion protein coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). The protein was coupled to the beads and washed according to the manufacturer’s directions. 5 ml of Ank3-R1 antiserum was diluted to 50 ml in 10 mM Tris-HCl (pH 7.5) and mixed gently with 1 ml of GST/Ank3 beads at 4°C overnight. The beads were washed (20 vol 10 mM TrisCl, pH 7.5, then 20 vol of the same buffer plus 500 mM NaCl), and the Ank3-R1 antibodies were eluted with 10 vol freshly prepared 100 mM triethylamine (pH 11.5). After washing the beads with 10 mM TrisCl (pH 8.8), Ank3-R1 antibodies were further eluted with 10 vol freshly prepared 100 mM triethanolamine (pH 11.5). After each elution, the antibodies were neutralized with 1 M N,N,N',N'-tetraacetate (Na\(^2\) EGTA), 1 ~g/ml pepstatin A, 2 ~g/ml leupeptin, 6.9 kD). Separated proteins were transferred in a Bio-Rad Trans Blot SD semi-dry apparatus to Immobilon P membranes (Millipore Corp, Bedford, MA) in 48 mM TrisCl, 39 mM glycine, pH 9.2, with 20% methanol and 0.0535% SDS. A portion of each gel was stained with Coomassie blue prior to transfer to verify the integrity of the proteins. Efficiency of transfer was monitored by the transfer of the pre-stained markers, by staining the gel after transfer, and by staining a portion of the filter with Ponceau S after transfer and prior to blocking. Filters were blocked in 5% BSA in TTBS (10 mM TrisCl, 0.05% Tween-20, 150 mM NaCl, pH 8.0) for a minimum of 1 h, incubated for 1-2 h with primary antibody (diluted 1:500 in TTBS without Tween-20). Unless otherwise indicated, when an antibody raised to a GST fusion protein was used, such as anti-Ank3-R1 or -R2, staining was performed in the presence of a 200-fold molar excess of purified GST to block any possible reactions with GST-cross-reacting epitopes. Bound antibody was visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate using a commercial kit (Bio-Rad). All incubations were performed at room temperature.

Immunocytochemistry

Mice were anesthetized with avertin and perfused through the left ventricle with 20 ml Bouin’s fixative. Tissues were excised, placed overnight in fresh fixative, and routinely processed to paraffin. For detection of Ank3 protein, sections were deparaffinized and hydrated in Tris buffer (0.2 M TrisCl, pH 7.2), incubated with 1% BSA in the same Tris buffer for 30 min at room temperature, washed in Tris buffer containing 0.1% BSA, and incubated overnight in a humidified chamber at 4°C with affinity purified anti-Ank3-R1 diluted in Tris buffer containing 0.1% BSA. Control slides were incubated in buffer alone. The following day, the slides were washed in Tris buffer containing “Super Block” (Seytck Laboratories, Logan, UT) at a final dilution of 1:30. Bound anti-Ank3-R1 was subsequently detected using a commercial kit (Kirkegaard and Perry Inc., Gaithersburg, MD) according to the manufacturer’s directions. Briefly, the slides were incubated for 45 min at room temperature with biotinylated anti rabbit IgG, washed in Tris buffer, and incubated for 45 min at 37°C with alkaline phosphatase-conjugated streptavidin. Positive reaction was seen as a red precipitate. The sections were counterstained with hematoxylin.

Results

Isolation of a Novel Ankyrin PCR Product

Comparison of amino acid sequences of human Ankl and Ank2, and mouse Ankl, with the evolutionarily distant unc44, a C. elegans ankyrin that functions in axonal guidance, reveals areas of extensive conservation in the distal portions of the spectrin-binding domains. For example, the sequence AQWEDITGT (V/E)DKT differs at only one amino acid (Fig. 1 a). To identify novel ankryns, degenerative oligonucleotide primers correspond-
to these sequences were designed (A and B, Table I) and used in PCR amplification of cDNA reverse transcribed from mouse kidney, brain, spleen, and thymus mRNA. Ank1 was recovered in abundance from the kidney (data not shown). PCR1 showed 61% and 76% amino acid identity to the corresponding regions of Ankl in kidney (data not shown). Ank2 was abundant in brain and detectable in kidney and thymus, while Ank3 was not detectable in kidney (data not shown). Ank2, therefore, is encoded by a gene distinct from Ank1 and Ank2, respectively (Fig. 1 b). We designated this apparent novel ankyrin Ank3 (gene symbol Ank3).

**Ank3 Is Located on Chromosome 10**

To prove that Ank3 is encoded by a gene distinct from Ank1 (mouse Chromosome 8) and Ank2 (mouse Chromosome 3), we genetically mapped Ank3 in the mouse using an interspecific backcross ([BALB/cJ × CAST/Ei]F1 × BALB/cJ) backcross set. The typing data obtained in this panel have been deposited in the Mouse Genome Database (Accession No. MGD-CREX-228) and can be accessed through the World Wide Web (http://www.jax.org). No recombinations occurred between Ank3 and the marker D10Bir10, confirming that Ank3 is on mouse Chromosome 10. The gene order (from the centromere) and map distances (given as % recombination ± SE, in order) are: D10Bir6 - 8.6 ± 2.9 - Egr2, D10Mit15 - 1.1 ± 1.1 - D10Bir10, Ank3 - 14.3 ± 3.8 - D10Mit11.3 The position of the SSLP marker D10Mit15 is estimated to be at ~31

3. Gene names and symbols (1) are: D10Bir6, DNA segment, Chromosome 10, Birkenmeier (Bir)-6; Egr2, Early growth response 2; D10Mit15, DNA segment, Massachusetts Institute of Technology (Mit)-15; D10Bir10, DNA segment, Chromosome 10, Bir-10; D10Mit11, DNA segment, Chromosome 10, Mit-11.

**Figure 3.** Cloning and organization of mouse Ank3. (a, CLONES) The organization of relevant Ank3 cDNA clones obtained by library screening (pA3-1, -2, -3, -4, -8, -13, -15, -19, -26, and -29) or by the polymerase chain reaction (PCR1-7) are shown. Solid lines indicate the portions of each clone/PCR product sequenced on both strands. All cDNA clones except pA3-1, pA3-3, and pA3-8 were completely sequenced. cDNA clones pA3-2 and pA3-29 contain potential translation start sites (arrows). (b, cDNA): The deduced organization of Ank3 cDNAs. Alternative splice and start sites are indicated. Restriction enzyme sites are marked above the cDNA schematic: A, AccI; B, BamHI; C, Clal; D, EcoRI; H, Hind III; K, KpnI; N, NdeI; S, SpeI; X, Xhol. The nucleotides at the beginning and end of major domains and the postulated translation start sites are numbered below the schematic. Hybridization probes described in the text are represented by filled boxes 1-4. (c, PROTEIN) The postulated domain structure of mouse Ank3 protein. Each domain and alternatively spliced isoform is indicated, with the amino acid numbers given above. The 24 repeats of the repeat domain are also shown. Sequences used to generate antibodies to the regulatory domain (Ank3-R1) and insert B (Ank3-B) are marked at the bottom.
The cDNA Sequence and Predicted Protein Structure of Mouse Ank3

We used PCR1 to screen two normal mouse (C57BL/6J-+/+) kidney cDNA libraries. Initially, three positive clones (pA3-1, pA3-2, pA3-4; Fig. 3) were obtained. An additional RT-PCR product, PCR2, was obtained from

Figure 4. Nucleotide sequence, deduced amino acid sequence and postulated domain structure of mouse Ank3. (a, Ank3-7kb) Amino acids that are identical in human and mouse Ank1, human Ank2, mouse Ank3, and Drosophila ankyrin are shaded. The 24 repeats of the repeat domain are underlined and numbered R1-R24. The two small inserts, A and B, at the junction of the repeat and spectrin-binding domains, and the alternatively spliced acidic sequence (insert C) within the regulatory domain are marked with a double underline. The two conserved sequences used to clone Ank3 (by degenerate oligonucleotide RT-PCR) are underlined with a single line.

(b, Ank3-5kb) The nucleotide sequence of the 5' untranslated region and first six deduced amino acids of the ~5-6 kb isoforms of Ank3, which lack the entire repeat domain. These sequence data are available from EMBL/Genbank/DDBJ under accession numbers L40631 (5-kb isoform) and L40632 (7-kb isoform).

cM on the consensus map (1). Hence Ank3 is located at ~32 cM on the consensus map using these markers. The data from both backcross sets are therefore in good agreement. Ank3 is not closely (~1 cM) linked to any mouse Chromosome 10 mutation. Based upon earlier mouse consensus maps, we investigated the possibility that an Ank3 defect could account for the mocha (gene symbol mh) mutation, a mouse platelet storage pool deficiency mutation located on Chromosome 10 (1) (Peters, L. L., E. M. Eicher, K. M. John, T. C. Hoock, M. Yialamas, and S. E. Lux. 1993. Blood. 82:340a). However, to date, no defect in the Ank3 sequence of mocha mice has been found (Peters, L. L., K. M. John, and S. E. Lux, unpublished observations).

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kinetics using a degenerate forward primer corresponding to conserved sequences at the 3' end of the repeat domains of Ank1 and Ank2 and reverse primers corresponding to conserved sequences in the spectrin-binding domains of Ank1, Ank2, and unc44. Subsequent screenings using PCR2 and the 5' NdeI restriction enzyme fragment of clone pA3-19 (probe 2, Fig. 3) were performed to obtain a complete Ank3 cDNA sequence.

The complete Ank3 cDNA is 7084 nucleotides (Fig. 4a) and encodes a 1,961-amino acid protein with a predicted mass of 214,144 D and a calculated isoelectric point of 6.68. The postulated translation start site is the first in-frame methionine and is the only start site that yields a structure similar to previous ankyrins.

Like Ank1 and Ank2, Ank3 consists of three domains. The 89-kD NH2-terminal “repeat” domain, corresponding to amino acids 1-873, is composed mostly of 24 repeats. Each contains 33 amino acids except for repeat 5, which has only 29. The Ank3 repeat consensus is: -G-T(P/A)LHAA--G---(V/I)(V/A)--LL--A--(N/D)---. The Ank3 repeats have the characteristic features that distinguish ankyrin repeats from the similar repeats found in many other proteins (10), such as the presence of a hydrophobic amino acid in repeat position No. 3 and an N or D in position No. 29 (53). When the ankyrins are aligned and compared to each other, 19 of the 24 Ank3 repeats are more homologous to the corresponding repeat in Ank2 than in Ank1. Overall the repeat domains of human Ank2 and mouse Ank3 are 76% identical (Fig. 5). Ank3 also shares significant identity to the repeat domain of Ank1 (Fig. 5). The entire domain is conserved except for the first 26 amino acids.

When Ank3 is compared to Drosophila ankyrin (24), the identity in the repeat domain falls to 56%. The overall identity is 51%. Hence, not unexpectedly, Ank3 resembles other mammalian ankyrins more than Drosophila ankyrin. Despite this, evidence of significant conservation of function between fly and mammalian ankyrins exists. When human Ank1 and Ank2, mouse Ank3, and Drosophila ankyrin are compared repeat 1 to repeat 1, repeat 2 to repeat 2, etc., the first 14 repeat positions are all conserved, including some positions (e.g., Nos. 1, 3, 8, 11, and 12) that vary widely from one repeat to the next, while the “variable” positions in the last half of the aligned repeats are not well conserved. Most likely the amino acids in positions Nos. 1, 3, 8, 11, and 12 have critical functions, such as protein binding, that vary from repeat to repeat. If so, the companion repeats in the various ankyrins must have retained similar functions throughout evolution.

Most of the 65-kD spectrin-binding domain (amino acids 874-1455) is also conserved (Fig. 5), including the basic RRRKFH(K/R) sequence (amino acids 1209-1215) that is required for spectrin-binding in Ank1 (55). (This basic peptide is also present in Ank2 and Drosophila ankyrin [24]). Some or all of the first 70 amino acids of the Ank1 spectrin-binding domain are also necessary for full erythroid spectrin-binding capacity (18), but a peptide containing just these amino acids does not bind spectrin, at least not when it is expressed as a fusion protein (55). Interestingly, the first 63 amino acids of the Ank3 spectrin-binding domain are reasonably well conserved compared to Ank2 but differ completely in Ank1 (Fig. 5). This supports the hypothesis (55) that this region is responsible for fine tuning the preferences of ankyrins for spectrin or fodrin (nonerythroid spectrin). Ank2 preferentially binds fodrin, whereas Ank1 favors spectrin (15). The spectrin/fodrin preferences of Ank3 are untested, but judging from where it is expressed (see below) the protein must interact mostly with fodrin. Spectrin binding is thought to be relegated to the first half of the spectrin-binding domain since a 32-40 kD tryptic peptide from that region of Ank1 binds spectrin (55). However, the latter half of the spectrin-binding domain is also well conserved (Fig. 5), suggesting that this region as well serves an important, albeit presently unidentified, binding or modulating function.

The COOH-terminal “regulatory” domain of Ank3 (amino acids 1456-1961), in contrast, is poorly conserved (<30% identical, Fig. 5), except for a segment of unknown function near the beginning of the domain (amino acids 1488-1555), which resembles portions of the cytoplasmic domain of the tumor necrosis factor alpha receptor, and a small segment in insert C (see below). Comparison of Ank1 and Ank2 reveals similar homologous and nonhomologous regions (52). Drosophila ankyrin shows no significant homology to other ankyrins in the regulatory domain (24).

The GCG program MOTIFS identifies 76 potential phosphorylation sites in Ank3. It is interesting that six of these (at residues 822, 830, 838, 844, 848, and 850) are clustered in or near insert A (see following section) at the

Figure 5. Amino acid homology between the three ankyrins. (Top) Schematic of the domain structure of Ank3. The + marks the location of a basic sequence believed to form part of the spectrin binding site (55). (Bottom) A comparison of the amino acid sequences of mouse Ank1 and human Ank2 with mouse Ank3 reveals extensive homology in the repeat domain and large segments of the spectrin-binding domain. Each point represents the percent identity of 10 aligned amino acids. The highest overall identity is with human Ank2. Notably, sequence conservation is lost at the beginning of the spectrin-binding domain and also falls dramatically in the last two-thirds of the regulatory domain.
junction of the repeat and spectrin-binding domains. This insert is one of the unique features of Ank3 and probably has special binding or regulatory functions.

The complete nucleotide sequence of the Ank3 cDNAs includes a number of alternatively spliced variants and suggests the use of two different start sites. As described later, northern and western blot analyses confirm the existence of multiple Ank3 isoforms.

**Ank3 Contains Two Alternatively Spliced Sequences between the Repeat and Spectrin-binding Domains**

The last 13 amino acids of clone pA3-19 are contained within the last 17 amino acids of clone pA3-26. Both differ from PCR2, where an alternative sequence of 18 amino acids is found in the same position (Figs. 3 and 4 a). This suggests the presence of at least two small inserts at the 3' end of the repeat domain of Ank3 where it joins the spectrin-binding domain. We designated the partial sequences present in cDNA clones pA3-19 and pA3-26 "insert A" and the 18-amino acid sequence present in PCR2 "insert B", and performed RT-PCR using mouse kidney RNA to amplify these and other sequences across the boundary of the repeat and spectrin-binding domains. Three new products, PCR3, PCR4 and PCR5, were obtained (Fig. 3). Neither insert A nor B is present in PCR3. Insert A alone is present in PCR4, and both inserts are present in PCR5. The complete insert A contains 21 amino acids. We conclude that two inserts are present at the junction of the first two domains of Ank3 and that all four possible splice variants are transcribed in the kidney. It is noteworthy that alternative splicing of Ankl in the rat brain (40) and mouse cerebellum (9) results in a 24-bp (8 amino acid) insert at precisely the same location as the A and B inserts of Ank3. Although the rat brain and mouse cerebellar inserts (TAHISLTG and TAMISIMG, respectively) resemble one another, there is no homology to Ank3 insert A or B. The functional role of the various inserts at the junction of the repeat and spectrin-binding domains remains to be defined for both Ankl and Ank3.

**Many Ank3 Transcripts Lack the Repeat Domain**

Ank3 clones pA3-2, pA3-13, and pA3-15 lack the repeat domain: a 5' untranslated region is connected directly to the spectrin-binding domain (Fig. 3). To determine if these cDNAs are transcribed, we performed northern analyses of mouse kidney mRNA using domain-specific probes (Fig. 6 a, lanes 1 and 2). Probes to sequences within the spectrin-binding (PCR1) and regulatory (probe 1) domains detect two closely migrating mRNAs of 7.5 kb (faint) and 6.9 kb (prominent), one mRNA of 6.3 kb (prominent), a broad band at ~5.7 kb (prominent), which may be heterogeneous, and minor bands of about 5.1 and 4.6 kb (Fig. 6 a, lane 2). However, a probe from the Ank3 repeat domain (probe 2) detects only the 6.9 and 7.5 kb mRNAs (Fig. 6 a, lane 1), indicating that the smaller transcripts and their encoded proteins lack the repeat domain, as suggested by the cDNA sequences obtained (Fig. 3).

Other data support this conclusion and indicate that at least two translation start sites are utilized in Ank3. For example, the 5' untranslated regions of the cDNA clones that lack a repeat domain (pA3-2, -15, and -13) overlap and are distinct from the 5' untranslated region attached to the repeat domain (clone pA3-29) (Figs. 3 and 4 b). When mouse kidney mRNA is hybridized to a cDNA from the unique 5' untranslated region of the clones lacking the repeat domain (probe 3, the EcoRI fragment of clone pA3-2, in Fig. 3), the 6.3- and 5.7-kb transcripts are strongly labeled, as expected, but at least one 6.9 kb transcript is also marked (Fig. 6 a, lane 3). This large transcript must contain sequences that are not present in clones pA-2, -13, -15 and more distal clones (collectively termed "Ank3-5 kb", Fig. 4 b) since the Ank3-5-kb sequence is too small. These could be sequences from the repeat domain or other, presently unknown sequences. Similarly, two of the transcripts that are assumed to represent "Ank3-5 kb" are larger (6.3 and 5.7 kb) than the cloned fragments (5.074 kb) and must contain additional sequences.

The only in-frame methionine in this 5' untranslated region adds six new amino acids to the spectrin-binding domain. The surrounding nucleotides only match the consensus sequence of vertebrate translation start sites (38) at positions -6 and +4; however, none of the other positions is statistically rare. There are stop codons in all frames upstream and the next in-frame methionine is 69-amino acids downstream.

**Some Ank3 Molecules Contain an Acidic Insert within the Regulatory Domain**

Two major products, PCR6 (1.0 kb) and PCR7 (1.6 kb), were obtained by RT-PCR of the Ank3 regulatory domain (Fig. 3). PCR7 contains an additional sequence of 588 bp (insert C), which appears to correspond to the "2.1 insert" of Ankl (41, 45). Both inserts are located in the same place in the aligned sequences, both are large (insert C = 21.5 kD, 2.1 insert = 17.3 kD), and both are highly acidic (pI of insert C = 4.0, 2.1 insert = 3.7). However, the sequences are quite different, except for a short segment (Fig. 5, amino acids 1652-1667), and there is only modest conservation of the organization of charged regions and predicted structural elements. Judging from recovered clones (Fig. 3) and from the yield of RT-PCR products, most Ank3 RNA transcripts lack insert C. The difference was not carefully measured but is about threefold. The multiple, COOH-terminal, alternatively spliced products of Ankl (27) are not observed in Ank3.

Northern blot data confirm that insert C is an alternatively spliced variant that is transcribed in mouse kidney. When kidney mRNA is probed with a sequence from within this exon (probe 4), the 6.9 and 7.5 kb transcripts are observed, and the 6.3 kb and minor 5.1 kb bands remain, but the 5.7 and 4.6 kb mRNAs are missing (Fig. 6 a, lane 4).

**Ank3 Is the Major Ankyrin in Kidney**

No Ankl or Ank2 is detected in mouse kidney mRNA by northern hybridization under conditions where Ank3 gives a strong signal (Fig. 6 e). With prolonged exposure faint bands of ~7 kb are barely discernable above background in the Ankl and Ank2 lanes (e.g., Fig. 6 e, lane 3). Whether these are true signals or cross-hybridization with Ank3 remains to be determined.
Ank3 mRNAs Are Widely Distributed in Mouse Tissues and Exist in Many Isoforms

In addition to kidney, Ank3 was detected in mRNA from skin, brain, lung, testis, intestine, heart, and liver by northern hybridization (Fig. 6, b–d). It was not detected in normal mouse spleen (Fig. 6, lane 2), in reticulocytes or spleen from mice with a phenylhydrazine-induced hemolytic anemia (not shown), in 16 d fetal liver erythroid precursor cells (not shown), or in the cultured lymphocyte cell lines JY and Jurkat (not shown).

In skin, lung, and testis, the overall pattern of Ank3 expression is similar to that of the kidney; at least one major transcript at ~7.0 kb is present, there is a broad band, which potentially encompasses several mRNAs, stretching from ~6.3 to ~5.7 kb, and some smaller mRNA species (~5.0 kb) are evident (Fig. 6, b and c). The ~7.0-kb mRNA predominates in cardiac muscle, but three additional discrete mRNAs (6.3, 6.0, and 5.6 kb) are also expressed (Fig. 6 d, lane 4).

In contrast, in the liver and intestine a 5.5-6.3-kb cluster of transcripts make up the major mRNAs (Fig. 6 d); little or no expression of the larger splice forms is seen. Brain, on the other hand, contains only large Ank3 transcripts: ~13, ~10 and ~7 kb (Fig. 6 b and c). The 5.7-6.3-kb mRNAs and other small species are not detected.

Immunoblots Confirm the Existence of Multiple Ank3 Isoforms in Different Tissues

The Northern analyses indicate Ank3 is widely distributed in mouse tissues and multiple protein isoforms exist. Using an antibody raised in rabbits to a GST/Ank3 regulatory domain fusion protein (antiAnk3-R1), multiple Ank3 protein isoforms are detected by Western blotting of crude kidney, brain, liver and testis membranes (Fig. 7 a, lanes 1-4). The blot was hybridized with a 2.8-kb fragment of clone pA3-2 that included all of pA3-2 except the 5’ sequences corresponding to probe 3. This corresponds to the entire spectrin-binding domain and most of the regulatory domain.

(d) Expression of Ank3 in intestine (lane 1, 5 μg mRNA), kidney (lane 2, 1 μg mRNA), liver (lane 3, 5 μg mRNA), and heart (lane 4, 1 μg mRNA) using PCR1 as the hybridization probe. Arrows indicate 7- and 6.3-kb mRNAs. (e) Comparison of Ank1, Ank2, and Ank3 expression in the kidney. Each lane contains 2 μg mouse kidney mRNA. Lane 1 was probed with Ank3 probe PCR1. Lane 2 was probed with a previously described (55) 4.6-kb mouse Ank1 cDNA. Both lanes were exposed for 22 h at ~−80°C with two intensifying screens. Lane 3 is a 3-d exposure of lane 2 and shows a faint band at ~6.9 kb. Lane 4 was probed with a 260-bp mouse Ank2 RT-PCR product, which was amplified from mouse brain cDNA with the same primers used to obtain PCR1. It was exposed for 22 h at ~−80°C. The 3-d exposure of lane 4 also showed a very faint band at ~7 kb but the dark background prevented a satisfactory photograph. Note that Ank1 and Ank2 mRNAs are barely detectable in mouse kidney by northern analysis, even with lengthy exposure times.

Figure 6. Expression of Ank3 in mouse tissues. (a) Northern blots of kidney Ank3 transcripts using domain-specific hybridization probes. Each lane contains 2 μg normal mouse kidney mRNA. Lines between lanes indicate corresponding mRNAs. The arrows in lane 1 indicate two mRNAs of 7.5 and 6.9 kb (mRNA sizes estimated relative to 18S and 28S ribosomal RNAs). The arrow in lane 2 is at ~6.3 kb. The probes used in each lane are as follows. Lane 1: probe 2, repeat domain-specific. Lane 2: probe 1, regulatory domain-specific. Lane 3: specific for the unique 5’ untranslated sequence of the Ank3 clones that lack the entire repeat domain. Lane 4: probe 4, corresponding to insert C, the alternatively spliced sequence in the regulatory domain. (b) Expression of Ank3 in normal mouse skin (lane 1, 2.5 μg mRNA) and brain (lane 2, 2.0 μg mRNA). The blots were hybridized with PCR1, specific to the spectrin-binding domain. Comparative experiments using several tissues reveal that PCR1 hybridizes to the same pattern of mRNAs as probe 1 (not shown). In the skin, at least one transcript at approximately 6.9 kb (arrow) is present in addition to mRNAs extending from ~6-3-5.7 kb and a smaller 4.7-kb species. In the brain, major transcripts of ~13 (arrow), ~10, and ~7 kb are present. (c) Expression of Ank3 in normal mouse brain (lane 7), spleen (lane 2), lung (lane 3), and testis (lane 4). Each lane contains 2 μg mRNA. The arrows are at 13 and 7 kb. The blot was hybridized with a 2.8-kb fragment of clone pA3-2 that included all of pA3-2 except the 5’ sequences corresponding to probe 3. This corresponds to the entire spectrin-binding domain and most of the regulatory domain. (d) Expression of Ank3 in intestine (lane 1, 5 μg mRNA), kidney (lane 2, 1 μg mRNA), liver (lane 3, 5 μg mRNA), and heart (lane 4, 1 μg mRNA) using PCR1 as the hybridization probe. Arrows indicate 7- and 6.3-kb mRNAs. (e) Comparison of Ank1, Ank2, and Ank3 expression in the kidney. Each lane contains 2 μg mouse kidney mRNA. Lane 1 was probed with Ank3 probe PCR1. Lane 2 was probed with a previously described (55) 4.6-kb mouse Ank1 cDNA. Both lanes were exposed for 22 h at ~−80°C with two intensifying screens. Lane 3 is a 3-d exposure of lane 2 and shows a faint band at ~6.9 kb. Lane 4 was probed with a 260-bp mouse Ank2 RT-PCR product, which was amplified from mouse brain cDNA with the same primers used to obtain PCR1. It was exposed for 22 h at ~−80°C. The 3-d exposure of lane 4 also showed a very faint band at ~7 kb but the dark background prevented a satisfactory photograph. Note that Ank1 and Ank2 mRNAs are barely detectable in mouse kidney by northern analysis, even with lengthy exposure times.
to 4). In the kidney, two closely spaced polypeptides of 215 and 200 kD and single bands at 170, 120, and 105 kD are observed when blots are probed with the antibody in the presence of a 200-fold excess of bacterially expressed GST protein. All five bands disappear when the antibody reaction is performed in the presence of a 200-fold excess of the GST/Ank3 fusion protein, indicating they are all Ank3 specific. In addition, the antibody does not cross-react with Ankl or Ank2. It does not react with Ankl (or other proteins) in red cell ghosts (data not shown), and it does not detect Ank2 on neuronal cell bodies or dendrites (see later section on Immunocytochemistry).

Multiple Ank3 isofoms are similarly detected in crude brain membranes (Fig. 7 a, lane 2), including a predominant polypeptide of 215 kD and minor isoforms of 180, 160, 145, 135, 120, and 105 kD. In testis and liver crude membranes, small amounts of the 120- and 105-kD isoforms are found (Fig. 7 b, lanes 3 and 4). The 105-kD isofom is the major species observed in crude platelet membranes (not shown).

The B Insert is Contained in a Single 170 kD Isoform

When antibody raised to a peptide sequence within the alternatively spliced B insert (antiAnk3-B) is used as the probe, a 170-kD isofom is present in crude membranes from kidney, liver, and testis but not brain (Fig. 7 b). It is also observed in platelet membranes (not shown). In the kidney it co-migrates with the 170-kD peptide detected with the Ank3 regulatory domain-specific antibody (anti-Ank3-R1). This antiseraum does not detect the 170-kD isoform in testis and liver (Fig. 7 b), but a companion antiserum (antiAnk3-R2), raised against the same GST/Ank3 regulatory domain fusion protein, does (not shown). We conclude that the B insert is expressed in a single isoform of 170 kD, and that it is present in kidney, testis, liver, and platelet membranes, but not in membranes prepared from brain.

Immunocytochemistry

We performed immunocytochemical staining of a variety of mouse tissues using affinity purified antiAnk3-R1, prepared against the GST/Ank3 regulatory domain fusion protein. In general, Ank3 is present in epithelial tissues, muscles, and axons (Figs. 8 to 10).

Epithelial Tissues

Skin. Intense staining is seen in the epithelium of the skin (Fig. 8, a and c), including that lining the sweat glands and hair follicles (Fig. 8 a, arrow).

Lung. Intense staining is also seen in the epithelium of the lung airways, where an apical-lateral concentration of reaction product is apparent (arrows) in addition to significant cytoplasmic staining (Fig. 8 d). The walls of the lung respiratory passages (alveoli) are negative. A subset of lung interstitial cells is clearly positive (Fig. 8 f, arrow). The identity of these cells is uncertain. The most likely possibility is lung macrophages, since the cells resemble macrophages and both peritubular and bone marrow macrophages contain Ank3 (Hoock, T. C., L. L. Peters, and S. E. Lux. 1994. Mol. Biol. Cell. 5[Suppl]:45a.)

Intestine. A diffuse (cytoplasmic) Ank3 staining pattern is seen in the intestinal absorptive epithelium (Fig. 8, g and i). Interestingly, there is significantly less intense staining of epithelial cells in the crypts as compared to the villi (Fig. 8 g), which suggests that Ank3 is a marker of intestinal epithelial differentiation and maturation. In addition, the intestinal smooth muscle is positive for Ank3, as are nerve ganglia (Fig. 8 g, arrow). In contrast, the connective tissue elements in the lamina propria do not stain.

Pancreas. In the pancreas, apical staining of acinar cells (arrow, Fig. 9 c) and intense staining of the epithelial lining of the ducts (arrow) is apparent.

Liver. Ank3 immunoreactivity is weak in the liver; only a faint diffuse staining pattern is observed (not shown).

Kidney. Intense Ank3 immunoreactivity is weak in the kidney (Fig. 10, d–i). In the cortex, the macula densa (Fig. 10 d, left arrow), distal convoluted tubule, and cortical collecting ducts (Fig. 10 d, right arrow) are strongly positive,
Muscle

Cardiac muscle (Fig. 9 e), skeletal muscle underlying the skin (Fig. 9 f), and smooth muscle in the intestine (Fig. 8 g) are all Ank3 immunoreactive. In the heart, the intercalated discs (arrows, Fig. 9 e) are clearly positive. Skeletal muscle shows a striated pattern that is consistent with localization at the transverse tubular membrane (26, 42).

Neural Tissues

In neural tissue, the axons of outer granular and pyramidal layers of the cerebral cortex and the hippocampus (not shown) are positive, particularly the initial segments (Fig. 10 a, arrow). Although it is difficult to discern at this magnification, the cell bodies and dendrites appear negative. [Ank2 is located in neuronal cell bodies and dendrites (35)]. The axons of cerebellar Purkinje cells are also positive (Fig. 10 b, arrow) and Purkinje cell bodies and dendrites are negative. It is noteworthy that in sections of the cerebellum stained with high concentrations of the Ank3 antiserum (1:25–1:50 dilution of antiAnk3-R1 vs the 1:100–1:250 dilution used in all other experiments), diffuse staining of the molecular layer of the cerebellum occurred, which resulted in visible outlining of the unstained Purkinje cell dendritic trees (not shown). Hence, it is clear that Purkinje cell dendrites do not contain Ank3 protein. However, it is difficult to be certain unmyelinated axons of the molecular layer of the cerebellum are really Ank3 positive, because of the high concentration of antibody required to stain this area. As noted earlier, peripheral nerves (Fig. 8 g, arrow) also stain for Ank3.

Other Tissues

Leydig Cells. In the testis, discrete areas within the cytoplasm of the interstitial (Leydig) cells are intensely positive (Fig. 9 a, middle and right arrows), while the Sertoli cells and seminiferous tubules stain very weakly or not at all.

Megakaryocytes. Although the majority of the cells in the spleen do not contain immunoreactive Ank3, particularly lymphocytes and reticuloendothelial cells, megakaryocytes are diffusely stained (Fig. 9 h, arrows).

Discussion

Ank3 Is the Major Ankyrin in the Kidney and Other Epithelia and Is Probably Involved in the Targeting/Maintenance of Na+/K-ATPase and Other Proteins in Plasma Membranes

Recent evidence shows that many integral membrane transporters and adhesive proteins bind ankyrins. The undoubtedly incomplete list already includes the α subunit of Na/K-ATPase in renal tubules (20, 50), intestinal epithelium (22), retinal pigment epithelium (30), choroid plexus (47), and brain (58); the voltage-dependent Na channel in neurons (61) and at the neuromuscular junction (25); the amiloride-sensitive Na channel in the kidney (60); the Na/Ca exchanger in the heart (42); the H/K-ATPase in gastric parietal cells (59); the hyaluronic acid receptor (CD44) (43) and inositol 1,4,5-triphosphate (IP3) receptor (11) in lymphocytes; a group of neurofascin/L1-like putative cell adhesion molecules in the brain (17); and, of course, the Cl/HCO3-anion exchanger (AE1, band 3) in erythrocytes (7). Ankyrins also bind a related anion exchanger (AE3) in the brain (48), and probably bind a shortened isoform of AE1 in the intercalated cells of the renal collecting duct (23). In all well studied cases, the integral membrane proteins bind to part of the ankyrin repeat domain and are in turn linked to the spectrin/fodrin-based membrane skeleton. These interactions appear to play a role in establishing and/or maintaining a polarized distribution of the ligands in the membranes of non-erythroid cells (22, 23, 25, 30, 31, 47, 49, 50, 60, 61).

Ank1 and Ank2 bind to many of the proteins listed above in vitro binding assays, but are unlikely to do so in vivo because they are expressed in different tissues. Ank1 is found in erythroid cells (53, 54), myocytes (9), en-

Figure 8. Immunocytochemical detection of Ank3 in mouse skin, lung, and intestine. In this and subsequent figures, red reaction product indicates the presence of Ank3 protein. The primary antibody is affinity purified antiAnk3-R1. Sections incubated in the absence of primary antibody serve as controls. All sections are counterstained with hematoxylin. (a) Skin. A positive reaction is seen in epithelial cells (arrow), including the epithelial cells lining the hair follicles, sebaceous glands and sweat glands. (b) Skin control. (c) High magnification of the skin showing intense Ank3 staining in all but the outermost, cornified layers of the epithelium. (d) Lung. Intense Ank3 reaction product is present in the epithelial cells of the conducting airways, where an apical–lateral concentration is observed (arrows). Positive staining is also seen in the smooth muscle layers (right). (e) Lung control. (f) High magnification of the lung showing an Ank3 immunoreactive interstitial cell (arrow), probably a macrophage, next to, but not abutting, alveolar airspaces. (g) Intestine. Diffuse Ank3 staining is present in the absorptive epithelia (E) of the intestinal villi. Within the intestinal crypts (C), less intense staining is seen. The smooth muscle (SM) of the muscularis layer is positive, and a nerve plexus between the muscle layers is intensely positive (arrowhead). (h) Intestine control. (i) High magnification of a portion of an intestinal villus showing the diffusely stained absorptive epithelium (E). Connective tissue components of the lamina propria (Lp) are negative. Bar, 10 μm.
dothelial cells (Lux, S. E., unpublished data), cerebellar and spinal cord neurons (9, 35, 54), and possibly hepatocytes (52). Ank2 is limited to neurons and glia (12, 35, 52, 54). In adult animals both ankrys are localized to neuronal cell bodies and dendrites. Fetal mice and rats express a giant isoform of Ank2 that is found in unmyelinated axons, but this protein disappears within a few days of birth. They also express an isoform of Ank2 in fetal liver precursors (53).

Numerous studies have identified “ankrys” in other tissues using antibodies raised to Ank1 or Ank2 (4, 5, 14, 15, 22, 23, 25, 26, 29, 34, 42, 60). However, in all cases the antibody was raised to whole Ank1 or Ank2 (8, 15) or to their conserved portions (the repeat and spectrin binding domains) (6), and presumably lacked specificity. In many of these studies the problem of specificity was accentuated by using an antiserum raised to an ankyrin of one species to probe tissues of another species, by immunizing with SDS-denatured proteins, or by using affinity chromatography to concentrate cross-reacting antibodies. For example, in one case (14) an antibody to pig brain ankyrin (Ank2) was affinity purified on a column containing bovine brain ankyrin, producing a reagent that reacted with all cells of the rat kidney. More recent surveys of ankyrin expression, using specific antibodies to Ank1 (52) or Ank2 (36, 52), show a much more restricted distribution, consistent with the expression of Ank1 and Ank2 detected by northern blotting (9, 52, 54). In particular the kidney does not react significantly with such sera (52).

These studies indicate that additional ankrys must exist in cells or sites that lack Ank1 or Ank2 but contain immunoreactive molecules. Ank3 is one such ankyrin. It is the major and possibly the only ankyrin in the kidney and other epithelial tissues, the major axonal ankyrin in adult neurons, and probably the major ankyrin in muscle. It is also found in some specialized cells, including megakaryocytes, macrophages, and the interstitial (Leydig) cells of the testis.

In the kidney the distribution of Ank3 (Fig. 10) correlates very closely with the activity of Na/K-ATPase in various segments of the nephron (33). Both proteins are expressed in the proximal convoluted tubule, the thick ascending limb of the loop of Henle, the distal convoluted tubule, and the collecting duct, and neither protein is detectable in the thin limbs of the loop of Henle (Fig. 10) (Piepenhagen, P.A., and W.J. Nelson. 1994. Mol. Biol. Cell. 5(Suppl.):367a). Na/K-ATPase is localized to the basal-lateral surfaces of the epithelial cells in these segments and, where its location can be discerned, Ank3 also has a basal-lateral distribution. Given these facts, and the fact that Na/K-ATPase can be isolated from kidney complexed with an ankyrin and fodrin (49-51), it is very likely that Ank3 is involved in the targeting/maintenance of Na/K-ATPase within plasma membrane domains in epithelial cells, and perhaps in other cells. Based on location, other ankyrin-binding molecules that may serve as ligands for Ank3 include the amiloride-sensitive and voltage-gated Na-channels, the H/K-ATPase, the Na/Ca exchanger, Cl/HCO₃ exchangers, and members of the neurofascin/L1-like family of cell adhesion molecules.

### Some Forms of Ank3 Lack the Repeat Domain

Ank3 exists in diverse forms. In the kidney the transcripts can be subdivided into four groups: two relatively discrete RNAs of ~7.5- and 6.9-kb, at least one RNA at 6.3 kb, a broad band of about 5.7 kb, and minor bands of 5.1 and 4.6 kb.

The 6.9- and 7.5-kb species (“Ank3-7kb”) are the only ones that are labeled by probes to all three domains, including the repeat domain (Fig. 6 a, lane 1). Several observations suggest these two transcripts encode full length kidney Ank3 with or without insert C (an acidic sequence, located in the regulatory domain that is analogous to the “2.1 insert” of Ank1 [45]). PCR products that differ by ~0.6 kb, the size of insert C, are observed when the entire coding sequence of Ank3-7kb is amplified (data not shown) and two proteins (215 and 200 kD) close to the expected sizes (214 and 193 kD) are detected on immunoblots (Fig. 7 a, lane 1). However the situation seems to be more complicated since the 6.9- and 7.5-kb transcripts both hybridize to a probe from insert C (Fig. 6 a, lane 4). Further investigations are required.

In contrast, the 6.3- and 5.7-kb RNAs lack the repeat domain and are tentatively classified as variants of “Ank3-5kb”. They fail to hybridize to probes from the repeat domain and they all contain the unique 5’ untranslated region found in cDNA clones in which the repeat domain is missing (Fig. 6 a, lane 3). They appear to encode a protein containing just the spectrin-binding and regulatory domains, with (6.3 kb) or without (5.7 kb) insert C. PCR surveys reveal the expected products and, as anticipated, a probe from insert C fails to hybridize to the 5.7-kb band (Fig. 6 a, lane 4). More importantly, two proteins (120 and 105 kD) very close to the predicted sizes (121 and 100 kD) are easily detected in crude renal membranes, and in fact are the major protein species (Fig. 7 a, lane 1). This strongly suggests that the truncated Ank3-5kb protein is actually synthesized, although additional evidence is needed to formally prove this point. Unfortunately, attempts to prepare an antibody specific to the six unshared NH₂-terminal amino acids of Ank3-5kb, or to isolate and sequence the NH₂-terminal ends of the 120- and 105-kD proteins have so far been unsuccessful.

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**Figure 9.** Immunocytochemical detection of Ank3 in mouse testis, pancreas, muscle, and spleen. (a) Testis. Large cytoplasmic inclusions containing abundant Ank3 are present within the testosterone-secreting interstitial cells of Leydig (middle and right arrows). Weak, diffuse Ank3 reactivity is seen in the rest of the cytoplasm. A portion of a seminiferous tubule is seen on the left. These stain very weakly or not at all. No Ank3 staining is seen in the endothelial lining of small blood vessels (top left arrow). (b) Testis control. (c) Pancreas. Acinar cells show apical staining for Ank3 (lower arrow). Epithelial cells lining the ducts are strongly positive (upper arrow). (d) Pancreas control. (e) Heart. Ank3 is present throughout cardiac muscle fibers and is concentrated at intercalated discs (arrows). (f) Skeletal muscle. Skeletal muscle of the skin is Ank3 immunoreactive, while the surrounding connective tissue is negative. (g) Skeletal muscle control. (h) Spleen. Megakaryocytes (arrows) show diffuse Ank3 staining. (i) Spleen control. Bar, 10 μm.
Figure 10. Immunocytochemical detection of Ank3 in mouse brain and kidney. (a) Cerebral cortex. Axon initial segments (arrow) are positive for Ank3. (b) Cerebellum. The molecular layer (M) is shown at top and the granule cell layer (G) is at the bottom. The unmyelinated neurons of the molecular layer appear negative while the axons of the Purkinje cells (arrow) are positive. (c) Brain (cerebral cortex) control. (d) Kidney cortex. Intense, basal-lateral Ank3 staining is seen in the macula densa (left arrow), distal convoluted tubules, and cortical collecting ducts (right arrow). Proximal convoluted tubules (P) show weaker and more diffuse Ank3 staining. A glomerulus (G) is negative. (e and f) Outer medullary collecting ducts show strong basal-lateral concentration of Ank3. (g) The junction of the outer and inner medulla. The epithelial cells of a thick ascending limb (lower arrow) and an outer medullary collecting duct (upper arrow) stain intensely for Ank3, while the inner medullary collecting ducts, at the bottom, are weakly stained. (h) High magnification photograph showing the abrupt transition (arrow) from heavy to light Ank3 staining of collecting ducts at the junction of the outer and inner medulla. (i) Kidney control. Bar, 10 μm.
The minor 5.1- and 4.6-kb mRNAs also lack the repeat domain and seem to differ by the presence or absence of insert C (Fig. 6 a, lane 4); however, it is not clear whether they are translated into stable proteins.

The fifth Ank3 protein in kidney, the 170-kD isoform, is more mysterious. This is the only Ank3 species that contains the B insert. Since it is detected by anti-Ank3-R1, which was made to a unique sequence near the COOH terminus of the regulatory domain, it must also contain sequences from that region. It is not clear what other parts of Ank3 are present in this isoform. The size of the related mRNA transcript is also unknown. However, it should be possible to use an antibody to the B insert to see which cells in the kidney express this protein and perhaps discover its ligands.

**The Truncated Ank3-5kb Protein May Be Associated with Cytoplasmic Structures**

It is notable that the 6.3/5.7 kb Ank3 RNAs and/or the presumptively related 120/105-kD proteins are the only forms of Ank3 detectable in intestine, testis, and liver (Figs. 6 and 7). In these tissues immunoreactivity is concentrated in the cytoplasm and is not evidently associated with the membrane (e.g., see Fig. 8, h and i). In contrast, in tissues containing the ~7-kb forms of Ank3, such as kidney, lung, heart and brain (Figs. 6 and 7), plasma membrane staining is observed, often in a polarized distribution (e.g., Fig. 8 or Fig. 10, e and f). The observations suggest that the repeat domain is required to attach Ank3 to its integral membrane ligands and, in its absence, that the protein remains in the cytoplasm or attaches to cytoplasmic structures. Preliminary studies of Ank3 in mouse bone marrow macrophages support this concept. The macrophages only have ~5.0- and ~3.6-kb Ank3 transcripts, neither of which contains the repeat domain. The related proteins (120 and 105 kD) are associated solely with the membrane of phase dense, late lysosomal vesicles, and not with the plasma membrane (Hocott, T. C., L. L. Peters, and S. E. Lux. 1994. *Mol. Biol. Cell.* 5 [Suppl]:45a). These preliminary observations suggest that ankynirs lacking the repeat domain may play a role in intracellular vesicle stabilization, sorting or targeting, or have other still undiscovered functions.

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