

Identification of a Small Cytoplasmic Ankyrin (Ank_{G119}) in the Kidney and Muscle that Binds β I Σ * Spectrin and Associates with the Golgi Apparatus

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Abstract. Ankyrins are a family of large, membrane-associated proteins that mediate the linkage of the cytoskeleton to a variety of membrane transport and receptor proteins. A repetitive 33-residue motif characteristic of domain I of ankyrin has also been identified in proteins involved with cell cycle control and development. We have cloned and characterized a novel ankyrin isoform, Ank_{G119} (GenBank accession No. U43965), from the human kidney which lacks part of this repetitive domain and associates in MDCK cells with β I Σ * spectrin and the Golgi apparatus, but not the plasma membrane. Sequence comparison reveals this ankyrin to be an alternative transcript of Ank_G, a much larger ankyrin recently cloned from brain. Ank_{G119} has a predicted size of 119,201 D, and contains a 47-kD domain I consisting of 13 ankyrin repeat units, a 67-kD domain II with a highly conserved spectrin-binding motif, and a truncated 5-kD putative regulatory domain. An Ank_{G119} cDNA probe hybridized to a 6.0-kb message in human and rat kidney, placenta, and skeletal muscle. An antibody raised to Ank_{G119} recognized an apparent 116-kD peptide in rat kidney cortical tissue and MDCK cell lysates, and did not react with larger

isoforms of ankyrin at 190 and 210 kD in these tissues, nor in bovine brain, nor with ankyrin from human erythrocytes. Ank_{G119} remains extractable in 0.5% Triton X-100, and assumes a punctuate cytoplasmic distribution in mature MDCK cells, in contrast to the Triton-stable plasma membrane localization of all previously described renal ankyrins. Ank_{G119} immunoreactivity in subconfluent MDCK cells distributes with the Golgi complex in a pattern coincident with β -COP and β I Σ * spectrin immunoreactivity. A fusion peptide containing residues 669–860 of Ank_{G119} interacts with β I Σ 1 spectrin in vitro with a $K_d = 4.2 \pm 4.0 (\pm 2 \text{ SD})$ nM, and avidly binds the β spectrin in MDCK cell lysates. Collectively, these data identify Ank_{G119} as a novel small ankyrin that binds and colocalizes with β I Σ * spectrin in the ER and Golgi apparatus, and possibly on a subset of endosomes during the early stages of polarity development. We hypothesize that Ank_{G119} and β I spectrin form a vesicular Golgi-associated membrane skeleton, promote the organization of protein microdomains within the Golgi and *trans*-Golgi networks, and contribute to polarized vesicle transport.

INTERACTIONS between integral membrane proteins and the underlying spectrin-actin cytoskeleton play key roles in activities such as cell motility, activation, proliferation, contact, and the maintenance of specialized membrane domains (Luna and Hitt, 1992; Bennett and Gilligan, 1993; Devarajan and Morrow, 1996; Morrow et al., 1996). Ankyrins are a family of large, membrane-associated proteins that have emerged as crucial adapter molecules mediating such linkages, since they possess recognition sites for various membrane proteins as well as for cytoskeletal elements (Bennett, 1992). Molecules using a 33-residue re-

petitive structure first identified in the 89-kD domain I of ankyrin display a wide tissue distribution (Bork, 1993; Chan et al., 1993; Axton et al., 1994; Diederich et al., 1994), and tissue-specific isoforms may be present in all cells (Lux et al., 1990a). Several distinct isoforms of ankyrin have been recognized by their immunological properties. The isoform associated with the membranes of red cells, neuronal cell bodies, and dendrites has been termed Ank_R. Antibodies to Ank_R also cross-react with an ankyrin found in the basolateral membrane of polarized epithelial cells (Davis et al., 1989; Morrow et al., 1989). Ank_R is encoded by the *ANK1* gene (Lux et al., 1990a,b), which transcribes mRNA species of 7 kb in erythrocytes (Lambert et al., 1990; Lux et al., 1990a) and 9 kb in brain (Lambert et al., 1990). Ank_B is the major isoform in brain,

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and is widely present in neuronal and glial cell membranes, as well as in a variety of nonneuronal tissues including the kidney. It is the product of the *ANK2* gene (Otto et al., 1991). At least two alternatively spliced mRNA transcripts of 9 and 13 kb are generated from this gene (Kunimoto et al., 1991; Otto et al., 1991). Ank_G is an immunologically distinct isoform found at the plasma membrane at the nodes of Ranvier and at the axon initial segments (Kordeli et al., 1990, 1995; Kordeli and Bennett, 1991). It is the product of the *ANK3* gene (Peters et al., 1995). Although two transcripts of the Ank_G gene (15 and 10 kb) are neural-specific, smaller, alternatively spliced isoforms may be expressed in the kidney and lung (Kordeli et al., 1995; Peters et al., 1995). In addition, a 72-kD truncated ankyrin that binds spectrin and is localized with the membrane protein GP85 has been detected immunologically in T lymphocytes; its gene of origin is unknown (Bourguignon et al., 1986).

Such isoform diversity may be critical to maintain a specific pattern of protein distribution in neurons and polarized epithelial cells such as those of the kidney tubules that directionally transport ions and nutrients. It is likely that such tissues harbor additional isoforms of ankyrin. Antibodies raised against Ank_R recognize a 210-kD product in erythrocytes and a 190-kD polypeptide in kidney tissue (Davis et al., 1989; Morrow et al., 1991). The Ank_R from both sources binds spectrin and Na,K-ATPase (Devarajan et al., 1994). Ank_R-deficient NB/NB mice, however, express the 190-kD renal ankyrin, indicating that it is encoded by a gene distinct from *ANK1*, presumably an *ANK3* gene (Bennett, 1992). Antibodies to Ank_B cross-react with a 220-kD peptide in kidney tissue, but Ank_B cDNA probes hybridize only weakly to renal RNA (Otto et al., 1991), suggesting that the kidney Ank_B peptide may also be the product of a distinct gene. Finally, antibodies to Ank_G recognize polypeptides in the 190–72-kD range in rat kidney (Kordeli et al., 1995), none of which have been further characterized.

In this study, we describe the cDNA cloning, characterization, and intracellular localization of a small 119-kD ankyrin isoform (Ank_{G119}) from kidney tissues. After this work was first submitted, a report describing Ank_G in the brain appeared (Kordeli et al., 1995). Comparison with that sequence reveals the ankyrin we have cloned to be an alternative transcript of the larger form that was identified in brain. Therefore, we now refer to this form as Ank_{G119}, rather than Ank_K, as had been previously reported in abstract form (Devarajan, P., A. Mann, T. Ardito, P. Stabach, M. Kashgarian, and J.S. Morrow. 1995. *Mol. Biol. Cell.* 6: 269a) and in the initial version of this report. Ank_{G119} possesses only part of the repeats domain characteristic of all previously described ankyrins, and also deletes almost the entire regulatory domain. Ank_{G119} is expressed in the kidney, placenta, and skeletal muscle, in MDCK cells (a collecting tubule line), and in cultured porcine proximal tubule cells (LLC-PK1). The distribution of this small ankyrin in rat kidney cells and in confluent MDCK and LLC-PK1 cells is cytoplasmic and Golgi associated, unlike the plasma membrane localization of all other previously described ankyrin isoforms. Ank_{G119} also specifically binds MDCK cell β I Σ * spectrin (erythroid-like) in vitro with nanomolar affinity, and is coincidentally distributed in the

Golgi apparatus with a previously described Golgi-associated β I Σ * spectrin (Beck et al., 1994). These findings significantly extend the known diversity of the ankyrins, confirm the presence of a Golgi-associated β I Σ * spectrin, and strongly imply that these two proteins function together as an integral part of the Golgi apparatus. We speculate that as with the plasma membrane, specialized variants of spectrin and ankyrin organize microdomains within the ER, Golgi, and *trans*-Golgi networks, and may play a pivotal role in the process of targeted membrane assembly.

Materials and Methods

Isolation of Renal Ankyrin Clones

All molecular biological procedures were carried out using standard methods (Sambrook et al., 1989). Oligonucleotides bracketing a 255-bp conserved region within the spectrin-binding domain of human erythrocyte ankyrin were used in standard PCR reactions (Innis and Gelfand, 1990). The sense primer was 5'-GCCAGTGGGAAGACATAACAGG-3'; the antisense primer was 5'-CTTGTCCACTTTATCATCTGTCATG-CAG-3'. Five templates were amplified: (a) human kidney cDNA library (Clontech Laboratories, Palo Alto, CA); (b) rat kidney cDNA library (Clontech); (c) rat kidney RNA reverse transcribed with random hexamer priming and avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN); (d) MDCK cell RNA reverse transcribed as described above; and (e) LLC-PK1 cell RNA reverse transcribed as described above. Water was used as a negative control for all PCR reactions. The absence of genomic DNA in the RNA samples was confirmed by the absence of amplified PCR products when no reverse transcriptase was used. PCR products were subcloned into the TA vector (Invitrogen, San Diego, CA), and both strands were sequenced by the dideoxynucleotide chain termination method (U.S. Biochemical Corp., Cleveland, OH). Subsequently, the random primer ³²P-labeled PCR product was used to screen the human kidney cDNA library to isolate several overlapping clones. One rescreening of the library was required to obtain the 5' end and complete the Ank_{G119} cDNA sequence.

Northern Blot Analysis of mRNA Expression

Human multiple-tissue nylon membrane blots containing poly-A-selected mRNAs (Clontech) were hybridized to a random primer ³²P-labeled cDNA probe (the 255-bp PCR product) encoding human kidney ankyrin or a control probe for actin and washed at high stringency. Hybridized bands were detected by autoradiography using X-OMAT film (Eastman Kodak Co., Rochester, NY) and fluorescent intensifying screens.

Preparation of Antibodies to Ank_{G119}

Antibodies to Ank_{G119} were produced from a recombinant peptide. The 255-bp PCR product from human kidney cDNA library was ligated in-frame to the expression vector pGEX-1N via the EcoR1 site. The pGEX vectors (Pharmacia Fine Chemicals, Piscataway, NJ) direct the bacterial synthesis of foreign proteins as a fusion peptide with glutathione S-transferase (GST)¹ (Smith and Johnson, 1988). Overnight cultures of transformed *Escherichia coli* were induced with 1 mM isopropyl β -D-thiogalactoside for 5 h at 37°C, pelleted, and resuspended in sonication buffer (containing 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM DTT, 1 mM benzamide, pH 8). After overnight storage at -80°C, the suspension was thawed, brought to 1% (vol/vol) Triton X-100, and sonicated at 70 W for 15 s, repeated three times, with a sonic power apparatus (Branson Ultrasonics Corp., Danbury, CT). The lysate was centrifuged at 48,000 g for 20 min, and the supernatant was affinity purified on a 2-ml glutathione-agarose column. The pelleted material was extracted by solubilization in 6 M urea, 50 mM glycine, pH 9.0, and successively dialyzed in 2, 1, 0.5, and 50 mM urea (each with 50 mM glycine, pH 9.0), and then affinity purified as described before. Extraction of the pellet yielded a 25-fold increase in the amount of fusion peptide obtained. The two fractions were pooled, rebound to a glutathione-agarose column, eluted with 50 mM Tris-HCl/5 mM reduced glutathione, pH 8.0,

1. Abbreviation used in this paper: GST, glutathione S-transferase.

and dialyzed into PBS. Aliquots were analyzed by SDS-PAGE, and 0.1 ml (at 40 mg/ml) was injected subcutaneously into New Zealand white rabbits in complete Freund's adjuvant for antibody production (Morrow et al., 1989). Antibody titers of hyperimmune sera were monitored by ELISA assay using recombinant Ank_{G119}.

Preparation of Cell and Tissue Lysates

The outer renal cortices of Sprague-Dawley rats were harvested in 5 ml of PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) containing 0.2% Triton X-100, 0.5 mM PMSF, 10 µg/ml leupeptin, and 0.1 mM DTT (Van Why et al., 1992). After homogenization with a Potter-Elvehjem homogenizer and centrifugation at 35,000 g for 14 min at 4°C, the pellets were resuspended in a volume of PHEM extraction buffer equal to that of the supernatants, and aliquots of each fraction were analyzed by SDS-PAGE and Western blotting (Molitoris et al., 1992).

MDCK cells grown to confluence in 150-cm² culture flasks in DME medium supplemented with 10% FBS and antibiotics were sequentially extracted *in situ* as described before (Morrow et al., 1989; Devarajan et al., 1994). Five extractions were performed: buffer 1 (yielding Fx1) contained 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, 1.2 mM PMSF, 0.5 mM Pefabloc SC; buffer 2 (yielding Fx2) substituted 250 mM ammonium sulfate for NaCl, but was otherwise identical to buffer 1; buffer 3 (yielding Fx3) was similar to buffer 1 but contained 50 mM NaCl, 100 µg/ml of DNase I, 100 µg/ml RNase, and 33 mg/ml ammonium sulfate; buffer 4 (yielding Fx4) was 10 mM Tris-HCl, pH 7.4, 9.5 M urea; buffer 5 (yielding Fx5) was 10 mM Tris-HCl, pH 7.4, 1% (vol/vol) 2-mercaptoethanol, 9.5 M urea. Protein in the MDCK cell extracts was assayed by Peterson's modification of the Lowry method (Peterson, 1983). Interfering buffer components were removed by precipitation of the protein with trichloroacetic acid in the presence of sodium desoxycholate (Bensadoun and Weinstein, 1976). Analysis of these fractions by Western blots has shown that ~65% of the immunodetectable spectrin is in Fx2, but a significant quantity (~30%) is also found in Fx1 (data not shown), as has been previously reported for the 210-kD isoform of ankyrin in MDCK cells (Devarajan et al., 1994).

Protease Digestions

Human erythrocyte ghosts and MDCK cell lysate soluble (fx1) and cytoskeletal (fx2) fractions were subjected to limited tryptic digestion (Weaver and Marchesi, 1984). Each sample at ~1 mg/ml total protein was exhaustively dialyzed against trypsin digestion buffer (5 mM NaHPO₄, 20 mM KCl, 1 mM EDTA, 0.5 mM 2-mercaptoethanol, pH 7.6) and then treated with trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated; Worthington Biochemical Corp., Freehold, NJ) at an enzyme/substrate ratio of 1:20 at 0°C for 30 min. Reactions were terminated by boiling with 4× solubilizing buffer containing 12% (wt/vol) SDS, 5 M urea, 250 mM Tris-HCl, pH 7.5. Aliquots were analyzed by SDS-PAGE and Western blotting with Ank_R⁻ and Ank_{G119}-reactive antiserum. Alternatively, the same substrates were subjected to digestion with µ-calpain (Harris et al., 1989) after dialysis into calpain digestion buffer (20 mM Tris, 25 mM NaCl, 5 mM DTT, pH 7.5). Purified µ-calpain from porcine erythrocytes (Nacalai Tesque, Inc., Kyoto, Japan) was diluted immediately before use 1:10 into 50 mM Tris, 1 mM DTT, pH 7.5, and added to each sample at a 1:100 (vol/vol) ratio. Before the addition of µ-calpain, each sample was brought to 100 µM in CaCl₂. Digestion was allowed to proceed for 20 min at 30°C, and was terminated by boiling as described above.

Expression of Recombinant Ank_{G119} Constructs

Three sets of oligonucleotides were designed as shown in Fig. 8a, so as to create three constructs spanning residues 457–692 (Ank W, 1595–2205 bp), 669–860 (Ank J, 2135–2709 bp), and 850–1058 (Ank Y, 2680–3303 bp), all within the putative spectrin-binding domain of Ank_{G119}. Using the clone HKA R as template (Fig. 1), these constructs were amplified by standard PCR, subcloned, and sequenced to verify their identity. They were then ligated in-frame into the pGEX vectors, expressed in bacteria as fusion peptides with GST, and purified on a glutathione-agarose column (Devarajan et al., 1994). GST alone was also expressed as a control peptide. The four recombinant peptides were eluted, dialyzed into spectrin-binding buffer (FBB; 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 mM Pefabloc SC), and aliquots were analyzed by SDS-PAGE and quantified by protein assay (Bio Rad Laboratories, Hercules, CA). Each fusion (50 µg at 1 mg/

ml) was conjugated to 50 µl of a 50% slurry of glutathione-agarose beads for 1 h at 4°C with gentle rotation and used in binding assays.

Binding Assays

The interaction of Ank_{G119} with spectrin in the soluble fraction (Fx1) of MDCK cell lysates was assayed *in vitro* by the ability of conjugated recombinant Ank_{G119} peptides to retain spectrin (Devarajan et al., 1994). Freshly prepared Fx1 was dialyzed into FBB and preadsorbed to glutathione-agarose beads conjugated with GST alone for 4 h at 4°C. After pelleting at 13,000 g for 1 min, the supernate (~500 µg total protein) was allowed to interact with each of the four fusion peptides (50 µg each, previously conjugated to GST and beads) at 4°C overnight with gentle rotation. The beads were pelleted and washed twice with FBB, and the aliquots were analyzed by SDS-PAGE. Bound spectrin was detected by Western blotting, with antibody directed against the β15* spectrin subunit.

The interaction of Ank_{G119} with β1 spectrin was measured quantitatively using spectrin purified from fresh human erythrocytes (Morrow and Marchesi, 1981) and biotinylated by reaction of a 3 mg/ml solution in PBS with a 12-fold molar excess of sulfo-*N*-hydroxysuccinimide-biotin (Pierce Chemical Co., Rockford, IL) at 0°C for 2 h. Excess biotin was removed by exhaustive dialysis against PBS. Recombinant Ank_{G119} peptide or control GST was incubated overnight in PBS with increasing amounts of labeled spectrin. Bound and free fractions were separated by absorption to glutathione-agarose as described before (Kennedy et al., 1994) and analyzed by SDS-PAGE. Free and bound spectrin were measured by an overlay assay using HRP-avidin and enhanced chemiluminescence (Kennedy et al., 1994). Each determination was compared to a series of standard protein loads using the same biotinylated spectrin and developed on the same transfer membrane. Binding results were analyzed by nonlinear regression after subtraction of the nonspecific binding to GST controls. All data was fitted as simple bimolecular binding, minimizing the degrees of freedom of the fit.

Immunofluorescence and Confocal Microscopy

Cells were fixed and labeled in a modification of a previously used protocol for intact tissues (Van Why et al., 1992). Briefly, cells grown to confluence in Lab-Tek eight-chamber slides were fixed by exchanging the tissue culture media with one rapid wash of PBS followed by paraformaldehyde-lysine-periodate fixative (PLP) for a minimum of 1 h at room temperature (McLean and Nakane, 1974). Cells were then washed three times in PBS and used immediately for labeling, or they were stored in holding buffer (PBS 0.2% formaldehyde) at 4°C for a maximum period of 10 d until labeling. On the day of labeling, the cells were gently permeabilized by freeze-thawing. In this procedure, the cells were treated with PBS, 10% DMSO for 15 min, and then rapidly frozen on an aluminum block at -35°C. They were then thawed by immersion in PBS, 1% BSA, 1% normal goat serum, and were allowed to remain in this solution to block nonspecific staining for 1 h at room temperature. Labeling of ankyrin antibodies was accomplished by exposing the cells to a titrated solution of preimmune and immune sera diluted in PBS, 1% BSA, 0.1% normal goat serum for 1 h at room temperature. Slides were gently agitated at 15-min intervals. The cells were then washed three times in PBS, and biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was applied at a 1:100 dilution in PBS/BSA solution, as done with the primary antibodies. This was followed by an identical third step of exposing the cells to FITC-conjugated avidin (Vector Laboratories) diluted 1:100 in PBS/BSA. Cells in adjacent chambers were labeled with a mAb for Na,K-ATPase using a two-step indirect labeling process involving exposure to the primary antibody, which was then followed after washing with rhodamine-conjugated goat anti-mouse antibody (Cappel/Organon-Teknika, Durham, NC) 1:200 in PBS/BSA. The slides were coverslipped in Vectashield (Vector Laboratories) to retard fading and were edge sealed with Cytoseal 60 (Stephens Scientific, Inc., Riverdale, NJ) mounting media. Confocal microscopy was performed using a Bio Rad MRC-600 scanning laser microscope attached to a standard microscope (Carl Zeiss, Inc., Thornwood, NY) with a Leitz 50× water immersion lens (Leica, Inc., Deerfield, IL). Labeled cells and negative controls were first visualized *en face* under identical microscope settings that were standardized with respect to illumination intensity, detector amplifier settings, and confocal aperture size. Z section microscopy was also performed on areas of interest to determine apical and basolateral domain staining. In this procedure, the confocal aperture was set at its minimum section thickness, ~1.0 µm with the objective lens used, and images were collected at 1.2-µm steps so that no overlap between sections would be observed. Alternatively, conventional indirect fluorescent microscopy

was performed using a microscope (AX70; Olympus Corp., Lake Success, NY) equipped for epillumination.

Other Methods

The isolation of total RNA from rat kidney, MDCK cells, and LLC-PK1 cells used standard methods (Chomczynski and Sacchi, 1987). SDS-PAGE and Western blotting methods were as described (Laemmli, 1970; Towbin et al., 1979). Immunodetection of transferred proteins was by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Antibodies to α -Na,K-ATPase, AnkR, and nonerythroid spectrin have been previously characterized (Morrow et al., 1989; Devarajan et al., 1994). The antibody to β -COP was a kind gift from Dr. Ira Mellman (Yale University, New Haven, CT) and has been previously described (Pepperkok et al., 1993; Whitney et al., 1995). The mAb to β I spectrin was mAb VIII C7, which reacts with epitopes near the center of β I spectrin (Harris et al., 1986).

Results

Cloning and Characterization of a Novel Ankyrin from Kidney Tissues (*Ank_{G119}*)

A comparison of published cDNA sequences encoding human erythrocyte (Lambert et al., 1990; Lux et al., 1990a) and brain (Otto et al., 1991) ankyrins revealed a region of sequence conservation in the spectrin-binding domain (between residues 1183 and 1268 of human erythrocyte ankyrin). Oligonucleotides bracketing this highly conserved region amplified a 255-bp product by PCR from five kidney tem-

plates: a human kidney cDNA library, a rat kidney cDNA library, reverse-transcribed MDCK cell RNA, reverse-transcribed LLC-PK1 cell RNA, and reverse-transcribed rat kidney RNA (Fig. 1 a). Nucleotide sequencing of several positive clones from each of the PCR reactions revealed that regardless of the templates that were used, just two families of sequences were detected (Fig. 1 b). One family included proteins that were highly homologous or identical to human brain ankyrin, reinforcing immunologic data that Ank_B is expressed in renal tissues (Otto et al., 1991). The other family of ankyrins (Ank_G) included sequences that were closely related to each other (>90% identity over the 255-bp region amplified by PCR) but were dissimilar to any recognized ankyrins from brain or erythrocytes. Comparing the human sequences over this region, Ank_{G119} displayed ~58 and ~64% identity at the nucleotide level (55–69% identity at the amino acid level) to Ank_R and Ank_B, respectively (Fig. 1 c). Subsequent comparisons to Ank_G, which were not available when these studies were carried out, reveal 100% identity at the amino acid and DNA levels over this region, confirming that it arises from the same gene as Ank_G (*ANK3*).

Using the 255-bp PCR product as a probe, three overlapping clones encoding a unique ankyrin sequence (HKA B, HKA S, and HKA R) were identified in the human kidney cDNA library (Fig. 1 d). Rescreening the library with HKA R yielded two additional overlapping clones (HKA Z

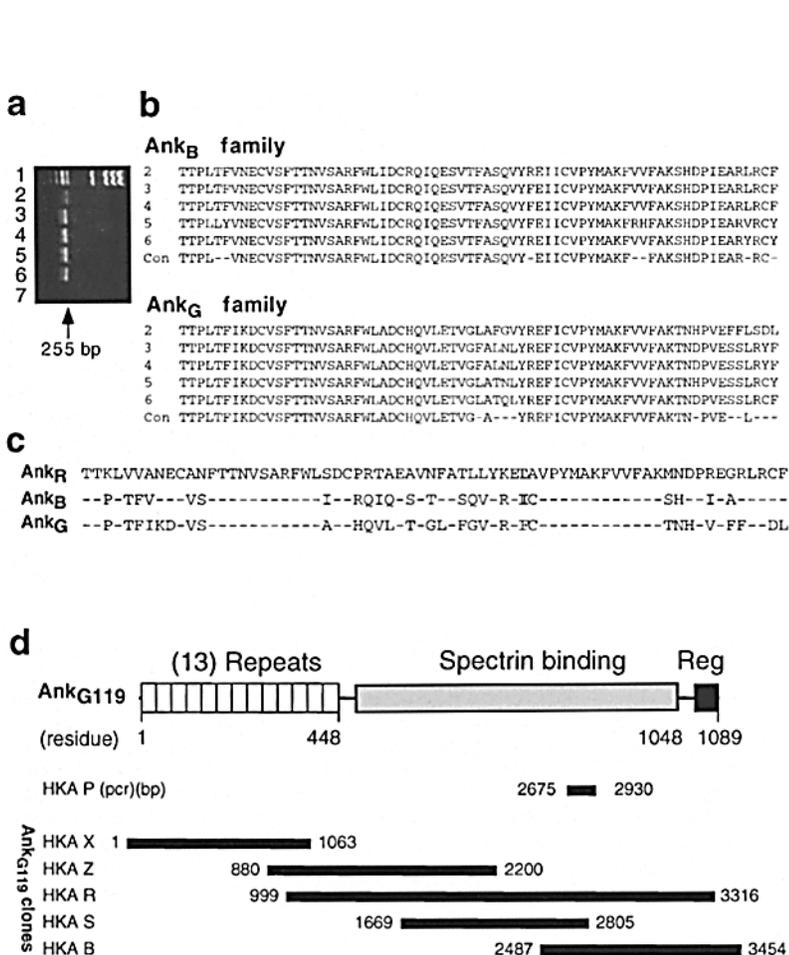


Figure 1. Cloning of Ank_{G119}. (a) Primers flanking a 255-bp conserved region in the spectrin-binding domains of previously described ankyrins were used to amplify a human kidney cDNA library (lane 2), rat kidney cDNA library (lane 3), reverse-transcribed rat kidney RNA (lane 4), reverse-transcribed MDCK cell RNA (lane 5), reverse-transcribed LLC-PK1 cell RNA (lane 6), and water (lane 7, negative control). Lane 1 contains a HaeIII digest of ϕ ×174 as a standard. The 255-bp PCR product is indicated by the arrow. Ethidium bromide-stained gel. (b) The derived amino acid sequences of the clones generated from the PCR amplified cDNA from each sample fell into two related categories. *Top*, those related to Ank_B; *bottom*, those related to each other but distinct from Ank_B, comprising the new type of ankyrin called Ank_{G119}. The origin of the numbered sequences correspond to the numbered lanes in a. *CON*, the consensus sequence. (c) Comparison of the derived human amino acid sequences of Ank_R, Ank_B, and Ank_{G119} over the amplified spectrin-binding domain. Conserved amino acids are indicated by dashes. Human Ank_B and Ank_{G119} share 66% and 56% amino acid identity, respectively, to human Ank_R over this region. (d) Overlapping Ank_{G119} clones obtained by screening a human kidney cDNA library first with the Ank_{G119} PCR product (HKA B, S, R) and then with HKA R (HKA Z, X). Comparison of the full-length deduced amino acid sequence with Ank_R and Ank_B allowed the delineation of a 47-kD domain consisting of 13 ankyrin repeats, a 67-kD spectrin-binding domain, and a very truncated 5-kD regulatory domain.

and HKA X), confirming a contiguous 3454-bp cDNA sequence for Ank_{G119} (GenBank No. U43965) with a putative polyadenylation signal (ATAAAA) 17 bp upstream of the poly(A)⁺ tail (Fig. 2). The cDNA sequence has a single open reading frame (with stop codons in all three reading frames upstream of the initiator methionine), and it encodes for a protein of 1,089 amino acids with a predicted molecular weight of 119,201 and isoelectric point of 8.2.

Comparison of the deduced Ank_{G119} amino acid sequence with previously described ankyrins allows for the delineation of three putative ankyrin domains (Fig. 2). After a unique 5'-flanking sequence, Ank_{G119} encodes just 13 33-residue repetitive motifs characteristic of the ankyrins, in contrast to the 22–24 copies of this motif found in all previously described ankyrins. As a result, domain I of Ank_{G119} is 47 kD. The Ank_{G119} repeats are identical to repeats 12–24 of Ank_G and homologous to repeats 11–22 in Ank_R and Ank_B, indicating that Ank_{G119} is devoid of the first 10–11 repeats found in other ankyrins. The Ank_{G119}

repeats domain is followed by a 67-kD putative spectrin-binding domain with strong homology to other ankyrins and a remarkably shortened 5-kD regulatory domain (starting with A-L-R as in Ank_R) (Lux et al., 1990a), but is then followed by unique sequence. This divergent regulatory domain cannot be an artifact of concatamer formation during library construction because it has been identified in two distinct clones (Fig. 1), and reverse transcription PCR amplification of RNA derived from kidney tissue using primers flanking the junction between the homologous ankyrin-like sequence and the novel 3' domain generated the predicted product (data not shown). Interestingly, these primers did not amplify any products from brain RNA, suggesting that this 3' end is unique to Ank_{G119}. Overall, Ank_{G119} is highly homologous to Ank_G, and partially homologous to Ank_B and Ank_R, suggesting that Ank_{G119} is a tissue-specific, alternatively spliced, and truncated isoform of Ank_G, but possesses unique 5' and 3' ends.

While the similarities noted above clearly indicate that it

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GGAGCTCTCTCACTCAAGCCGAGTTTCATGTATGTCACAGATAGTACATTCATCACTGTCTCTCCAGBATTGGGAAGTCTGGCAAAACACCATTCCAGTACGT 105
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TTEKHEKHMNVPEITMNEVLDHMSDDEEDAMTGDTDK 482
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TLNRSYATGACAGGATGAGTAEELLVPSKEOHLTFTRFDS 652

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GGS MRGSRRHGMRIIPPRKCTAPTRITCRLLVKRH 822
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KLANPPPHGERRRGIS SRLVEHMGPA G A D F L G P V I V E 657
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I P H F G S M R G K E R E L I V L R S E N G E T W K E H Q F D S K N E 692
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DLTELELLNGMD E E L D S P E E L G K K R I C R I I T K D F P Q Y 727
TTTTGAGTGGTTCCCGGATTAAGCAGAAAGCAACAGATGGTCTTCAAGGTTGAAATCTGAGCAGCAGCAGTCCCTTGTCAAGCATTTCCCGA 2415
FAVVSRIKVGESNDIGPEGSI L S T V P L V Q A S F P E 762
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GALTKRIRLVGLDQAPVPDEIVK K I L G N K A T F S P I V 797
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T P N L R L L C S I J T G C S I T G G T S P A Q W E D I T G T T P L I F I K D C V 887
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S F T T N V S A R F W L A D C H Q V L E T V G L A T D L Y R E L I C V 902
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P Y M A K F V V F A K M N D P V E S S L R C F C M T D D K V D K T L E 937
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Q O E N F E E V A R S K D I E V L E G K P I Y V D C Y G N L A P L T K 972
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G G Q O U L V F N F Y S F K E N R L P F S I K I R D T S O E P C G R L S 1007
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F L K E P K I T K G L P D T A V C N L I T L P A H K K I E K T D R 1042
ACAGAGCTTGCATCTTACGTTACGTAAGCGTACAGCTACTGACTGAGCTGGAATGAGTGGTTCCTTGCACAGCTCAACCTCCGGCTCACTTTAG 3380
G S F A S L A L R K R Y S V L T E P G M S E F P D T S T N P G Q C F R 1077
GAGAAGACATTTTCTTATGCTCAAAATATGATGTTGTTGCAAAATAAGCGCCCTGGCCCAAAAAA A A A A A A A A A A A A A A A A A A A A 3454
R R D I F S M R S K L * 1089

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Figure 2. Nucleotide sequence, deduced amino acid sequence, and putative domain structure of human kidney Ank_{G119}. The beginning of each repeat is marked. The stop codons in all three reading frames upstream of the initiator methionine are underlined. The putative polyadenylation site is in bold and underlined. (GenBank accession No. U43965).

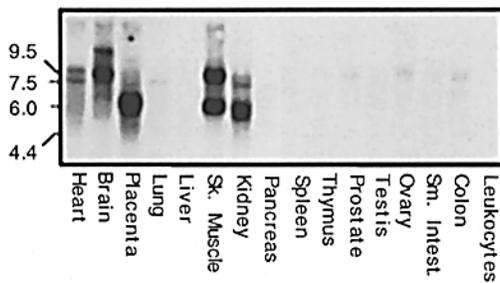


Figure 3. ANK_{G119} is expressed in a restricted subset of human tissues. A Northern blot containing poly-A-selected mRNAs from human tissues was hybridized to a human ANK_{G119} probe. Transcript sizes in kilobases are indicated on the left; tissue sources are as indicated. Note the presence of a prominent 6.0-kb band in the kidney, placenta, and skeletal muscle. The larger bands seen in the brain and some other tissues reflect cross-hybridization with other transcripts of Ank_G (Kordeli et al., 1995). Hybridization of this blot with a human γ -actin probe revealed equal loading of all lanes (not shown).

arises from the same gene, Ank_{G119} differs from brain Ank_G in several respects. Pairwise sequence alignment of Ank_{G119} vs brain Ank_G using either Bestfit (University of Wisconsin Genetics Computer Group) (Devereux et al., 1984) or MegAlign (DNASTAR, Inc., Madison, WI) reveals five specific regions of deleted or altered sequence. The first six residues of Ank_{G119} are unique, as are the first 385 residues of brain Ank_G. Compared to Ank_G, Ank_{G119} deletes (a) 18 residues between positions 850–869 of Ank_G; (b) 6 residues between positions 912–919 of Ank_G; and (c) 9 residues between Ank_G positions 1,441–1,451. In addition, after residue 1477 of Ank_G, Ank_{G119} displays a unique 27-residue (amino acids 1063–1089) COOH terminus vs the 901 residue (amino acids 1477–4378) COOH-terminal domain of Ank_G.

Ank_{G119} Is Expressed Primarily in Kidney, Placenta, and Skeletal Muscle

Northern blot analysis of Ank_{G119} mRNA expression using the 255-bp PCR product as a probe revealed a major 6.0-kb transcript in the human kidney, placenta, and skeletal muscle, and in rat kidney tissue. Upon longer exposures, the Ank_{G119} cDNA probe also hybridized with a 7-kb message in kidney tissue, 9- and 14-kb species in the brain, 8- and 9-kb messages in the heart, and faint 7–8-kb messages in intestinal tissues and ovary (Fig. 3). No message was detected in leukocytes or in the liver. Presumably, these bands represent either cross-hybridization with other ankyrins (given the strong sequence conservation between different ankyrins over some part of their structure), or more likely, other alternatively spliced products of the Ank_G gene (Kordeli et al., 1995; Peters et al., 1995). Hybridization of these blots after stripping of the Ank_{G119} cDNA probe with a human γ -actin cDNA probe revealed equal loading of the actin message in all lanes.

Antibodies to Ank_{G119} Recognize a 116-kD Peptide in the Soluble Pools of Renal Cell Lysates

A recombinant peptide representing the 255-bp PCR product of Ank_{G119} (Fig. 1, clone HKA P) was prepared and

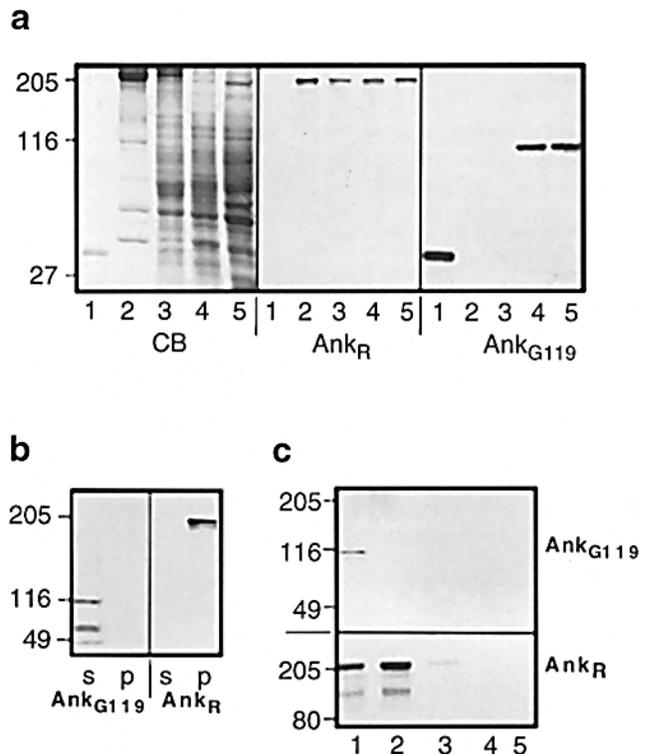


Figure 4. Antibodies to Ank_{G119} identify a 116-kD protein in kidney and muscle lysates. (a) Polyclonal antibodies raised to a 37-kD recombinant human GST-Ank_{G119} fusion peptide or antibodies raised to Ank_R were used to identify immunoreactive bands in various tissues. Lane 1, GST-Ank_{G119} peptide alone; lane 2, human erythrocyte ghosts; lane 3, bovine brain (cortex); lane 4, MDCK cells; lane 5, mouse C2C12 (muscle) cells. *Left panel*, Coomassie blue stain; *center panel*, anti-Ank_R Western blot; *right panel*, anti-Ank_{G119} Western blot. (b) Distribution of Ank_{G119} and Ank_R in rat renal cortical lysates. The Triton-soluble (s) and -insoluble (p) fractions of rat renal cortical lysates were analyzed for the presence of Ank_{G119} (*left panel*) or Ank_R (*right panel*) by Western blot. Note that Ank_{G119} distributed to the soluble fraction, while Ank_R reacted with an ~190-kD peptide in the pellet fraction. (c) Distribution of Ank_{G119} and Ank_R in MDCK cell lysates. Western analysis as described above was carried out on sequentially extracted MDCK cell lysates (see Materials and Methods). The fraction numbers are given. Note that all immunoreactive Ank_{G119} was in the soluble fraction (Fx1, lane 1) (*top panel*), while Ank_R (~215 kD in these canine cells) was predominantly in the cytoskeletal fraction (Fx2, lane 2), as previously noted (Devarajan et al., 1994).

purified as a fusion protein with GST. This peptide was used to generate a polyclonal antibody that specifically recognized this fusion protein on Western blots (Fig. 4 a). This antibody did not react with any proteins in erythrocyte ghosts or in the bovine brain. A protein with an apparent molecular mass of 116 kD was detected in MDCK cells and in C2C12 cells, a myoblast cell line. By comparison, an antibody to Ank_R detected immunoreactive bands between 190 and 220 kD in all cell samples, representing previously described ankyrin species. These observations were refined by examining the solubility properties of the peptide being detected by anti-Ank_{G119}. Unlike the 190-kD ankyrin recognized by Ank_R antibodies, the 116-kD Ank_{G119}

was present only in the Triton-soluble fraction of rat kidney cortical tissue (Fig. 4 b).

A similar segregation of the 116-kD peptide into a soluble pool was also apparent in cultured confluent MDCK cells. Under conditions of sequential extraction of various cellular elements, we have previously demonstrated that the 210-kD immunological counterpart of Ank_R in MDCK cells is found predominantly in the "cytoskeletal" fraction (Fx2) (Devarajan et al., 1994). Under identical extraction conditions, the 116-kD Ank_{G119} is found exclusively in the soluble pool (Fig. 4 c).

*Ank_{G119} Codistributes with Golgi Markers and β I Σ * Spectrin*

Immunofluorescence confocal microscopy with antibodies to Ank_{G119} produced a largely diffuse but punctuate cytoplasmic staining in stable and highly confluent MDCK cells (Fig. 5, c and f), as well as in LLC-PK1 cells and intact renal tubule cells (data not shown). In contrast, antibodies directed against ANK_R demonstrated a basolateral and somewhat apical membrane distribution (Fig. 5, b and e), while the distribution of the α subunit of Na,K-ATPase (Fig. 5, a and d) was basolateral, as expected in these mature MDCK cells. The α II β II spectrin (fodrin) in these cells was also predominantly localized to the basolateral membrane (data not shown) (Nelson and Veshnock, 1986; Koob et al., 1988; Morrow et al., 1989).

When isolated or subconfluent MDCK cells were examined, a different intracellular distribution became apparent (Figs. 6 and 7). Under these conditions, in which full epithelial cell polarity had not yet been established, intense anti-Ank_{G119} staining was concentrated eccentrically near the nucleus, with less punctuate cytoplasmic staining apparent (Figs. 6, a and d, and 7 a). This pattern was strongly suggestive of Golgi staining and was confirmed as such by its coincidence with the staining pattern of β -COP (Fig. 6, e and g). β -COP is the best-characterized component of the cytoplasmic coatamer proteins that assemble on COP I vesicles involved in the transport of newly synthesized proteins between the ER, the Golgi, and the trans-Golgi network (Pepperkok et al., 1993; Griffiths et al., 1995). Recent data has also implicated β -COP in the function of some types of endosomes (Whitney et al., 1995). Ank_{G119} was also found to be colocalized in the Golgi with β I Σ * spectrin, as revealed by its coincident immunostaining with mAb VIIIIC7 (Figs. 6, b and h, and 7 b).

The 116-kD Ank_{G119} Is Not a Proteolytic Fragment of a Conventional Ankyrin

The size of the Ank_{G119}-immunoreactive peptide, as well as its cytoplasmic and Golgi distribution, are novel for a member of the ankyrin superfamily. Previous work has demonstrated that proteolysis of ankyrin from either brain or red cells yields fragments in the size range of 40–120 kD, and that many of these fragments lack the putative membrane-binding domain (Davis and Bennett, 1984; Weaver et al., 1984). Therefore, the reactivity of the anti-Ank_{G119} antibody with proteolytic fragments of conventional renal ankyrins was of concern. To exclude the possibility that the anti-Ank_{G119} was recognizing only a new epitope created by the proteolysis of a conventional ankyrin, extracts

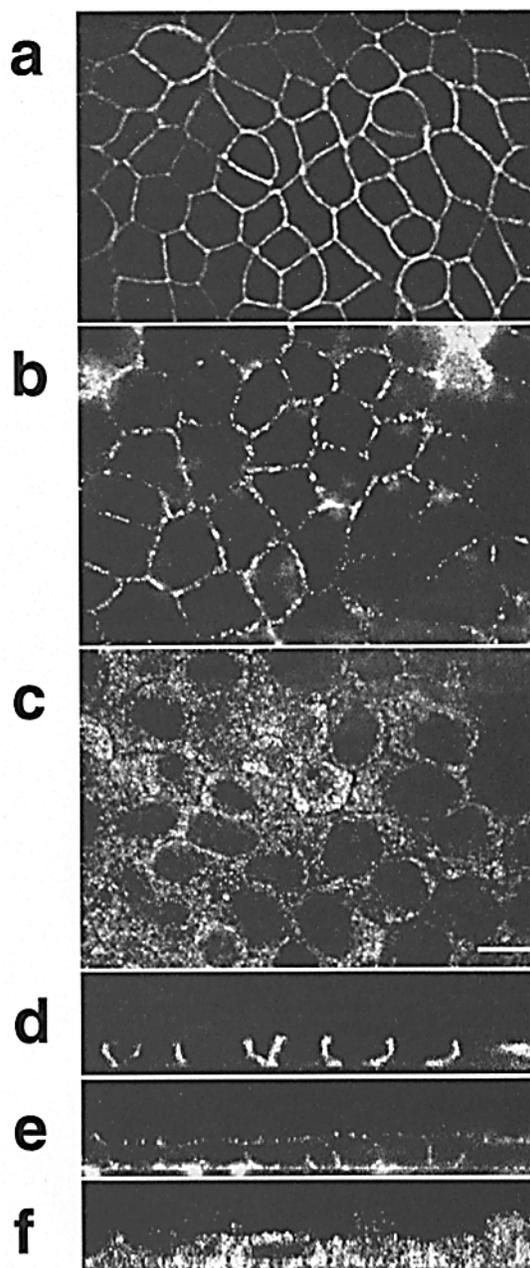


Figure 5. The distribution of Ank_{G119} is cytoplasmic and distinct from that of Ank_R by indirect immunofluorescent microscopy. Cultured MDCK cells were fixed and immunostained after several days at confluence to determine the distribution of (a and d) α -Na,K-ATPase, (b and e) Ank_R, and (c and f) Ank_{G119}. Note the punctate cytoplasmic distribution of Ank_{G119} compared to the plasma membrane compartmentalization of both Ank_R and Na,K-ATPase. In such confluent and stably polarized cells, the association of Ank_{G119} with Golgi membranes is largely inapparent (compare with Fig. 6). The z-axis micrographs in d–f were constructed from a series of z-axis line scans recorded at 1.2- μ m levels using the standard BioRad 600 confocal microscope software. Bar, 10 μ m.

of MDCK cells were subjected to proteolysis with either μ -calpain or trypsin, and the resulting breakdown products were examined by Western blotting with either the Ank_{G119} or Ank_R antibodies. Erythrocyte ghosts were also

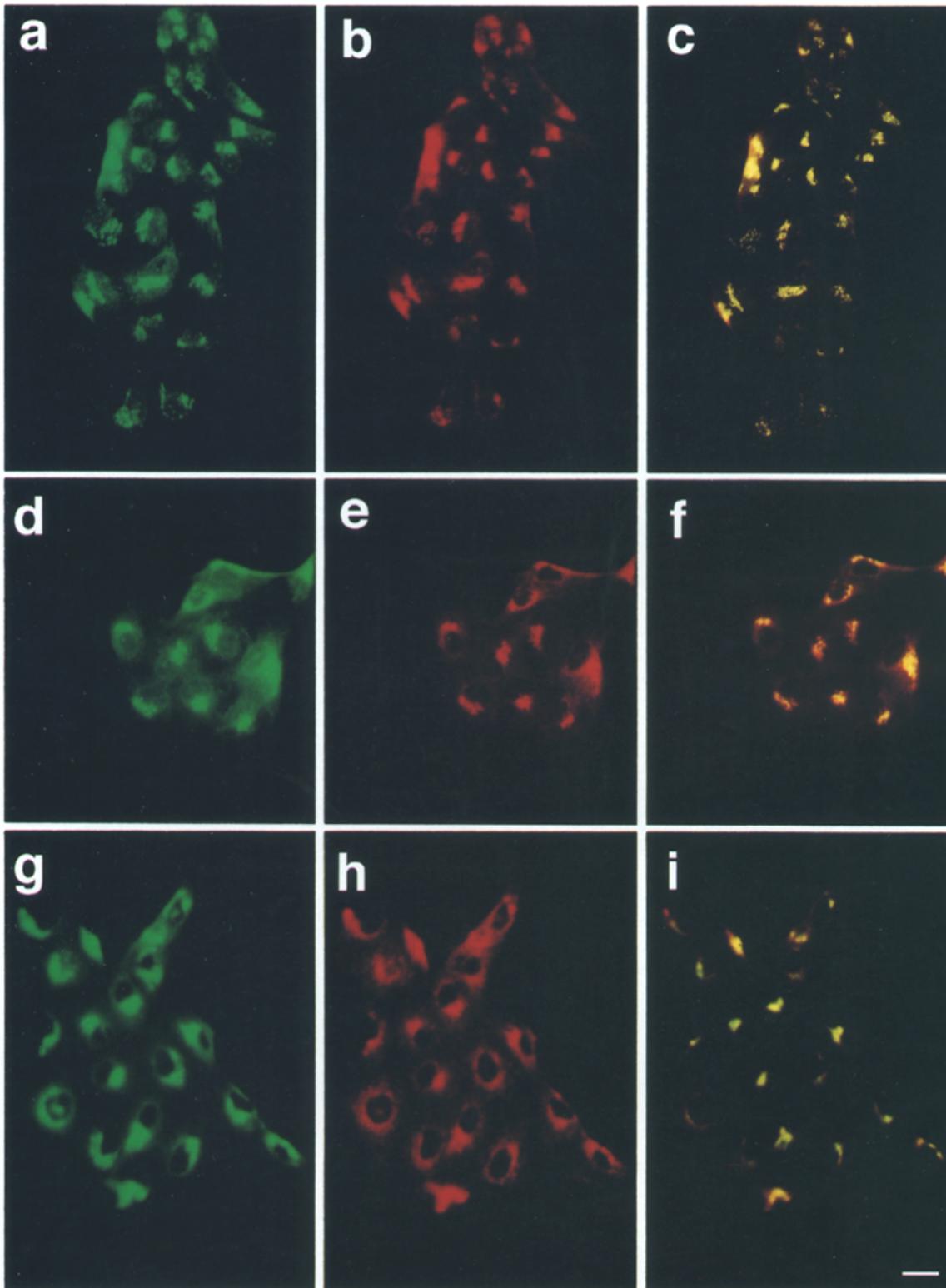


Figure 6. In subconfluent MDCK cells, Ankg₁₁₉ and βΣ* spectrin codistribute with β-COP, a Golgi marker. Cultured MDCK cells were sparsely plated, fixed, and immunostained before achieving confluence. The distribution of Ankg₁₁₉ (*a* and *d*), βΣ* spectrin (*b* and *h*), or β-COP (*e* and *g*) were determined by indirect immunofluorescence. Each row represents double-immunostained preparations. The rightmost column (*c*, *f*, and *i*) represents the appearance of the preparation in each row when viewed through a filter that passes the emission of both CY-2 (green) and CY-3 (red). Areas of absolute coincidence are revealed as yellow. Note the strong coincident staining over the Golgi complex. As seen in fully confluent MDCK cells, there is no staining at the plasma membrane. Bar, 50 μm.

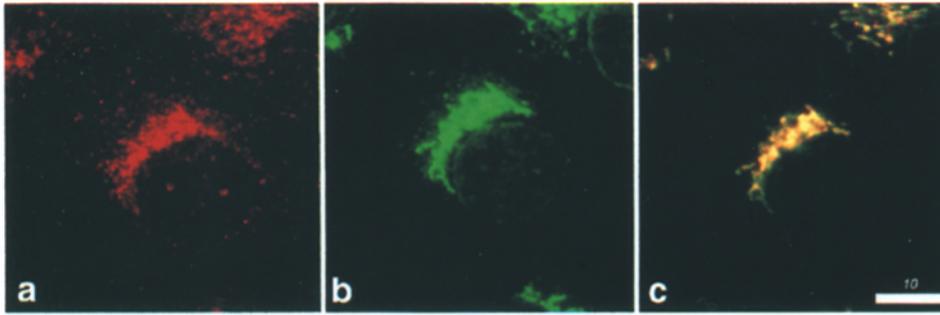


Figure 7. Ank_{G119} and β II* spectrin are closely codistributed in subconfluent MDCK cells. Sparsely plated MDCK cells were stained for either β II* spectrin (mAb VIII C7) (a) or Ank_{G119} (b), as described in Fig. 6. Their overlapping distributions are depicted by the yellow signal in c. Note that with few exceptions, the two proteins are highly coincident. Bar, 10 μ m.

examined in these assays. Both the conventional 210-kD renal ankyrin (recognized by anti-Ank_R in the cytoskeletal fraction, Fx2) and the 116-kD Ank_{G119} (in the soluble fraction, Fx1) were degraded by these proteases (Fig. 8). The Ank_{G119} antibody reacted with a breakdown product at \sim 20 kD in the soluble fraction after trypsin treatment. No breakdown products were detected after μ -calpain treatment. The Ank_R antibody recognized an array of breakdown products after μ -calpain and trypsin treatment in erythrocyte membranes and in the cytoskeletal fraction of MDCK cells, although the fragmentation patterns were not identical (Fig. 8). Importantly, no 116-kD peptide that would react with the Ank_{G119} antibody was generated at any point in the proteolysis of the cytoskeletal fraction of MDCK cells or of red cell ghosts. The pattern of fragments generated from the proteolysis of Ank_{G119} was also not a subset of the fragments generated by similar conditions of proteolysis of the Ank_R, based on their molecular weights. These distinct patterns of proteolytic breakdown and immunoreactivity between renal Ank_{G119} and Ank_R indicate that the 116-kD Ank_{G119}-immunoreactive protein observed in the cytoplasmic pool cannot be merely a proteolytic fragment of a conventional, membrane-associated renal ankyrin.

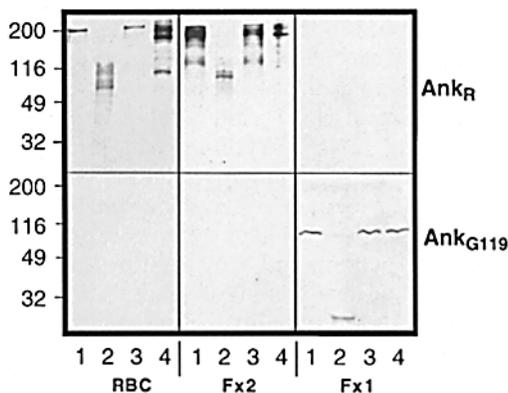


Figure 8. The protease digestion pattern of Ank_{G119} is distinct from Ank_R. Erythrocyte ghosts (left panel) or the MDCK cells extracts fx1 (right panel) or fx2 (center panel) were partially digested with either μ -calpain or trypsin. Lanes 1 and 3, starting material; lane 2, trypsin digest; lane 4, μ -calpain digest. Each digest was immunoblotted with either anti-Ank_{G119} or anti-Ank_R antibodies, as indicated. Note that a 116-kD peptide that is immunoreactive with Ank_{G119} antibodies is not generated by proteolysis from any extract, and that the protease digestion pattern of Ank_{G119} is distinct from that of Ank_R.

Ank_{G119} Binds to MDCK Cell β I- and β II-Spectrin *In Vitro*

Since Ank_{G119} was identified based on homologies in ankyrin's spectrin-binding domain, it was of interest to determine if the 116-kD Ank_{G119} retained bonafide spectrin-binding activity. The putative spectrin-binding domain of Ank_{G119} was expressed in bacteria as three overlapping GST fusion peptides (Fig. 9 a), purified over a bioaffinity column, and assayed for its ability to retain spectrin from fx1 (soluble) of MDCK cell lysates. Each of the recombinant peptides was soluble, stable, and of the predicted molecular weight by SDS-PAGE analysis (Fig. 9 b). Only peptide pGEX-HKA J specifically and reproducibly bound both β II-spectrin (Fig. 9 b) and β I-spectrin (Fig. 9 c). This peptide represents residues 669–860 of Ank_{G119} (Fig. 9, shaded area), a region highly conserved between all known ankyrin isoforms (100% homology with Ank_G, 80% with Ank_B, and 67% with Ank_R). Interestingly, the spectrin-binding domain previously identified in residues 1136–1160 of Ank_R (Platt et al., 1993) is highly homologous to Ank_{G119} residues 801–825 contained within the spectrin-binding recombinant peptide pGEX-HKA J.

Given the coincident localization of Ank_{G119} with β II* spectrin in MDCK cells (Fig. 8), the strength of the interaction between these proteins was of interest. The β II* spectrin in kidney cells remains incompletely characterized although it is immunologically most similar to the β II1 spectrin from erythrocytes (Beck et al., 1994), a point further confirmed by its reactivity with mAb VIII C7 reported here. Therefore, the ability of the Ank_{G119} spectrin-binding domain to associate with β II1 spectrin from red cells was measured (Fig. 10). These two proteins interacted strongly, with an apparent $K_d = 4.2 \pm 4.0$ (± 2 SD) nM.

Discussion

These studies identify a novel truncated spectrin-binding ankyrin, Ank_{G119}, in kidney and muscle tissue that is associated with the Golgi apparatus. This molecule has many unusual features. Specifically: (a) cDNA cloning of Ank_{G119} reveals strong sequence homology within the spectrin-binding domain of conventional ankyrins; (b) the putative regulatory domain of conventional ankyrins is markedly truncated in Ank_{G119}, and what sequence exists in this domain is unique and unrelated to any other known ankyrin; (c) a large part of the 33-residue ankyrin repeat structure characteristic of this family of proteins is absent; (d) Ank_{G119} expression is largely restricted to or at least most

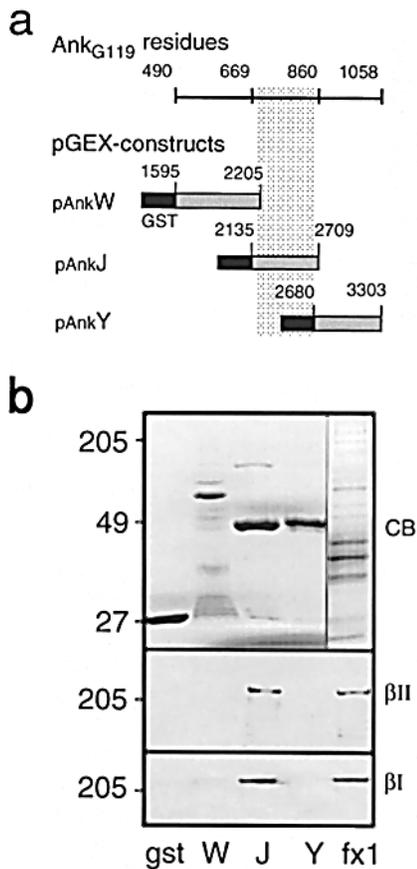


Figure 9. Ank_{G119} binds to MDCK cell spectrin *in vitro*. (a) The putative spectrin-binding domain of Ank_{G119} was expressed as three overlapping GST fusion peptides (pGEX2T-Ank W at 54 kD; pGEX2T-Ank J at 48.5 kD; pGEX1N-Ank Y at 50 kD). The basepair positions of each construct with respect to the full-length clone (Fig. 2) is depicted. The relationship of these constructs to the full-length Ank_{G119} amino acid sequence is depicted in the bar at the top of the figure with the residue numbers indicated. The shaded area represents the minimal spectrin-binding domain that is identified in these studies. (b) *Top*, Coomassie blue-stained SDS-PAGE gel showing, respectively, from left to right GST alone at 27.5 kD, the Ank W, Ank J, and Ank Y fusion peptides, and the soluble fraction (fx1) from MDCK cell lysates. *Bottom*, Western blot of the lysate retained by each fusion peptide and of Fx1 alone, using an antibody directed against β II spectrin. Note that only the AnkJ construct interacted with β II spectrin in MDCK cell fx 1. (c) Western blot of the same lysate as in b that was retained by each fusion peptide and of Fx1 alone, now using mAb VIIIC7 antibody directed against β I* spectrin. Again, note that the AnkJ peptide also interacts with this form of β spectrin from the MDCK cell fx1.

abundant in the kidney, placenta, and skeletal muscle; (e) Ank_{G119} binds spectrin with high affinity; (f) Ank_{G119} does not associate with the plasma membrane; (g) Ank_{G119} and β I* spectrin are colocalized with β -COP in incompletely polarized (subconfluent) MDCK cells; and (h) Ank_{G119} is a substrate *in vitro* for trypsin but resists μ -calpain digestion. Collectively, these studies substantively expand our understanding of the ankyrin gene superfamily, and they suggest that members of this family of proteins, together with other components of the spectrin-actin skeleton, may play

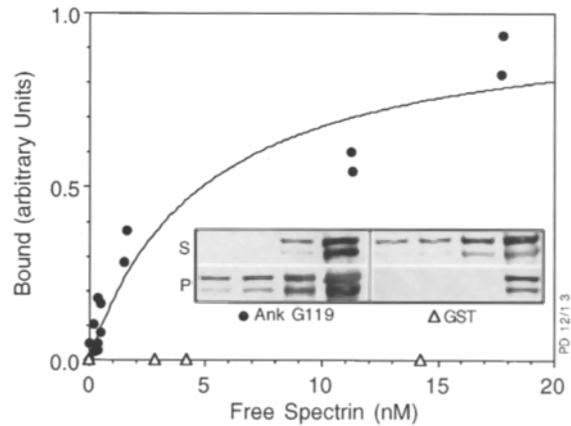


Figure 10. Ank_{G119} binds β I* spectrin avidly. The interaction of Ank_{G119} with biotinylated α β I spectrin was measured quantitatively by incubating increasing amounts of biotinylated erythrocyte spectrin with a fixed amount of the pGEX2T-AnkJ construct or GST alone, after which the AnkJ peptide with any bound spectrin was separated by absorption to glutathione-Sepharose, analyzed by SDS-PAGE, and the amount of bound spectrin was determined (in arbitrary units) by overlay with HRP-avidin. The extent of binding is expressed as a fraction of the maximal binding (B_{max}) estimated by nonlinear regression analysis of the binding isotherm. The fitted curve yielded an estimated $K_d = 4.8 \pm 4.0$ nM (± 2 SD). (*Inset*) Western blots of the supernatant (S) or pellet fractions (P) from one of the experiments demonstrating the specific binding of spectrin to the Ank_{G119} construct.

a role in intracellular vesicle trafficking or other processes that transcend plasma membrane “anchoring” functions.

The possible relationship of Ank_{G119} to the only other known “small ankyrin,” the 72-kD ankyrin-like molecule previously described in T lymphocytes (Bourguignon et al., 1986), is provocative. In that study, a molecule with immunologic and peptide map similarities to ankyrin was found to bind both spectrin and the membrane protein GP85. It is unlikely that this molecule was Ank_{G119}, since Ank_{G119} is larger in size, is not found in lymphocytes, and is not associated with the plasma membrane. However, other alternative transcripts of Ank_G appear to exist (Kordeli et al., 1995; Peters et al., 1995), and in several other respects, the lymphocyte 72-kD ankyrin appears similar to Ank_{G119}. While sequence or cloning data on the lymphocyte ankyrin is lacking, certain conclusions can be surmised. Both molecules are smaller than conventional ankyrins. Both bind spectrin, and thus must presumably conserve the central spectrin-binding domain of ankyrin. Both lack part or all of the repetitive 33-residue domain that is characteristic of the conventional ankyrins. This region is responsible for membrane binding, and its partial absence in Ank_{G119} may explain the solubility of Ank_{G119}. We postulate that the defining characteristic of these unconventional ankyrins will be their spectrin-binding capacity, and that linked to the spectrin-binding motif by exon splicing or other mechanisms will be additional protein cassettes bestowing alternative functionality on different members of this putative family. Indeed, the putative basic minimal spectrin-binding motif RRRKFH(K/R) first identified in Ank_R (Platt et al., 1993) is strongly conserved in all known ankyrin sequences irrespective of their tissue

distribution, including Ank_B (Otto et al., 1991), Ank_G (Kordeli et al., 1995), *Drosophila* ankyrin (Dubreuil and Yu, 1994), and Ank_{G119} (residues 802–808, Fig. 2). Also supportive of this notion is the marked diversity in alternative 5' and 3' transcripts being recognized that originate from the *ANK1* (erythroid) and *ANK3* genes despite complete conservation of the spectrin-binding domain (Gallagher et al., 1992; Birkenmeier et al., 1993; Kordeli et al., 1995; Peters et al., 1995).

The structural basis for the failure of Ank_{G119} to associate with the plasma membrane may be inferred from its deduced amino acid sequence. It has been shown that the plasma membrane-binding domain of conventional ankyrins consists of 22–24 tandemly arrayed repeats that fold into four independently folded subdomains of six repeats each (Michaely and Bennett, 1993). All four sets of six repeats are required for proper folding into a membrane-binding globular configuration, with removal of even a single repeat resulting in a 40% loss of α helicity. All four of the six-repeat subdomains have also been implicated in the binding of ankyrin to the anion exchanger (AE1) (Davis et al., 1991; Michaely and Bennett, 1993). The loss of the first 11 repeat units in Ank_{G119} is therefore anticipated to render Ank_{G119} unable to bind AE1. It remains undetermined whether Ank_{G119} retains binding capacity for other integral proteins, such as Na,K-ATPase, which may interact at different sites within ankyrin (Morrow et al., 1989; Davis and Bennett, 1990; Devarajan et al., 1994). However, Ank_{G119} almost certainly retains some membrane-binding properties, since it appears to be closely associated with various intracellular membrane compartments involved with membrane protein biogenesis and polarized assembly.

The association of Ank_{G119} (and β I Σ * spectrin) with the Golgi is unexpected and provocative, and suggests that these proteins play a role in the function of this structure. Work over the past two decades has established a comprehensive picture of many aspects of intracellular vesicle trafficking (reviewed in Kreis and Pepperkok, 1994; Matter and Mellman, 1994; Robinson, 1994). At least three classes of transport vesicles are derived from the ER, the Golgi, and the plasma membrane, respectively. Each of these is characterized not only by specific integral membrane proteins displaying either apical or basolateral targeting signals, but also by the assembly of peripheral protein coats that tend to be distinct for each vesicle type. These coats are believed to control the budding of nascent transport vesicles and the sorting of specific components to each vesicle. The best understood of these coats is the clathrin complex characteristic of plasma membrane-derived endosomes, as well as one class of vesicles budding from the *trans*-Golgi network (Robinson, 1994). The other two classes of vesicles, COP I and COP II, mediate transport between the ER and through the Golgi to the *trans*-Golgi network. COP I vesicles form by the recruitment of a coatamer that consists of at least seven distinct subunits, of which β -COP is one component. These coatamer components have also been recently identified on certain endosomes derived from the plasma membrane, suggesting that their function may be more general than first envisioned (Whitney et al., 1995). The assembly of COP I vesicles is disrupted by brefeldin A treatment. The results reported here, together with earlier findings that brefeldin A dis-

rupts the association of β I Σ * spectrin with the Golgi of MDCK cells (Beck et al., 1994), suggest that Ank_{G119} and β I Σ * spectrin must now be added to the list of coatameric proteins that are characteristic of this class of vesicles.

The functional significance of a β I Σ * spectrin and Ank_{G119} association with components of the ER and Golgi remains uncertain. At the plasma membrane, the spectrin-actin and ankyrin complex acts to control the lateral organization of integral membrane proteins and to organize membrane microdomains (reviewed in Bennett and Gilligan, 1993; Morrow, et al., 1996). This is probably its most fundamental role, rather than direct membrane support (Morrow et al., 1996). By analogy, one would expect that the Golgi- β I Σ * spectrin and Ank_{G119} complex acts to organize microdomains within these membrane compartments and perhaps link them to the filamentous actin cytoskeleton or to other filamentous components via spectrin's well-established binding capacities. Such a linkage, if required for polarized vesicular transport, would establish a molecular basis for the heretofore unexplained sensitivity of polarized vesicular transport to agents that disrupt actin filaments (Kelly, 1991). A problem with this hypothesis is the relative paucity of Ank_{G119} in other epithelia, such as enterocytes or hepatocytes (see Fig. 3). Perhaps other isoforms of ankyrin exist in these lines. Alternatively, and perhaps more likely, the abundance of Ank_{G119} in different cell types may vary greatly, perhaps reflecting the balance between a cell's use of the *trans*-Golgi network for sorting vs transcytotic pathways, with only those that sort predominantly in the *trans*-Golgi network using Ank_{G119}. Consonant with this hypothesis would be the predominance of this ankyrin in MDCK and kidney cells (in which *trans*-Golgi network sorting pathways predominate, Matter and Mellman, 1994) and the diminution of Ank_{G119} in Golgi under confluent [steady-state] conditions in which transcytotic pathways may assume greater importance (Mays et al., 1995). It will be of interest to explore these novel and testable hypotheses in future studies.

We are grateful to Ms. Carol Cianci for expert advice and for the provision of an antibody to Ank_R, and to Dr. Ira Mellman for suggesting the β -COP studies and for insights into the mechanisms of vesicle trafficking.

This work was supported by grants to P. Devarajan and J.S. Morrow from the National Institute of Diabetes and Kidney Disease and by a Donaghue Foundation New Investigator Award to P. Devarajan.

Received for publication 16 February 1995 and in revised form 26 February 1996.

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