Characterization of ANKRA, a Novel Ankyrin Repeat Protein that Interacts with the Cytoplasmic Domain of Megalin

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Abstract. Ankyrin-repeat family A protein (ANKRA) is a novel protein that interacts directly and specifically with the cytoplasmic tail of megalin in the yeast two-hybrid system and glutathione-S-transferase pull-down assays. ANKRA has three ankyrin repeats and shows 61% overall homology to regulatory factor X, ankyrin repeat-containing protein. Mapping studies show that the three ankyrin repeats and C-terminus of ANKRA are required for binding to a unique juxtamembrane, 19-amino acid sequence on the megalin tail. Point mutational analysis reveals that a proline-rich motif (PXXPXXP) within this region is the site of ANKRA binding. ANKRA interacts with megalin but not with low-density lipoprotein receptor related protein, in keeping with the fact that the sequence of the megalin tail is unique. By cell fractionation, ANKRA is found both in the cytosol and associated with membranes enriched in megalin in L2 cells and proximal tubule cells. By immunofluorescence, ANKRA is also expressed in connecting tubule cells and more broadly expressed than megalin, and by immunofluorescence ANKRA is also expressed in connecting tubule cells and principal cells of collecting ducts.

Megalin (approximately 600 kD), a member of the low-density lipoprotein receptor (LDLR) superfamily, is found in clathrin-coated vesicles in glomerular and proximal tubule epithelial cells of the kidney (1). Recent studies show that megalin is the main endocytic receptor in the proximal tubule and specifically binds and internalizes a number of proteins that are filtered by the glomerulus (2,3). These proteins include insulin (2); apolipoprotein H/I complexes; carriers for vitamins D₃, B₁₂, and A (retinol); and parathyroid hormone, among others (3,4). Megalin knockout mice exhibit proximal tubule reabsorption deficiency and a significant reduction in the number and size of organelles (clathrin-coated pits, endosomes, dense apical tubules, and lysosomes) associated with endocytosis in the proximal tubule (3,4). Thus, megalin expression is directly correlated with endocytic activity in the proximal tubule. In other systems, megalin has been shown to bind a number of other ligands from the lipoprotein and protease/protease inhibitor families, plasminogen, lactoferrin, thyroglobulin, Ca²⁺, and receptor-associated protein (RAP), a chaperone for members of the LDLR gene family (1,3).

Although considerable work has been done on ligand binding to the extracellular domain of megalin, the role of its cytoplasmic domain in regulating megalin’s endocytic or exocytic trafficking pathways (1,6) or putative Ca²⁺-sensing function (7) is completely unknown. Megalin has a unique cytoplasmic tail that shows little sequence similarity to other LDLR family members except for conserved NPXY motifs (8). The fact that the cytoplasmic tail of receptors regulates their trafficking and signaling suggests that megalin has trafficking itineraries or intracellular signaling pathways distinct from those of other LDLR family members. The presence of dileucine, YXXØ, PXXP, and (S/T)XV motifs as well as NPXY motifs, provides clues to the function of the megalin tail (7,8). Tyrosine-based motifs (NPXY, YXXØ) and dileucine motifs serve to direct receptors to clathrin-coated pits for rapid internalization or sorting within the trans-Golgi network by binding selectively to adaptor proteins AP-1, AP-2, or AP-3 (9–11). This is in keeping with the fact that megalin, like other members of the LDL family, is internalized via clathrin-coated pits (1,6,12). PXXP motifs bind to SH3 domains, (S/T)XV to PDZ domains, NPXY to PTB domains, and YXXØ to SH2 domains (13). These interactions have been shown to be important for subcellular localization (14,15), endocytic trafficking (16–19), and signaling (13–15,20) of some transmembrane proteins. However, nothing is known concerning the functions or molecular interactions of these motifs in the case of megalin.
To gain insight into the interactions of the megalin tail and thereby into megalin’s functions, we used the two-hybrid system to identify proteins that bind to the cytoplasmic domain of megalin and therefore might be involved in directing megalin traffic or signaling. In this article, we report the initial characterization and localization of a novel protein, ANKRA, which specifically binds to the cytoplasmic domain of megalin.

**Materials and Methods**

**cDNA and Antibodies**

Two incomplete expressed sequence tags (ESTs) of human ANKRA were obtained from the I.M.A.G.E. Consortium (Washington University-Merck EST project, L. Hillier, unpublished data) and sequenced by automated DNA sequencing. A complete human cDNA sequence (1507 bp long) including the poly A tail was assembled from overlapping human ESTs (GenBank accession numbers aa418089, aa442702, N6431, and AW079850). A plasmid containing the entire sequence of human low-density lipoprotein receptor related protein (LRP) was obtained from Joachim Herz (University of Texas Southwestern).

Rabbit megalin antiserum (459) was raised against a peptide from the cytoplasmic tail of megalin as described (21). Mouse monoclonal (mAb) anti-megalin IgG 20B was prepared as described previously (22). Polyclonal anti-β1 integrin (130) was a generous gift from Richard O. Hynes (MIT). The following antibodies were purchased: anti-HA mAb (BAbCO), goat anti-rabbit or goat anti-mouse IgG (Sigma, St. Louis, MO) or automated sequencing (DNA Sequencing Facility, Scripps Research Institute, San Diego, CA).

**Expression and Purification of Fusion Proteins**

Sequences of mouse ANKRA were subcloned by PCR into pET28a(+) vector (Novagen, Madison, WI). His-tagged fusion proteins were expressed in BL21(DE3) bacteria (Stratagene, La Jolla, CA). Because the fusion proteins were insoluble in 1% Tween-20, they were solubilized from inclusion bodies with 8 M urea (in 20 mM Tris [pH 7.8], 0.5 M NaCl, and 5 mM Imidazole), bound to Ni-NTA agarose beads (Pharmacia, Piscataway, NJ), and cross-absorbed goat anti-rabbit Alexa 488 or anti-mouse Alexa 594 (Molecular Probes, Eugene, OR).

Antisera were raised in rabbits against purified histidine (His)-tagged full-length ANKRA and were affinity purified by coupling purified His-tagged ANKRA (1-181) to AffiGel 15 beads (BioRad, Hercules, CA) according to the manufacturer’s instructions. Bound IgG was eluted with 0.1 M glycine (pH 2.5). By immunoblotting, 0.6 μg/ml affinity-purified anti-ANKRA (1-181) IgG detected 10 ng purified His-tagged ANKRA.

**GST-Precipitation Assays from Cell Lysates**

Mouse ANKRA was subcloned by PCR behind an HA-epitope in the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). For each assay, a 100-mm dish of HEK293 cells was transiently transfected with 15 μg HA-tagged ANKRA in pcDNA3 by calcium phosphate precipitation. Forty-eight h posttransfection, cells were solubilized in 10 mM CHAPS (3-[3(cholamidopropyl)-dimethylamno-nio-1-propanesulfonate] in buffer A [20 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM CaCl2] containing protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin A (Sigma)] and centrifuged (14,000 rpm). Lysates were then incubated for 2 h at 4°C with 15 μg of purified GST-tagged fusion proteins immobilized on glutathione beads. In some cases, the GST-MT fusion protein was preincubated with purified (His)6-tagged ANKRA fusion protein, or the lysates were preincubated with anti-ANKRA(FL) serum or preimmune serum for 1 h at 4°C. Beads were washed (4X with lysis buffer, incubated (2X 5 min) with 20 μl of elution buffer (20 mM glutathione + 100 mM Tris-HCl [pH 8.0]), and boiled in 4X Laemmli sample buffer. Eluted proteins were separated on 10%
with affinity-purified anti-ANKRA(1-181) IgG (approximately 1 ng/μl) and centrifuged at 100,000 g for 5 min. Bound antibodies were detected by chemiluminescence using Super Signal (Pierce).

Interactions in the Yeast Two-Hybrid System

Bait and prey constructs were co-transformed into yeast strain HF7c (Clontech), and one-to-one interaction was analyzed qualitatively by a colony lift assay for β-gal using 5-bromo-4-chloro-3-indoly1 (X-gal) (Clontech). For mapping interaction sites, deletion mutants of the MT and ANKRA were subcloned into the pGBT9 “bait” vector and pACT “prey” vectors (Clontech), respectively. Except for the mutants described below, clones were subcloned by PCR using MT- or ANKRA-specific primers (sequences available upon request). Complementary oligonucleotides containing point mutations were annealed to form mutants MT(1-19), MT(117/A), and MT(PPP/AAA). The deletion mutants MT(1-58) and ANKRA(138-254) were made by ligation of a sequence with stop codons in all three frames into blunt-ended NotI sites present in MT(1-213) and ANKRA(138-312). All constructs were co-transformed with pGBT9 or pACT2 to show that they did not activate transcription by themselves.

In Vitro Pull-Down Assays

In vitro transcription/translation of HA-tagged ANKRA in pCDNA3 was carried out using a TNT-T7-coupled reticulocyte lysate system (Promega, Madison, WI) in the presence of Amersham RediPrime-[35S]methionine (1000 Ci/mmol) according to the manufacturer’s instructions. Ten μg of GST-fusion proteins immobilized on glutathione beads were incubated with 5 μl of reaction mixture containing [35S]-labeled, in vitro translated HA-tagged ANKRA in 500 μl of 0.1% Tween-20/phosphate-buffered saline (pH 7.4) for 2 h at 23°C with rotation. Beads were washed (3X) in the same buffer, resuspended in 25 μl of Laemmli buffer, and boiled for 5 min. Proteins were separated on 12% SDS polyacrylamide gels and probed for β-gal using 5-bromo-4-chloro-3-indolyl β-galactosidase (B-Gal) (Clontech). Ten μg of GST-fusion proteins immobilized on glutathione beads were incubated with 5 μl of reaction mixture containing [35S]-labeled, in vitro translated HA-tagged ANKRA in 500 μl of 0.1% Tween-20/phosphate-buffered saline (pH 7.4) for 2 h at 23°C with rotation. Beads were washed (3X) in the same buffer, resuspended in 25 μl of Laemmli buffer, and boiled for 5 min. Proteins were separated on 12% SDS polyacrylamide gels and probed for β-gal using 5-bromo-4-chloro-3-indolyl β-galactosidase (B-Gal) (Clontech).

Preparation and Analysis of Cell Fractions

Membrane (100,000 × g pellet) and cytosolic (100,000 × g supernatant) fractions were prepared from postnuclear supernatants (PNS) of rat kidney cortex, L2 cells, or HEK293 cells transfected with HA-tagged full-length mANKRA as described previously (28,29). To prepare a PNS from kidneys, the cortical region was trimmed from frozen kidneys (obtained from Pelfreeze Biologicals, Rogers, AK), minced, homogenized in buffer A containing protease inhibitors using a Tissumizer (Tekmar Co.) for 3 min, and centrifuged twice at 600 × g for 10 min. To prepare a PNS from cultured cells, cells were homogenized in ice-cold Tris-buffered saline containing protease inhibitors and centrifuged at 600 × g for 10 min. The resultant PNS were centrifuged at 100,000 × g for 29,30). Proteins in the pellet and supernatant were separated by 12% or 6% SDS-PAGE and immunoblotted with affinity-purified anti-ANKRA(1-181) IgG (approximately 1 μg/ml), anti-HA IgG (1:1000), or anti-MT(459) serum (1:2000), followed by HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:3000). After enhanced chemiluminescence detection, protein bands were quantitated by densitometry using Alpha-imager software.

Membranes were subjected to alkaline extraction as described previously (29,30). Briefly, the 100,000 × g pellet prepared from rat kidney cortex was resuspended in 0.2 M Na2CO3 (pH 11.5), incubated on ice for 30 min, and centrifuged at 100,000 × g. The resulting pellet was lysed in 10 mM CHAPS in buffer A containing protease inhibitors, and proteins from the supernatant and pellet were separated by 10% SDS gel electrophoresis.

Brush border membranes, basolateral membranes, and heavy endosome- and light endosome-enriched fractions were isolated from the cortices of kidneys of Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as described (31). Brush border (32,33) and microvillar fractions (34,35) were prepared from the kidneys of Sprague-Dawley rats or frozen rat kidneys by standard procedures. Brush border fractions consist of the entire apical region of proximal tubule epithelia (33,36), whereas microvillar fractions consist of vesiculated microvilli, apical plasma membrane, and clathrin-coated pits (34,35).

Immunocytochemical Localization of Endogenous ANKRA

Kidneys of Sprague-Dawley rats were flushed with Cellgro DME medium followed by perfusion with 4% paraformaldehyde (PFA) in 100 mM phosphate buffer (pH 7.4) for 15 min and immersion in 8% PFA for 1 h. L2 cells were fixed in 4% PFA for 15 min followed by 8% PFA for 45 min in phosphate buffer. Samples were cryoprotected and frozen in liquid nitrogen (37). Semithin cryosections (0.5 to 1.0 μm) were cut on an Ultracut UCT microtome (Leica) equipped with an EM FCS cryoattachment at −100°C, placed on gelatin-coated microscope slides, and incubated for 2 h at room temperature with affinity-purified anti-ANKRA(1-181) IgG (0.06 μg/μl) and/or anti-megalin mAb 20B (1:100), followed by incubation for 1 h with goat anti-rabbit Alexa 488 and/or anti-mouse Alexa 594 IgG as described previously (6,29,30). Cells were observed with a Zeiss Axioshot microscope equipped for epifluorescence. Images were captured by a Cohu camera and processed as TIFF files using SCION Image (NIH) software. Images were colorized and superimposed using Adobe Photoshop 5.0 software to show overlap in the fluorescence pattern. Final images were printed on Kodak Ektachrome XLS paper.

Results

Identification of Mouse and Human ANKRA

To identify intracellular proteins that interact with megalin, we used the cytoplasmic tail of megalin to screen a mouse kidney cDNA library in the yeast two-hybrid system. We obtained an interacting clone that encoded the C-terminal half of a novel protein, which we named ANKRA for ankyrin-repeat protein, family A. To obtain the full-length mouse ANKRA sequence, we screened a mouse pituitary cDNA library and identified overlapping and full-length cDNAs. Figure 1A depicts the 312-aa sequence of ANKRA deduced from its nucleotide sequence, which predicts a 33-kD protein. ANKRA has three consecutive ankyrin repeats, two regions with low homology to ankyrin repeats, four putative casein kinase II phosphorylation sites, and two putative protein kinase C phosphorylation sites. The ankyrin repeat is a common 33-aa motif, originally identified in the cytoskeletal protein ankyrin, that is implicated in protein-protein interactions (38). Sequence analysis indicates that ANKRA lacks putative membrane-spanning domains or targeting motifs, suggesting that it is most likely a cytosolic or peripheral membrane protein. To obtain the amino acid sequence of human ANKRA, we assembled a full-length sequence from ESTs. Comparison of human and mouse ANKRA reveals 96% identity at the amino acid level (Figure 1B).
Homology Between ANKRA and Regulatory Factor X Ankyrin Repeat-Containing Protein

By searching protein sequence databases using BLAST, we found that ANKRA has significant homology to a protein known as regulatory factor X ankyrin repeat-containing protein (RFXANK) in all regions except the N-terminal domain (Figure 1, B and C). RFXANK (33 kD) functions in the assembly of the RFX-5 transcription complex that upregulates expression of MHC class II proteins (39,40). RFXANK is also a substrate of Raf-1, a serine/threonine kinase, and enhances Raf-1 activation by epidermal growth factor (41). Amino acids 126 to 308 of ANKRA are 66% identical to aa 67 to 260 in RFXANK (Figure 1, B and C).

The unique N-terminal domain of ANKRA (aa 1 to 122) has a predicted α-helical/loop secondary structure in contrast to the N-terminus (aa 1 to 64) of RFXANK, which contains a PEST domain (Figure 1C) (40). Interestingly, the N-terminus of ANKRA contains one putative casein kinase II phosphorylation site (thr 132) (Figure 1A), also found in RFXANK, which is known to be phosphorylated in vivo (40,41).

The next closest protein to ANKRA is integrin-linked kinase, where the three ankyrin repeats in ANKRA are 30% identical to ankyrin repeats in integrin-linked kinase. The lack of any significant (>50%) sequence homology between ANKRA and any protein other than RFXANK suggests that ANKRA and RFXANK are members of a novel gene family.

ANKRA mRNA Transcripts Are Widely Expressed and Alternatively Spliced

By Northern blotting, three transcripts were detected; a major mRNA transcript of approximately 2 kb was detected in all tissues tested: brain, heart, spleen (less abundant), lung, liver, skeletal muscle, kidney, and testis. In addition, a transcript of approximately 2.7 kb was detected in brain, spleen, lung, liver, and kidney, and a transcript of approximately 2.5 kb was detected in testis (Figure 2A). Because megalin is expressed in epithelial cells of the kidney, lung, testis, and brain (1,8), ANKRA and megalin mRNA are found together in these tissues. However, ANKRA has a broader tissue expression than megalin.

Detection of three mRNA transcripts by Northern blotting suggested that ANKRA is alternatively spliced. This was confirmed by library screening and searching EST databases, where we identified three alternatively spliced ANKRA cDNAs: ANKRA(FL), ANKRA Long 3'UTR, and ANKRA Spliced C-terminus (Figure 2B). ANKRA(FL) (1774 bp) corresponds in size to the major mRNA of approximately 2 kb (Figure 2A), and ANKRA Long 3'UTR (approximately 2374 bp) may correspond to the minor mRNA of approximately 2.5 or 2.7 kb (Figure 2A). ANKRA Spliced C-terminus is alternatively spliced at aa 295 and encodes a protein that is 8 aa shorter than ANKRA.

Figure 1. ANKRA is a novel protein. (A) The amino acid (aa) sequence of ANKRA contains three ankyrin repeats (ank 1 to 3) as well as two adjoining regions with low homology to ankyrin repeats (ank). It also contains four putative casein kinase II (**) and two putative protein kinase C (') phosphorylation sites. A vertical line indicates a C-terminal alternative splice site found in a library clone and in expressed sequence tags (EST; GenBank AC# AI060551 and 731339), and an arrow marks the beginning of the two-hybrid clone. (B) ANKRA has significant homology to RFXANK. Identical amino acids are highlighted in black, and homologous amino acids are highlighted in grey. mANKRA, mouse ANKRA; hANKRA, human ANKRA; mRFXANK, mouse RFXANK; hRFXANK, human RFXANK. (C) Schematic diagrams of ANKRA and RFXANK showing the conserved ankyrin repeats (I to III) (■) and ank regions (□) as well as the nonconserved N-termini. The unique N-terminal domain of ANKRA (■) is predicted to form several α-helices separated by loops and β-turns, whereas RFXANK contains a PEST domain (□). The percentage of amino acid identity between the conserved domains is indicated.
ANKRA Interacts Specifically with the MT in Cell Lysates

To verify the interaction between MT and ANKRA, we performed precipitation assays on cell lysates prepared from cells transfected with HA-tagged ANKRA with MT immobilized on glutathione beads (GST-MT). We found that ANKRA bound to GST-MT (Figure 3A, lane 2) but not to GST alone (Figure 3A, lane 1). When GST-MT was preincubated with equivalent or 10-fold amounts of recombinant His-tagged ANKRA before incubation with cell lysate, binding of HA-tagged ANKRA was reduced (Figure 3A, lanes 3 and 4). Preincubation with less than equivalent amounts of His-tagged ANKRA (Figure 3A, lanes 5 and 6) had little or no effect. Moreover, preincubating cell lysates with anti-ANKRA(FL) polyclonal antiserum abolished binding to GST-MT (Figure 3B, lane 3), whereas an equivalent amount of preimmune serum (Figure 3B, lane 4) did not. These results demonstrate the specificity of ANKRA binding to MT and confirm the two-hybrid results.

The Ankyrin-Repeat Domain and C-Terminus of ANKRA Are Required for Interaction with the MT

To map the megalin binding site in ANKRA, we tested the ability of MT to interact with ANKRA deletion mutants in the yeast two-hybrid system. When truncation was carried out from the N-terminus of ANKRA (Figure 4A), we observed strong interaction of MT with constructs containing the ankyrin repeats and C-terminus (aa 138 to 312) but not with the C-terminal domain alone (aa 254 to 312). When truncation was carried out from the C-terminus, we found that ANKRA (aa 138 to 254) containing ankyrin repeats 1 and 2 did not bind to MT. Moreover, when the extreme C-terminus (aa 290 to 312) was deleted, the N-terminal domain and ankyrin repeats (aa 1 to 290) were not capable of interacting with MT. Together, these results suggest that both the ankyrin repeats and the last 22 aa of ANKRA, which encompass the ank domain and the alternatively spliced C-terminus, are necessary for interaction with the MT.

The N-Terminal Domain of the MT (aa 1 to 19) Binds to ANKRA and Contains a Proline-Rich Motif PXXPXXP Necessary for the Interaction

Next we mapped the MT binding site for ANKRA in the two-hybrid assay. We carried out truncation from the C-terminus of MT (Figure 4B) and narrowed the binding site to aa 1 to 58. The region C-terminal of this binding site, aa 58 to 213, did not interact with ANKRA, demonstrating that NPXY motifs in this region of MT are not required for the interaction.

Next we generated a smaller mutant, MT(1 to 19), which contains a dileucine and a proline-rich motif, and found that it also bound to ANKRA. To determine whether the dileucine or proline-rich motifs are required for the interaction of MT with ANKRA, we mutated the dileucine motif to alanines MT(LL/AA) or the prolines P10 XXP 13 XXP 16 to alanines MT(PPP/AAA) and found that ANKRA bound to MT(LL/AA) but did not interact with MT(PPP/AAA). From these data, we conclude that the proline but not the dileucine motif is required for binding of MT to ANKRA.

To verify the interaction between ANKRA and MT (1 to 19), we deleted aa 1 to 20 of MT and tested its binding to ANKRA using in vitro pull-down assays. Figure 4C shows that an MT(20 to 213) (lane 3) or GST alone (lane 1). Thus, both in the yeast two-hybrid system and by in vitro pull-down assays, we found that each interaction with ANKRA.

ANKRA Binds to Megalin but not to LRP

We also used in vitro GST-pull-down assays to determine whether ANKRA binds to the cytoplasmic tail of LRP. LRP has little sequence homology to the MT except for NPXY motifs (8) and therefore would not be expected to bind to ANKRA. HA-tagged ANKRA protein (44 kD) did not bind to...
GST-LRP tail (Figure 4C, lane 4) or GST-RAP (binds to the ectodomain of megalin and LRP) used as a negative control (Figure 4C, lane 5). These results indicate that ANKRA binds specifically to megalin but not to LRP. We were unable to assess the binding between the LRP tail and ANKRA using the yeast two-hybrid system because, as previously reported (42), the LRP-tail bait is weakly self-activating.

ANKRA Is Found in Both Cytosolic and Membrane Fractions of Rat Kidney Cortex and L2 Cells

Next we investigated whether endogenous ANKRA is associated with membrane or cytosolic fractions in rat kidney cortex and L2 yolk sac cells. We used L2 cells because the distribution of megalin in these cells has been well characterized (1,25,26). First, we carried out immunoblotting on cytosolic (100,000 g supernatant) or membrane (100,000 g pellet) fractions using anti-ANKRA(1 to 181) IgG that had been affinity purified on the unique N-terminus of ANKRA to eliminate antibodies that might cross react with ankyrin-repeats of other proteins. In fractions of rat kidney cortex, we detected two bands, 46 and 44 kD, which presumably represent post-

Figure 3. ANKRA interacts specifically with the GST-MT in GST precipitation assays. (A) Lysates from HEK 293 cells overexpressing HA-tagged ANKRA were incubated with GST (lane 1) or GST-MT immobilized on glutathione-agarose beads (lanes 2 to 6) in the presence or absence of varying concentrations of purified His-tagged ANKRA for 2 h. Interacting proteins were separated by SDS-PAGE, and ANKRA was detected by Western blotting using anti-HA mAb. HA-tagged ANKRA binds to GST-MT (lane 2) but not to GST alone (lane 1). Addition of 80 µg (lane 3) or 8 µg (lane 4) of purified His-tagged ANKRA to GST-MT eliminates interaction, whereas addition of 0.8 or 0.08 µg (lanes 5 and 6) has minimal or no effect. Lane 7: 3% of total cell lysate. (B) Cell lysates prepared as in A were preincubated with ANKRA immune or preimmune antisera (1 h), followed by incubation with GST (lane 1) or GST-MT (lanes 2 to 4) for 2 h as in A. HA-tagged ANKRA binds to GST-MT (lane 2) but not to GST alone (lane 1). Addition of 2.5 µl of anti-ANKRA(FL) serum (lane 3) but not preimmune serum (lane 4) eliminates binding to GST-MT.

GST-LRP tail (Figure 4C, lane 4) or GST-RAP (binds to the ectodomain of megalin and LRP) used as a negative control (Figure 4C, lane 5). These results indicate that ANKRA binds specifically to megalin but not to LRP. We were unable to assess the binding between the LRP tail and ANKRA using the yeast two-hybrid system because, as previously reported (42), the LRP-tail bait is weakly self-activating.

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Figure 4. Mapping the interacting domains on ANKRA and the MT using the yeast two-hybrid system. (A) Ankyrin repeats (I to III) and the C-terminus (aa 290 to 312) of ANKRA are required for interaction with MT in the yeast two-hybrid system. MT interacts strongly with full-length ANKRA, ANKRA (138 to 312), the original two-hybrid clone, and ANKRA (177 to 312)—all of which contain ankyrin repeats (I to III) and the C-terminal domain. MT does not interact with the C-terminal domain alone (ANKRA 254 to 312), ankyrin repeats (I to II) (ANKRA 138 to 254), or the N-terminal domain plus ankyrin repeats (ANKRA 1 to 290). (B) The N-terminal domain of MT (aa 1 to 19) is required for interaction with ANKRA in the yeast two-hybrid system. ANKRA interacts strongly with MT(1 to 172), MT(1 to 58), and the short sequence MT(1 to 19). Deletion of aa 1 to 58, MT(58 to 213), abolishes interaction with ANKRA. Point mutational analysis of MT(1 to 19) shows that ANKRA interacts with MT(LLL/AAA) but not MT(PPP/AAA), demonstrating that the proline-rich motif (PXXPXXP) but not the dileucine motif (LL) is necessary for the interaction. (Note: aa 1 to 213 of the MT correspond to aa 4423 to 4635 of megalin.) The β-gal filter assay was performed on (Leu-, Trp-) plates and the color intensity was qualitatively scored at 4 to 7 h as the following: −, no color; +, weak color; ++, intermediate color; +++, strong color; ++++, strongest color.

(C) Deleting aa 1 to 20 abolishes the interaction with MT in in vitro pull-down assays. Radiolabeled, in vitro translated HA-tagged ANKRA binds to GST-MT (lane 2) but not to GST-MT(20 to 213) (lane 3) or GST alone (lane 1). ANKRA also does not bind GST-LRP tail (lane 4) or GST-RAP, a negative control (lane 5). Lane 6: 10% of the total in vitro-translated product. Equivalent amounts of GST fusion proteins were bound to glutathione beads and incubated with 35S-labeled, in vitro-translated HA-tagged ANKRA for 2 h. Bound proteins were separated by SDS-PAGE and detected by autoradiography.
translationally modified and/or alternatively spliced forms of ANKRA (Figure 5A). The 46-kD band was found in both cytosolic (40%) and membrane (60%) fractions, whereas the 44-kD band was found exclusively in the cytosol. In L2 cells, we detected a single, 46-kD band in both cytosolic (80%) and membrane (20%) fractions (Figure 5B). These results show that the number of bands and the percentage of ANKRA found in the cytosolic and membrane pools vary depending on the source.

To determine the nature of ANKRA’s association with membranes, we carried out alkaline extraction (pH 11.5) on membrane fractions from rat kidney. We found that ANKRA was released into the supernatant after alkaline treatment (Figure 5C), indicating that ANKRA is peripherally associated with membranes and is not an integral membrane protein.

**ANKRA Is Found in Apical Membrane Fractions of Proximal Tubules**

Megalin is found in glomerular epithelia and is distributed on the apical domain of proximal tubule epithelia (35,43). To determine whether ANKRA co-distributes with megalin in proximal tubules, we analyzed fractions enriched in brush border membranes and basolateral membranes (31). By immunoblotting, we detected ANKRA in fractions enriched in light and heavy endosomes (data not shown) and brush border membranes but not basolateral membranes (Figure 6A). We also prepared purified brush border (32) and microvillar fractions (34) from rat kidney cortex and detected ANKRA in both fractions by immunoblotting (Figure 6B). Collectively, these results suggest that ANKRA is enriched in apical membranes of the proximal tubule.

**Localization of Endogenous ANKRA in L2 Cells and Rat Kidney**

Next we carried out double labeling by immunofluorescence on semithin cryosections of L2 cells using affinity-purified anti-ANKRA(1 to 181) IgG (Figure 7A) and anti-megalin mAb 20B (Figure 7B). ANKRA was found in the cytoplasm concentrated predominantly along the plasma membrane (Figure 7B), where it partially overlapped with megalin (Figure 7C).

In rat kidney cortex, we detected ANKRA in glomeruli (Figure 8, A and C), proximal tubules (Figure 8, A and B), connecting tubules (Figure 8, A and D), and collecting ducts (data not shown). In some proximal tubule cells, ANKRA was concentrated near the brush border where megalin is located (Figure 8B); in others, ANKRA was distributed throughout the cytoplasm. ANKRA was also distributed throughout the cytoplasm in glomerular epithelial cells (Figure 8C), which express megalin, and in selected cells of connecting tubules (Figure 8D) and in principal cells of collecting ducts.

**Discussion**

To date, no cytoplasmic proteins that bind to megalin have been identified. Because the cytoplasmic tail of megalin has putative tyrosine and dileucine-based sorting signals (10,11) as well as putative motifs for binding SH3, SH2, and PDZ domains (13), it can be anticipated to bind multiple intracellular proteins. Using the cytoplasmic domain of megalin as bait in the yeast two-hybrid system, we screened a mouse kidney cDNA library and identified a novel 312 aa, mammalian protein, ANKRA, which has three ankyrin repeats and is highly conserved (approximately 96%) in mouse and human. ANKRA shows 61% overall homology to another ankyrin repeat–containing protein, RFXANK, indicating that ANKRA and RFXANK are members of a novel gene family. Ankyrin
repeats are a common motif, originally identified in ankyrin, that have subsequently been found in more than 400 proteins with a variety of functions (44). Ankyrin, a cytoskeletal protein with 24 tandem ankyrin repeats, functions to link spectrin-actin networks to the cytoplasmic domains of transmembrane proteins (44), such as Na⁺K-ATPase (45).

Using GST in vitro pull-down and GST-precipitation assays on cell lysates, we demonstrated specific interaction between ANKRA and megalin in vitro and in cell lysates. Moreover, we have shown by immunofluorescence that ANKRA is present in cells known to express megalin, including rat L2 yolk sac cells and rat kidney proximal tubule and glomerular epithelial cells, where it is concentrated near megalin along the plasma membrane. There are two pools of ANKRA—one cytoplasmic and the other membrane associated. The membrane-associated pool co-distributes with megalin in purified brush border and microvillar fractions and is peripherally associated with membranes as shown by alkaline extraction. These collective results indicate that ANKRA interacts with megalin and probably plays a role in megalin’s functions. In addition, ANKRA most likely has interactions and functions in kidney epithelia and other organs. This is suggested by our findings that ANKRA can undergo alternative splicing, is more broadly expressed in tissues and cells than megalin, and is abundantly expressed in connecting and collecting tubules of the kidney cortex.

Figure 6. ANKRA is enriched in brush border and microvillar (MV) fractions. (A) Fractions of brush border membranes (BBM) and basolateral membranes (BL) were prepared from fluorescein dextran-loaded kidneys by magnesium precipitation according to the method of Hammond et al. (31). ANKRA is enriched in BBM but not BL. Megalin is found both in the BBM fraction and in the BL fraction. β1-integrin, a basolateral membrane protein, is enriched in the BL fraction. (B) ANKRA and megalin are detected in purified BB and MV fractions. The 34-kD band in the MV fraction is believed to be a degradation product of ANKRA. Brush border (BB) fractions were prepared from kidney homogenate (H) according to Thuneberg and Rostgaard (32). MV fractions were prepared from kidney by calcium precipitation (34). Fractions were immunoblotted with approximately 1 μg/ml affinity-purified anti-ANKRA(1 to 181) IgG and anti-β1-integrin or anti-megalin 459 serum (1:2000).

Figure 7. Immunofluorescence localization of ANKRA and megalin in a semithin cryosection of L2 cells. (A) ANKRA is detected in the cytoplasm and concentrated at the cell periphery along the plasma membrane (PM). (B) Localization of megalin in the same section showing its characteristic distribution in a punctate pattern at the cell surface reflecting its coated pit localization. Note that L2 cells express variable levels of megalin (25). (C) When A and B are merged, the distribution of ANKRA and megalin partially overlap. Cells were fixed in paraformaldehyde, and semithin cryosections were incubated with affinity-purified anti-ANKRA(1 to 181) IgG and anti-megalin 20B mAb followed by incubation in cross-absorbed goat anti-rabbit Alexa 488 and anti-mouse Alexa 594 IgG. Bar, 10 μM.
Mapping studies reveal that the three ankyrin repeats and the C-terminus of ANKRA are required for optimal interaction with MT. As ANKRA is alternatively spliced, incorporation of different C-termini into ANKRA could contribute to the binding specificity and/or the structural stability of ANKRA because the binding specificity of ankyrin repeat regions has been shown to depend on adjacent nonankyrin repeat regions as well as the number of stacked ankyrin repeats and the surface contact residues (44). It is interesting that the ankyrin repeat region that is highly conserved in both ANKRA and RFXANK facilitates homodimerization of RFXANK and its binding to Raf-1 kinase (41). Dimerization of ANKRA could allow si-

Figure 8. Immunofluorescence localization of ANKRA in rat kidney cortex. Phase-contrast (left panel) and immunofluorescence (right panel) of semithin sections fixed with paraformaldehyde and incubated with affinity-purified anti-ANKRA(1 to 181) IgG. (A, A') ANKRA is seen in the glomerulus (G), proximal tubule (PT), and connecting tubule (CNT). (B, B') ANKRA is concentrated in the apical region of a PT cell between the nucleus (N) and brush border (arrows). (C, C') High-magnification view of a portion of a glomerulus showing ANKRA distributed throughout the cytoplasm of glomerular epithelial cells (GEC). (D, D') CNT showing expression of ANKRA in connecting tubule cells. L, lumen; MV, microvilli; N, nucleus. Bar: 50 μm in A, 10 μm in B through D.
recently discovered that the fourth ankyrin repeat of ankyrinR directs ubiquination and subsequent degradation in proteosomes (47). The N-terminal domain of ANKRA does not show direct ubiquination and subsequent degradation in proteosomes (47). The N-terminal domain of ANKRA does not show any homology to other mammalian proteins and most likely has a unique function.

What is the role of ANKRA in megalin’s function? Several possibilities come to mind. Ankyrin repeats commonly have a proline-rich region. Ankyrin repeat domains have the potential to bind multiple proteins simultaneously because they have three potential surface contacts (β-hairpins, ankyrin groove, and outer surface of the α-helical bundle) (44).

Mapping studies demonstrate that interaction between ANKRA and aa 1 to 19 of the MT requires a proline-rich motif (PXXP), which may provide surface contacts or contribute to the conformation of the binding domain on megalin. Three protein modules—SH3, WW, and EVH1 domains—are known to recognize proline-rich consensus sequences, but only SH3 domains have been shown to interact with the PXXP consensus sequence (13). In this article, we identified a novel protein-protein interaction between an ankyrin repeat domain and a proline-rich region.

ANKRA is highly homologous to RFXANK (alias Tvl-1 and RFX-B) (39,40) between aa 122 and 308 (see Figure 1C), which suggests that an early gene duplication gave rise to ANKRA and RFXANK. In addition, several genes, including ANKRA and RFXANK, are duplicated on chromosomal bands 5q12–13 (46) and 19p12 (39), respectively. Although there is extensive sequence homology between the ankyrin repeat regions and C-termini of ANKRA and RFXANK, they have unique N-termini. ANKRA has a predicted α-helical/loop domain, whereas RFXANK has a PEST domain, a sequence that directs ubiquination and subsequent degradation in proteosomes (47). The N-terminal domain of ANKRA does not show any homology to other mammalian proteins and most likely has a unique function.

What is the role of ANKRA in megalin’s function? Several possibilities come to mind. Ankyrin repeats commonly have a scaffold function and are required for the interaction with MT. This raises the possibility that ANKRA could facilitate endocytosis by linking megalin to components of the cytoskeleton or endocytic machinery. In yeast, ankyrin repeat-containing protein 1 (Akr1p) interacts with the cytoplasmic tails of pheromone receptors and is required for their constitutive and/or ligand-mediated endocytosis (48). It has been hypothesized that Akr1p together with other proteins (e.g., iqg1p) regulates actin organization during endocytosis (5). In addition, it was recently discovered that the fourth ankyrin repeat of ankyrinR interacts with high affinity with the heavy chain of clathrin and plays a role in internalization of LDL (49).

Although ANKRA does not contain an SH3 domain, it binds to a proline-rich motif on MT that is a consensus sequence for the binding of SH3 proteins. Another possibility is that ANKRA may affect megalin endocytosis by inhibiting binding of the MT to an SH3-containing protein. Recent studies have shown that removal of the SH3-domain binding sites from the third intracellular loop of the dopamine D4 receptor promotes receptor internalization (50). It has also been shown that the third intracellular loop of the β2-adrenergic receptor interacts with the SH3 domain of endophilin 1 and that overexpression of endophilin 1 enhances internalization of the receptor (16). The latter effect may be because the SH3 domain of endophilin binds to proteins of the endocytic machinery and functions in late steps of clathrin-coated vesicle formation (16,17,50).

Alternatively or simultaneously, ANKRA could act as a signaling molecule. ANKRA has significant homology to RFXANK, which contributes to the assembly of the RFX-5 transcription complex and is a substrate of Raf-1, a cytosolic serine/threonine kinase (39–41). It also potentiates Raf-1 activation induced by epidermal growth factor (41). ANKRA may interact with Raf-1 because the RFXANK binding domain for Raf-1 kinase (41) is highly conserved (77% homologous) in ANKRA.

Future studies will be aimed at identifying other interacting partners of ANKRA and determining ANKRA’s role in megalin’s function.

Note added in proof: Two articles (Biochem J 347: 613–621, 2000, and J Biol Chem 275: 25616–25624, 2000) appeared while this manuscript was in press, reporting additional cytosolic proteins that interact with the megalin tail.

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