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Ankyrin binds to two distinct cytoplasmic domains of Na,K-ATPase α subunit

(cytoskeleton/protein interaction/Madin–Darby canine kidney/spectrin/actin)

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ABSTRACT **Ankyrin has emerged as a ubiquitous protein linking integral membrane transport proteins such as Na,K-ATPase to an underlying spectrin cytoskeleton. This interaction is mediated by the α subunit of Na,K-ATPase; however, the nature of the ankyrin binding site in Na,K-ATPase is unknown. As a step to determine the mechanism of this interaction, the ankyrin binding region of human erythrocyte spectrin and each of five putative cytoplasmic domains of the Na,K-ATPase α subunit have been prepared as recombinant fusion proteins in bacteria and analyzed for their interaction with erythrocyte and kidney ankyrin (Ank1 and Ank3, respectively) *in vitro*. Spectrin binds both Ank1 and Ank3 avidly, as expected. Two of the Na,K-ATPase domains, immobilized on a bioaffinity column, also interact specifically with both of these ankyrins. These ATPase domains are encoded by codons 140–290 (domain II) and 345–784 (domain III), with domain II displaying the greatest apparent affinity. Sequences in domain II are highly conserved between species and isoforms of Na,K-ATPase and are homologous to a cytoplasmic domain in H,K-ATPase and to a limited region of sequence in Ca-ATPase. Conversely, domain II shares no significant homology with other ankyrin binding proteins such as band 3 and Na^+-channel proteins. These results identify a clear function for a conserved but previously not understood region of the α subunit of Na,K-ATPase and suggest that the interaction of ankyrin with membrane transport proteins may involve complex tertiary structural determinants not easily deduced from the primary sequence.**

Interactions between the cytoskeleton and the cytoplasmic domains of transmembrane proteins play fundamental roles in several cellular activities (1–3). These associations are probably critical for the maintenance of epithelial and neuronal cell polarity and are mediated by a large number of biochemical mechanisms (4, 5). General cellular organizational concepts have begun to emerge from the study of these diverse mechanisms, yet, beyond the codistribution of various proteins in different assays, there is little detailed understanding of the molecular basis for many of these interactions. One such interaction is the attachment of integral membrane transport proteins such as Na,K-ATPase to the spectrin-based cortical cytoskeleton. In most cells, the spectrin cytoskeleton is a submembranous spectrin–actin network joined to integral membrane proteins by at least four or five specific interactions: an ankyrin-mediated linkage; a protein 4.1-mediated linkage; adducin- and/or α-catenin-mediated linkages; and at least two direct "ankyrin-independent" linkages (D. L. Rimm, P. Kebriaei, C. R. Lombardo, and J.S.M., unpublished data; refs. 3 and 6). Of these, ankyrin appears to be the most important for tethering integral membrane transport proteins.

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The ankyrins are a family of proteins that link the spectrin cytoskeleton and integral membrane proteins. Multiple isoforms of ankyrin have arisen both by gene duplication as well as by alternative transcription (7–10). The polarized distribution of Na,K-ATPase in epithelial cells appears to be maintained via its direct anchorage to ankyrin, presumably ANK3, and both Na,K-ATPase and ankyrin are colocalized to the basolateral membranes of Madin–Darby canine kidney (MDCK) cells and renal tubular epithelial cells (11, 12). The detergent extractability of a spectrin–ankyrin–Na,K-ATPase complex in MDCK cells is also reduced by cell–cell contact (13) and by cell adhesion molecules (14), suggesting synchronized assembly of these proteins.

In the present study, we demonstrate the direct binding of recombinant peptides of the α1 subunit of Na,K-ATPase to both erythrocyte ankyrin (ANK1) and its immunologic counterpart in MDCK cells (ANK3). Two of the five putative cytoplasmic domains of Na,K-ATPase α subunit account for this activity. The active sequences are highly conserved within all known Na,K-ATPase α subunits, both across species and between isoforms, but they are distinct from sequences in other ankyrin binding proteins. We conclude that the interaction between ankyrin and integral membrane transport proteins may utilize multiple sequence motifs, but we postulate that different ankyrin binding sequences share similar secondary and possibly tertiary structures.§

MATERIALS AND METHODS

Construction of Expression Plasmids. Full-length cDNA encoding Na,K-ATPase α1 subunit (17) was used as template in a series of standard PCRs (18). Oligonucleotides were targeted as shown in Fig. 1, designed to bracket each of the five putative cytoplasmic domains. The numbers indicated below the oligonucleotides in Fig. 1 denote the codon positions according to published sequences (17, 20). Amplification products were subcloned into the TA vector (Invitrogen) and sequenced by the dideoxynucleotide chain-termination method (United States Biochemical) to verify their identity. *Eco*RI digestion released the cDNA encoding each cytoplasmic domain from the TA vector and enabled its in-frame insertion into the expression vector pGEX-1N. The pGEX vectors (Pharmacia) direct the bacterial synthesis of foreign proteins as a fusion peptide with glutathione *S*-transferase (GST). Other constructs used as controls included pGEX-3X/β28-XhoI, representing the 15th repetitive unit of human erythrocyte β-spectrin (19); the construct pGEX-2T/CAD, representing the cytoplasmic domain of human E-cadherin (GenBank data base, accession no. L08599); and the vector pGEX-1N alone.

Abbreviations: MDCK, Madin–Darby canine kidney; GST, glutathione *S*-transferase.

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§The nomenclature for spectrin used in this manuscript follows recent recommendations (15, 16).

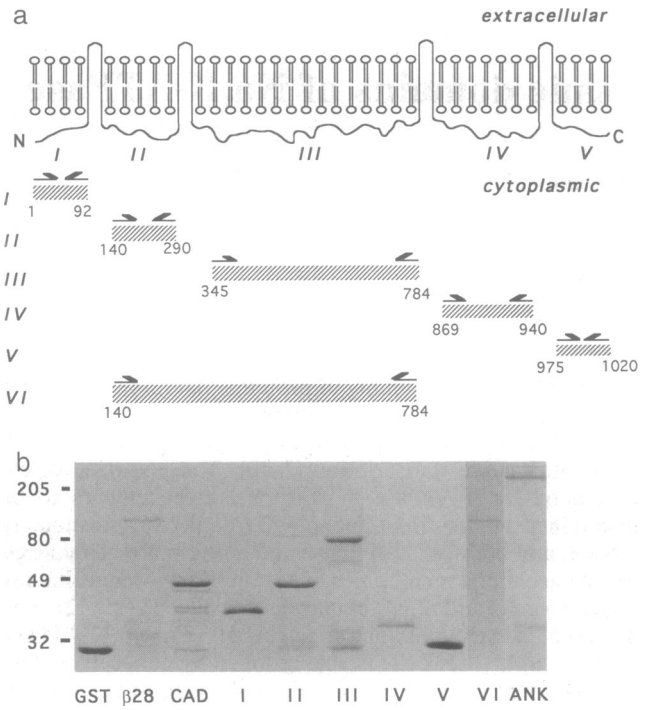

FIG. 1. Five putative cytoplasmic domains of Na,K-ATPase α subunit were prepared as recombinant peptides. (*a*) Schematic diagram of the recombinant peptides generated for this study. Codon positions defining each peptide are indicated. Peptide VI includes a putative transmembrane segment but remained soluble in aqueous buffer and active in these studies. (*b*) SDS/PAGE analysis of the various peptides and proteins used in this study (Coomassie blue stained). Positions of molecular size markers (kDa) are as indicated. β28, C terminus of βIΣ1 spectrin, encompassing its ankyrin binding domain (19); CAD, cytoplasmic domain of human E-cadherin; I–VI, recombinant Na,K-ATPase peptides representing the cytoplasmic domains; ANK, human erythrocyte ankyrin.

Expression and Purification of Recombinant Fusion Proteins. 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, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 8.0). All subsequent steps were performed at 0°C–4°C as described (19). Briefly, leupeptin (10 μg/ml), pepstatin A (1 μg/ml), aprotinin (10 μg/ml), 1 mM dithiothreitol (DTT), 1 mM benzamidine, and 1% (vol/vol) Triton X-100 were added, and the suspension was rotated gently. After sonication (70 W for 15 sec, repeated three times using a Branson sonic power apparatus), the lysate was centrifuged at 48,000 × *g* for 20 min and affinity purified on a 2-ml reduced glutathione-agarose column (19). Peptides were eluted with 50 mM Tris·HCl/5 mM reduced glutathione, pH 8.0, dialyzed, and stored in ankyrin binding buffer (50 mM Tris·HCl, pH 6.9/50 mM NaCl/1 mM DTT/1 mM EDTA/1 mM EGTA/1 mM PMSF/1 mM benzamidine/10 μg of leupeptin per ml/1 μg of pepstatin A per ml/10 μg of aprotinin per ml). Aliquots of fusion proteins were analyzed by SDS/PAGE. The yield of soluble fusion protein from each of the constructs was similar (≈2 mg per liter of bacteria). All peptides were used in binding assays within 2 weeks of preparation, during which time no proteolytic degradation was apparent by SDS/PAGE analysis.

Preparation of MDCK Cell Extracts. MDCK cells were grown to confluence in 150-cm^2 culture flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (11). After confluence, they were sequentially extracted *in situ* by using a modification of previously described methods (21) until only their nuclear matrix and intermediate filament network remained. After rinsing with ice-cold PBS (pH 7.5), five sequential extracts were performed with a gently rocking platform. The following buffers were used: buffer 1, 10 mM Pipes, pH 6.8/100 mM NaCl/300 mM sucrose/3 mM $MgCl_2$/0.5% Triton X-100/1.2 mM PMSF/0.5 mM Pefabloc SC [a serine protease inhibitor manufactured by Pentapharm (Basel) and sold in the United States by Centerchem (Stamford, CT)]; buffer 2, identical to buffer 1 except that 250 mM ammonium sulfate was substituted for 100 mM NaCl; buffer 3, same as buffer 1 except it contained 50 mM NaCl, 100 μg of DNase per ml, 100 μg of RNase A per ml, with the addition of 33 mg of ammonium sulfate per ml as the extraction progresses; buffer 4, 10 mM Tris·HCl, pH 7.4/9.5 M urea; buffer 5, 10 mM Tris·HCl, pH 7.4/1% 2-mercaptoethanol/9.5 M urea. The first extraction (Fx1) was for 10 min on ice with buffer 1. This yielded a "soluble" fraction. Next, the cells were extracted on ice for 10 min with buffer 2. This high-salt buffer solubilized the "cytoskeletal" fraction (Fx2) of proteins. The cells were then extracted at room temperature for 20 min with buffer 3. Ammonium sulfate crystals (33 mg/ml) were added, and the cells were rocked for an additional 5 min at room temperature. This step extracted the chromatin-associated proteins (Fx3) and left the salt-resistant nuclear matrix–intermediate filament network intact. This network was solubilized at room temperature for 15 min in buffer 4, to extract the "nuclear matrix and non-disulfide cross-linked keratins" (Fx4). Finally, the cells were rocked at room temperature for 15 min in buffer 5 to isolate the disulfide cross-linked keratins (Fx5). After all extractions, no residual protein was detectable in the pellet fraction. All extracts were dialyzed overnight into ankyrin binding buffer and all fractions remained soluble. Protein in the MDCK cell extracts was assayed by Peterson's modification of the Lowry technique (22, 23). Interfering buffer components were removed by precipitation of the protein prior to protein determinations with trichloroacetic acid in the presence of sodium desoxycholate (24).

Na,K-ATPase Binding to Ankyrin. For the studies with erythrocyte ankyrin, each fusion peptide (25 μg at 1 mg/ml) was conjugated to 50 μl of a 50% slurry of glutathione-agarose beads in ankyrin binding buffer for 1 h at 4°C with gentle rotation. Comparable (>95%) amounts of each fusion peptide bound to the beads under these conditions, as determined by SDS/PAGE analysis. Erythrocyte ankyrin (25 μg) was then added to the beads, and the total volume of the mixture was brought immediately to 500 μl with ankyrin binding buffer. This slurry was gently rotated at 4°C overnight and then at 20°C for 4 h. The beads were pelleted at ≈13,000 × *g* for 1 min and washed twice with ankyrin binding buffer at 4°C; aliquots were analyzed by SDS/PAGE. Bound ankyrin was detected by Western blotting. To assess the approximate affinity of ankyrin for the peptides, the above protocols were repeated using ankyrin concentrations ranging from 20 to 0.02 μM. The K_d in these studies was approximated by the point at which half-saturation of the peptide was achieved.

For the studies with the MDCK cell extracts, the same procedure was used, except that twice the amounts of fusion peptide and glutathione agarose beads were used, and these were incubated with 300 μg of total protein from fraction 2 (cytoskeletal fraction) from MDCK cells.

Other Procedures. Ankyrin was isolated from human erythrocyte ghosts (42) and was desalted and concentrated with Microsep microconcentrators (Filtron Technology, Northborough, MA). After adjustment to a concentration of 1 mg/ml, it was stored at 4°C in ankyrin binding buffer. Primary affinity-purified rabbit polyclonal antibodies to human erythrocyte ankyrin and αII spectrin (fodrin) have been characterized (11). Protein determinations except where otherwise noted used the method of Bradford with Coomassie brilliant blue G-250 reagent (Pierce) (25). SDS/PAGE analysis and Western blotting after transfer to nitrocellulose membranes

(Whatman) were as described (26, 27). Immunodetection of transferred protein bands utilized either colorimetry with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega) or enhanced chemiluminescence (Amersham) with comparable results.

RESULTS

Expression of Recombinant Cytoplasmic Domains of Na,K-ATPase α1 Subunit. Previous studies have identified three isoforms of the Na,K-ATPase α subunit (28). We chose the α1 isoform for this study because it is ubiquitously expressed and is the predominant form in epithelial cells (20). The hydropathy profile of the α1 subunit revealed five distinct cytoplasmic domains (17, 20, 28, 29). The predicted boundaries of each of these domains (labeled I–V) were used to identify oligonucleotide primers for the preparation of each domain, either individually or in tandem (construct VI) (Fig. 1*a*). By SDS/PAGE analysis the GST fusion proteins representing these domains were of the predicted molecular mass, and each was found to be stable to further breakdown and highly soluble in ankyrin binding buffer (Fig. 1*b*). Present in each preparation were variable amounts of smaller peptides. In no instance did these exceed 30% of the total protein as estimated by their Coomassie blue staining intensity, and further purification was not attempted since any inactive peptides if present would not interfere with the binding studies. Other peptides used in these studies are also shown in Fig. 1*b*. These include GST alone at 27 kDa, the ankyrin binding half of βI spectrin as a 95-kDa GST fusion protein (GST-β28-XhoI) (19), the cytoplasmic domain of human E-cadherin also as a GST fusion protein of 44 kDa (30), and purified human erythrocyte ankyrin at ≈210 kDa.

Na,K-ATPase Domains II and III Bind Erythrocyte Ankyrin. Ankyrin binding by each fusion peptide was examined by measuring the ability of each GST fusion peptide to retain erythrocyte ankyrin (ANK1) on a glutathione matrix. The bound ankyrin was measured by Western blotting and densitometry after SDS/PAGE analysis of the bound fractions (Fig. 2). A fusion peptide containing the 15th repetitive unit of human erythrocyte β-spectrin (GST-β28-XhoI) had been previously shown to bind with full affinity to ankyrin *in vitro* and served as a positive control (19). No binding was detected with GST alone, or with the cytoplasmic domain of E-cadherin (uvomorulin). Of the five cytoplasmic domains of Na,K-ATPase α1 subunit, only domain II (aa 140–290) and domain III (aa 345–784) consistently bound erythrocyte ankyrin. This binding was specific, since these peptides did not bind bovine serum albumin, nor did they bind to ankyrin heat denatured by incubation at 95°C for 10 min (data not shown).

Although these binding assays were not designed to be quantitative, it was apparent that for the same concentration of ankyrin (≈0.2 μM) domain II bound considerably more ankyrin than domain III. We also tested whether a fusion protein containing both cytoplasmic domains II and III in tandem would exhibit an even greater ankyrin binding ability than domain II alone. The tandem fusion peptide (labeled VI in Fig. 1) was stable and soluble despite the presence of a probable transmembrane loop. However, it did not bind greater quantities of ankyrin when compared to domain II alone (Fig. 2). Finally, concentrations of ankyrin ranging from 2 to 0.002 μM were examined for their ability to bind to 10 μg of domain II. Half-saturation of binding was achieved between 0.2 and 0.02 μM ankyrin (data not shown). This result suggests that both domain II and the combined domains II and III tandem peptide possess an ≈100 nM affinity for ankyrin. This binding affinity is exactly comparable to that previously observed for erythrocyte ankyrin (ANK1) binding to purified intact rat kidney Na,K-ATPase (11).

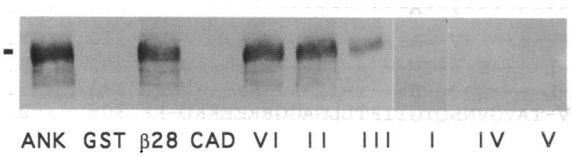

FIG. 2. Erythrocyte ankyrin binds to domains II and III of Na,K-ATPase. The ankyrin bound to immobilized recombinant peptides was analyzed by SDS/PAGE and Western blotting with anti-ankyrin antibodies. Autofluorograms after chemiluminescent detection are shown. Lane designations are as in Fig. 1. Note that only spectrin β28 and peptides II, III, and VI bound ankyrin.

Most Ankyrin Is in the Cytoskeletal Fraction in Confluent MDCK Cells. An operational definition of cytoskeletal-associated proteins is their extractability in nonionic detergents such as Triton X-100. Confluent MDCK cells were subjected to a series of extraction conditions, which resulted in five fractions that have been operationally defined as follows: Fx1, soluble; Fx2, cytoskeletal; Fx3, chromatin associated; Fx4, nuclear matrix and non-cross-linked keratins; Fx5, cross-linked keratins (21). The total relative amount of protein in each fraction was constant in four separate experiments (Fig. 3). The soluble fraction contained 54% ± 3.6% of the total protein, while the cytoskeletal and nuclear fractions contained 18% ± 2.8% and 19% ± 3.2% of the total protein, respectively.

Each fraction was analyzed for the presence of ankyrin by Western blotting (Fig. 3). The antibody recognized a dominant band at ≈215 kDa and a fainter band at ≈190 kDa. These species of ankyrin are similar to those detected by others in renal tissues (13). The majority of immunodetectable ankyrin was found in the cytoskeletal fraction. Data from four separate Western blots, representing four different extractions, are summarized in Fig. 3. The majority (63% ± 11%) of the total immunodetectable ankyrin was present in Fx2 (cytoskeletal pool), while the soluble fraction contained 29% ± 9%. Western blot analysis with an antibody to αII spectrin

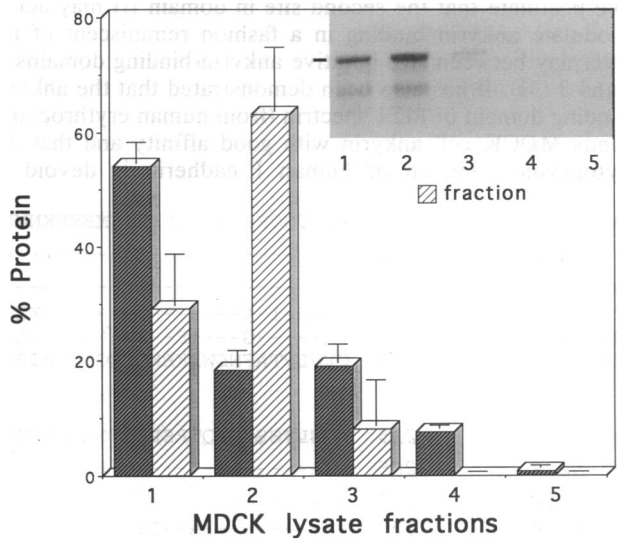

FIG. 3. Most ankyrin in confluent MDCK cells is found in the cytoskeletal fraction. MDCK cells were sequentially extracted as described in the text, and the distribution of total protein (■) and immunodetectable ankyrin (▨) was determined. Error bars represent ±2 SD of four determinations. (*Inset*) Western blots of the immunoreactive MDCK cell ankyrin in each fraction. The most intense band was at ≈215 kDa (marked). Less intense ankyrin bands are present at ≈190 and ≈150 kDa. Presumably, both of these bands represent alternatively spliced forms of Ank3.

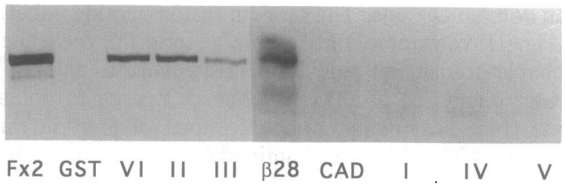

FIG. 4. MDCK cell ankyrin also binds domains II and III of Na,K-ATPase. An experiment identical to the one described in Fig. 2 was carried out with the cytoskeletal fraction of the MDCK cell lysate. As with erythrocyte ankyrin, strong binding was again detected to β28 spectrin and to peptides II, III, and VI derived from Na,K-ATPase. Experiments from two separate SDS/polyacrylamide gels are shown. Other nomenclature is as in Fig. 1.

(fodrin) also revealed a majority of that protein in Fx2 (data not shown).

Domains II and III of Na,K-ATPase and GST-β28 Bind MDCK Cell Ankyrin. Each of the cytoplasmic domains of Na,K-ATPase α1 subunit was incubated as described above with Fx2 of the MDCK cell extracts to determine which domains would interact with renal ankyrin (Fig. 4). These experiments also demonstrated the ability of human erythrocyte βIΣ1 spectrin, as represented by the GST-β28-XhoI fusion peptide, to bind avidly to MDCK cell ankyrin. Of the five putative cytoplasmic domains of Na,K-ATPase α1 subunit, domains II and III were again found to be the only sequences with avidity for renal ankyrin. The relative strength of MDCK cell ankyrin binding to these domains was also exactly parallel to that for erythrocyte ankyrin, as was the binding activity of peptide VI compared to peptide II.

DISCUSSION

The results presented here identify two distinct cytoplasmic domains of Na,K-ATPase that mediate its binding to ankyrin and establish that at least two distinct types of ankyrin can utilize these receptor sites in Na,K-ATPase. Of the two sites, the one in cytoplasmic domain II, residues 140–290, accounts for most of the overall avidity of Na,K-ATPase for ankyrin. We postulate that the second site in domain III may act to modulate ankyrin binding in a fashion reminiscent of the interplay between two putative ankyrin binding domains in band 3 (31). It has also been demonstrated that the ankyrin binding domain of βIΣ1 spectrin (from human erythrocytes) binds MDCK cell ankyrin with good affinity and that the cytoplasmic domain of human E-cadherin is devoid of ankyrin binding capacity, at least in the assays used here. These results mirror previous observations that at least two distinct domains of erythrocyte ankyrin are involved in its association with Na,K-ATPase (32) and that Na,K-ATPase binds to ankyrin through its α subunit (11).

Three additional findings come from these studies. First, the same cytoplasmic domains of Na,K-ATPase that bind erythrocyte ankyrin (domains II and III) also interact with ankyrin from MDCK cell lysates. Thus, the sequence divergence between human erythrocyte and dog kidney ankyrin (P.D. and J.S.M., unpublished observations) does not alter their specificity for the same site on Na,K-ATPase. Second, a recombinant peptide encoding the ankyrin binding domain of erythrocyte β-spectrin consistently bound to ankyrin from MDCK cells, lending support to the hypothesis that erythroid and nonerythroid β-spectrin share structural and functional homologies in their ankyrin binding domains (19). Finally, it has been postulated, based on their cosedimentation (2, 14), that ankyrin may interact with the cytoplasmic domain of E-cadherin. In our assays, no direct binding between the recombinant cytoplasmic domain of E-cadherin and erythrocyte or MDCK ankyrin could be demonstrated. We suggest that associations between ankyrin and E-cadherin are indirect and are mediated by intervening molecules such as spectrin, actin, and the catenins (D. L. Rimm and J.S.M., unpublished observations).

We have searched nucleotide and protein sequence banks (GenBank, EMBL, Swiss-Prot) for proteins that may share homologies with the Na,K-ATPase domain II (Fig. 5). No significantly homologous sequences were present in any other known ankyrin binding proteins, including the erythrocyte anion exchanger (32), the amiloride-sensitive sodium channel in renal epithelial cells (35), the voltage-sensitive sodium channel in brain (36), the cardiac Na^+/Ca^{2+} exchanger (37), and ankyrin binding glycoproteins in brain (38). Conversely, domain II of Na,K-ATPase is very highly conserved both across species and between isoforms (28). Significant homology was also noted between Na,K-ATPase domain II and a cytoplasmic domain of two other ATPases, specifically H,K-ATPase (75% homology) (33) and Ca-ATPase (52% homology over a limited region; residues 195–247) (34). Although the latter two proteins have not been directly tested for ankyrin binding, it is intriguing to note that, like Na,K-ATPase, both of these are critically polarized integral membrane enzymes involved in the ATP-dependent transport of ions across cell membranes and that H,K-ATPase has recently been found associated with ankyrin and

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α1-Na,K      140  VIITGCFSYYQEAKSSKIMESFKNMVPQQALVIRNGEKMSINAEDVVVGDLVEVKGGDRIPADLRIISANGCKVD
α2-Na,K      130  ----------T----------E-D--QV---E--------I-----V--E------HR----
α3-Na,K      130  ----------E-----V---E--------I-----V---------H-----
Col-H,K      152  --L--I-A-------TN--A--SKI---------DA--KV-S--QL----V--I----Q----I-LVFSQ-----
Gas-H,K      152  -------G----F--TN--A----L-----T---D-K-FQ---DQL-------M-----V---I-L---Q-----
Ca-ATPase    165  CVVLVTAFNDWSKEKQFRGLQSRIEQE-KFT---G-QVIQ-PVA-IT---IAQ--Y--LL---GIL-QG-DL-I-

α1-Na,K      215  NSSLTGESEPQTRSPDFTNENPLETRNIAFFSTNCVEGTARGIVVYTGDRTVMGRIATLASGLEGG 280
α2-Na,K      205  ----------C-HD--------T----------V--A---------- 270  (86.8%)
α3-Na,K      205  ----------C-HD--------T----------V--A----------V- 270  (89.4%)
Col-H,K      227  ----------A--TE--H---------G-Y--T-L----T---IN-----II----S----VGSE 292  (69.5%)
Gas-H,K      227  ----------EC-H-S----------M-L----Q----S-----II----S----V-NE 292  (75.0%)
Ca-ATPase    240  E-------DHVKK-L-KDPLLLSG-HVMEGSGRMV-TAVGVNSQTGIIFTLLGAGGEEEEKKD-KK 305  (52.0%)
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FIG. 5. Sequence alignment of the ankyrin binding domain II with other transport proteins. Dashes represent residues identical to the Na,K-ATPase α1-subunit sequence. Numbers in parentheses represent the degree of sequence identity over the depicted region, except for the Ca-ATPase. For this protein, the region of comparison is at residues 195–247. Note the strong conservation of this sequence among various Na,K-ATPases as well as its presence in other putative ankyrin binding transporters. Separate alignments with other ankyrin binding proteins such as band 3 failed to demonstrate homology. α1-Na,K, Na,K-ATPase α1 subunit (17); α2-Na,K, Na,K-ATPase α2 subunit (28); α3-Na,K, Na,K-ATPase α3 subunit (28); Col-H,K, rat colonic H,K-ATPase (33); Gas-H,K, rat gastric H,K-ATPase (33); Ca-ATPase, bovine Ca-ATPase (34).

spectrin in gastric parietal cells (39). These three proteins also share other regions of sequence homology associated with the binding and hydrolysis of ATP (28).

Collectively, these findings suggest that ankyrin may interact in a pleiotropic way with different transmembrane proteins and/or that the ankyrin binding motif involves complex tertiary structural determinants not easily deduced from the primary sequence. Both possibilities probably are true. In a similar way, the interaction of spectrin with ankyrin involves a relatively large and conformationally sensitive region of spectrin (19), and studies on erythrocyte band 3 have identified at least two distinct regions in the cytoplasmic domain of this protein that interact with ankyrin (31). Conversely, studies of the interaction of ankyrin with band 3 and Na,K-ATPase (32, 40, 41) delineate large and distinct regions in ankyrin necessary for binding activity, even though the cytoplasmic domain of band 3, which shares no sequence homology with the ankyrin binding domain of Na,K-ATPase, will inhibit the binding of ankyrin to Na,K-ATPase (11). In future studies, it will be important to delineate the minimal sequences necessary for binding and to evaluate the functional consequences of a loss of ankyrin binding function on the sorting and stability of Na,K-ATPase in epithelial cells.

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