Ankyrin Links Fodrin to the Alpha Subunit of Na,K-ATPase in Madin–Darby Canine Kidney Cells and in Intact Renal Tubule Cells

Jon S. Morrow, Carol D. Cianci, Thomas Ardito, Andrea S. Mann, and Michael Kashgarian

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. In nonerythroid cells the distribution of the cortical membrane skeleton composed of fodrin (spectrin), actin, and other proteins varies both temporally with cell development and spatially within the cell and on the membrane. In monolayers of Madin–Darby canine kidney (MDCK) cells, it has previously been shown that fodrin and Na,K-ATPase are codistributed asymmetrically at the basolateral margins of the cell, and that the distribution of fodrin appears to be regulated posttranslationally when confluence is achieved (Nelson, W. J., and P. I. Veshnock. 1987. *J. Cell Biol.* 104:1527–1537). The molecular mechanisms underlying these changes are poorly understood. We find that (*a*) in confluent MDCK cells and intact kidney proximal tubule cells, Na,K-ATPase, fodrin, and analogues of human erythrocyte ankyrin are precisely colocalized in the basolateral domain at the ultrastructural level. (*b*) This colocalization is only achieved in MDCK cells after confluence is attained. (*c*) Erythrocyte ankyrin binds saturably to Na,K-ATPase in a molar ratio of ~1 ankyrin to 4 Na,K-ATPase's, with a kD of 2.6 μM. (*d*) The binding of ankyrin to Na,K-ATPase is inhibited by the 43-kD cytoplasmic domain of erythrocyte band 3. (*e*) ^{125}I-labeled ankyrin binds to the alpha subunit of Na,K-ATPase in vitro. There also appears to be a second minor membrane protein of ~240 kD that is associated with both erythrocyte and kidney membranes that binds ^{125}I-labeled ankyrin avidly. The precise identity of this component is unknown. These results identify a molecular mechanism in the renal epithelial cell that may account for the polarized distribution of the fodrin-based cortical cytoskeleton.

THE compartmentalization of the plasma membrane into specialized structural and functional domains is a universal cellular phenomenon (e.g., see Gumbiner and Louvard, 1985). The epithelial cells of the renal tubule are segregated by the tight junction into basolateral and apical domains. Associated with each of these regions are unique integral membrane and cytoskeletal proteins, as exemplified by the basolateral distribution of Na,K-ATPase (Louvard, 1980; Kashgarian et al., 1985) or the apically directed brush border and terminal web of the proximal tubule cell. The mechanisms responsible for establishing and maintaining this high degree of order are poorly understood, although it seems likely that an interaction between the cytoplasmic domain of certain integral membrane proteins and the cytoskeleton may be involved (e.g., see reviews by Rodriquez-Boulan, 1983; Simons and Fuller, 1985; Rindler et al., 1985; also study by Puddington et al., 1987).

Significantly, the actin-containing cortical cytoskeleton of epithelial cells is also highly polarized. In addition, it has recently been demonstrated at the level of the light microscope that fodrin, an actin-binding protein analogous to erythrocyte spectrin (e.g., see Marchesi, 1985 and references therein), is codistributed in the basolateral domain of confluent MDCK cells with Na,K-ATPase (Nelson and Veshnock, 1986). Significantly, the distribution of spectrin between cytoplasmic and cytoskeletal pools is regulated posttranslationally by cell contact (Nelson and Veshnock, 1987*a*), an observation reminiscent of the temporal relationship between targeted membrane protein insertion and tight junction assembly (Balcarova-Stander et al., 1984). In the erythrocyte, ankyrin and protein 4.1 mediate the attachment of the spectrin-actin cortical cytoskeleton to the integral membrane proteins band 3 and glycophorin (for reviews, Bennett, 1985; Morrow and Anderson, 1986). Analogues of ankyrin, protein 4.1, and band 3 also enjoy a wide tissue distribution (e.g., see Bennett, 1985; Spiegel et al., 1984; Cohen et al., 1982; Granger and Lazarides, 1985; Drenckhahn et al., 1984). Much less is known about the ability of ankyrin to mediate the attachment of the spectrin-actin skeleton to other integral membrane proteins unrelated to band 3.

In this report we demonstrate that immunoreactive analogues of ankyrin and spectrin (fodrin) are codistributed at the basolateral domain with Na,K-ATPase in the cells of the rat renal proximal tubule and in confluent but not sparse cultures of Madin–Darby canine kidney (MDCK) cells. Evidence is presented that ankyrin binds specifically to the alpha subunit of Na,K-ATPase by a mechanism that must involve the same region of ankyrin that binds band 3 in the erythrocyte, and that there is a second, much less abundant protein in kidney membranes that may also bind ankyrin. These studies therefore suggest that renal analogues of ankyrin may mediate the organization of the cortical cytoskeleton via a di-

rect interaction with the alpha subunit of Na,K-ATPase, and at least one other membrane protein.

A portion of these results have been previously reported in abstract form. While this manuscript was in revision, independent studies reporting the interaction of ankyrin with Na,K-ATPase appeared (Nelson and Veshnock, 1987*a*; Koob et al., 1987). The results reported here confirm and extend the qualitative conclusions of these studies, but differ in several quantitative aspects, as discussed.

Materials and Methods

Purification of Proteins

Ankyrin was prepared from fresh human erythrocyte membranes by extraction of spectrin depleted erythrocyte vesicles with 1 M KCl, followed by ion-exchange chromatography using DE-52 cellulose (Tyler et al., 1980). The purified protein was stored at 4°C in buffer containing 3% sucrose and used within 1 wk of its preparation for all experiments.

The cytoplasmic domain of band 3 was solubilized by alpha-chymotryptic digestion of the ankyrin depleted erythrocyte membrane vesicles that had been stripped of any residual peripheral proteins by washing with 1.0% acetic acid (Bennett, 1983). This procedure yields predominately a 43-kD fragment, with minor components at 45 and 41 kD which have been previously shown to be closely related to the major cleavage fragment (Bennett, 1983).

Membrane vesicles enriched in Na,K-ATPase were prepared from fresh frozen canine kidneys (Pel-Freez Biologicals, Rogers, AK) by homogenizing the outer medullas in 0.25 M sucrose, 0.03 M histidine, pH 7.2, followed by isolation of the membrane fraction by sedimentation using standard methods (Jorgensen, 1982). Freshly prepared membranes were stored at 0°C and used within 72 h of their purification or frozen at −20°C for subsequent use. Na,K-ATPase activity was measured according to Forbush (1983). The SDS activatable purified membranes used for these experiments had a specific activity of at least 24 units (μM Pi released/mg protein per minute).

Preparation of Antibodies

Antibodies to human brain spectrin (fodrin) and to human erythrocyte ankyrin were prepared in New Zealand white rabbits by multisite intradermal injection of the purified proteins in complete Freund's adjuvant, followed by three intradermal boosting injections in incomplete Freund's adjuvant at biweekly intervals. The preparation and characterization of the anti-fodrin antisera has been previously described (Harris et al., 1985). This antisera reacts predominately with the alpha subunit of human brain fodrin, although it does contain some reactivity with the beta subunit (Fox et al., 1987). Ankyrin was purified for immunization by preparative SDS-PAGE (Knowles and Bologna, 1983). The preparation of the ankyrin antisera was otherwise similar to that of the fodrin antisera. Before use, the antisera were affinity purified using cyanogen bromide-activated Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) to which had been coupled the purified proteins (Harris et al., 1985).

The antibody to the Na,K-ATPase was monoclonal antibody C62.4, which has been previously characterized (Kashgarian et al., 1985).

Immunoelectron Microscopy

Rat kidneys were obtained immediately after retrograde perfusion with periodate-lysine-paraformaldehyde fixative and then postfixed in the same solution (McLean and Nakane, 1974). MDCK cells were stabilized by immersion in the same fixative. The samples were then cryoprotected with 10% DMSO, frozen, and the tissue samples sectioned at 16 μM. The specimens were incubated with the primary antibody for 2 h at room temperature after blocking for 15 min with 1% BSA in PBS, followed by a similar incubation with the secondary antibody (anti-rabbit or anti-mouse) conjugated to horseradish peroxidase (BioSys, Compiegne, France). At each step, the unbound antibodies were washed from the sample with PBS containing 1% BSA. The samples were then postfixed with 1% glutaraldehyde in 0.1 M sodium cacodylate, 5% sucrose, pH 7.4, for 1 h at room temperature. The presence of the bound antibody was detected by reaction with 0.1% diaminobenzidine in 50 mM Tris-HCl, pH 7.4, 7.5% sucrose, at room temperature for 5–10 min. After generation of the reaction product, the samples were rapidly washed and further fixed with reduced osmium tetroxide, dehydrated with graded ethanols, embedded in Epon 812, and thin sectioned (Kashgarian et al., 1985).

Samples were viewed without further counterstaining using a Zeiss 10B electron microscope at 60 KV.

Binding Assays

The binding of ^{125}I-labeled ankyrin to the dog kidney membranes was measured by cosedimentation of the bound ankyrin with the membranes through a 10% sucrose barrier in binding buffer (20 mM KCl, 50 mM Tris, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% BSA, 1 mM PMSF, pH 7.6) at 12,000 *g*. These assays were done exactly as previously described for the binding of spectrin to erythrocyte membrane vesicles (Howe et al., 1985). Briefly, 20–40 μg of dog kidney membranes were incubated with various amounts of ankyrin in a total volume of 180 μl, and then layered atop a 150 μl cushion of 10% sucrose in binding buffer less the BSA at 4°C. The samples were sedimented for 1 h at 12,000 *g*, quick frozen in liquid nitrogen, and the pellet harvested by cutting off the tip of the plastic centrifuge tube with a heated razor blade. Both supernatant and pellet fractions were gamma counted, and the binding isotherms calculated using conventional procedures. Estimates of kD, B_{max}, and their standard errors were determined by nonlinear regression analysis (Bevington, 1969).

Binding inhibition studies were performed by incorporating increasing amounts of the 43-kD cytoplasmic domain of band 3 into the incubation medium in the above assay. Generally, the 43-kD fragment was added to the ankyrin solution 30 min before the addition of the kidney membranes.

The ability of solubilized and ^{125}I-labeled Na,K-ATPase to bind to ankyrin was investigated by comparing its ability to bind ankyrin vs. BSA when both were immobilized on CNBr–Sepharose. Sepharose CL-4B (Pharmacia Fine Chemicals) was activated with CNBr (15 ml Sepharose, 700 mg CNBr) for 10 min at room temperature at pH 11, after which it was exhaustively washed with 0.2 M sodium bicarbonate, pH 8.6, at 0°C. Identical aliquots (300 μl) of the activated gel were added to either 300 μg of freshly prepared ankyrin or 300 μg of BSA in the same buffer, and allowed to react for 24 h at 4°C. The reaction was terminated by 1% ethanolamine, pH 8.6, and the unbound protein removed by exhaustive washing into the same buffer used for these binding experiments (20 mM imidazole, 100 mM NaCl, 10 mM KCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.0). Nearly complete coupling was achieved for both proteins. In control experiments, the CNBr–Sepharose was blocked with ethanolamine alone, without the addition of any protein. Kidney membranes were labeled with ^{125}I using the Bolton-Hunter reagent as described for ankyrin, except that 700 μg of Na,K-ATPase–enriched kidney membranes, identical to the preparations used for the direct ankyrin binding studies, were incubated with 500 μCi of reagent in 25 mM imidazole, 1 mM EDTA, 8.5% sucrose, pH 7.5, for 4 h at 0°C. Unreacted Bolton-Hunter reagent was removed by washing the membranes using a 1-h 30,000 *g* sedimentation at 4°C. Na,K-ATPase was solubilized from these membranes using octaethylene glycol dodecyl monoether (C12E8; Calbiochem-Behring Corp., La Jolla, CA) at a ratio of 4:1 ([wt/wt] detergent/protein) in the imidazole binding buffer, followed by sedimentation at 200,000 *g* for 1 h at 4°C (Esmann, 1988). For the actual binding measurement, aliquots of solubilized Na,K-ATPase were incubated for 3 h with 25 μl of the Sepharose coupled proteins all in the presence of 0.1 mg/ml BSA. Bound Na,K-ATPase was recovered by centrifugation at 30,000 *g* for 1 min, followed by three washes with imidazole binding buffer. The Sepharose pellet was then stripped of noncovalently bound protein with SDS solubilizing buffer and analyzed by SDS-PAGE and autoradiography.

The direct binding of ^{125}I labeled ankyrin to the alpha subunit of Na,K-ATPase was detected after the transfer of the Na,K-ATPase (or control proteins) to BA85 (0.45 μM) nitrocellulose sheets (Schleicher & Schuell, Keene, NH), according to the methods established for Western blotting (Towbin et al., 1979). Samples of human erythrocytes, kidney membranes, or the purified proteins were subjected to electrophoresis in 5–15% SDS polyacrylamide gels (Laemmli, 1970), after which they were transferred electrophoretically to nitrocellulose sheets in a 25-mM Tris-glycine 20% methanol buffer. The transfers were stained briefly with a 0.2% solution of Ponceau S in 3% TCA to establish the completeness of the protein transfer, after which they were rinsed for 30 min in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% sodium azide, pH 7.4) and blocked by a 48-h incubation at 37°C with a 3% solution of BSA in wash buffer. After blocking, the transfers were incubated with a 50-nM solution of ^{125}I-labeled ankyrin (1.44 E7 cpm/nmol) in isotonic KCl buffer containing 1.0% BSA and 1 mM EDTA, pH 7.2–7.6 for 2 h at 0°C, followed by exhaustive washing with PBS, 0.1% BSA, 0.1% Tween 20. Isotonic KCl buffer was also used in lieu of

PBS in some experiments, without effect. The binding of ankyrin was detected by autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY) with fluorescent intensifying screens at −60°C. Experiments were considered valid if the erythrocyte ghost sample demonstrated binding only to beta spectrin and band 3, and if the binding was inhibited by partially solubilized erythrocyte band 3 (see below). Problems with nonspecificity were only encountered in this assay if the radiolabeled ankyrin was >7 d old. These problems were presumed to arise from radiolytic damage to the ankyrin, although the precise cause of nonspecificity when it was encountered was not investigated.

Inhibition of ankyrin binding in the Western blot assays was performed by incubating the ^{125}I-labeled ankyrin solution with increasing amounts of human erythrocyte band 3 from stripped human erythrocyte membranes, rendered partially soluble by treatment with 0.1% Tween 20 at room temperature in isotonic KCl buffer containing 0.1% BSA, 0.1 mM EDTA, pH 7.2. for 30 min at room temperature. The preparation of stripped membranes has been described (Howe et al., 1985). The ratio or detergent to band 3 in these experiments was ~250:1 (wt/wt), assuming that in the typical preparation ~80% of the protein is band 3 by Coomassie Blue. The ankyrin–band 3 suspension was then added to the nitrocellulose transfers, and incubated for an additional 12 h in the same buffer at 4°C. Care was taken throughout all subsequent steps to avoid vigorous agitation or treatments that might lead to shearing of an ankyrin–band 3 complex from the transfer. Subsequent processing and development were as described above.

Cell Culture

Madin–Darby canine kidney cells (ATCC-MDCK-CCL34) were obtained from the American Type Culture Collection (Rockville, MD), subcloned, and maintained in modified MEM with 10% FCS and 100 IU/ml of penicillin and 100 μg/ml streptomycin. Trypsinized cells were either cultured in suspension or seeded onto Millicel-NA membranes (Millipore Corp., Bedford, MA) coated with purified native laminin derived from the murine EHS tumor. (The purified laminin was a kind gift of Dr. J. A. Madri, Yale University.)

Other Procedures

All chemicals were of reagent grade, and in general were purchased either from Sigma Chemical Co. (St. Louis, MO) or from Bio-Rad Laboratories (Richmond, CA). Protein determinations were carried out by the method of Lowry (1951). Ankyrin was iodinated by reaction of 500 μg of protein (0.5–2.0 mg/ml) with 1 mCi of ^{125}I-labeled Bolton-Hunter reagent (ICN Radiochemicals, Irvine, CA) in 100 mM sodium phosphate, 1 mM EDTA, 0.05 mM PMSF, 3% sucrose, pH 8.5, for 2–5 h at 0°C. Unbound reagent was removed from the labeled ankyrin by gel-filtration on a 5 × 150-mm column of Sephadex G-15 (Pharmacia Fine Chemicals), equilibrated with binding buffer containing 3% sucrose, 1 mM 2-mercaptoethanol, 0.5 mM PMSF, pH 7.2, at 0°C. Two-dimensional gel analyses was done according to the method of O'Farrell (1975). Proteins were detected by either Coomassie Blue staining, or by silver staining (Wray et al., 1981). Quantitative densitometry of both stained electrophoretic gels and autoradiograms was performed using a Visage 2,000 analytical imaging instrument (BioImage Inc., Ann Arbor, MI).

Results

Fodrin, Ankyrin, and Na,K-ATPase Are Codistributed in Renal Tubule Epithelial Cells

The distribution of fodrin, ankyrin, and Na,K-ATPase was examined in thin sections of rat kidney proximal tubules by immunoelectron microscopy. Separate experiments established the reactivity of our antisera with the expected proteins in rat kidney tissues (Kashgarian et al., 1985; data not shown). The results are shown in Fig. 1. As previously noted (Louvard, 1980), Na,K-ATPase is confined to the basolateral domain of the cell and, in particular, to the basolateral infoldings (Fig. 1 *a*). It is excluded from those portions of the cell that appear to be involved in substratum attachment (Kashgarian et al., 1985). A similar asymmetric distribution

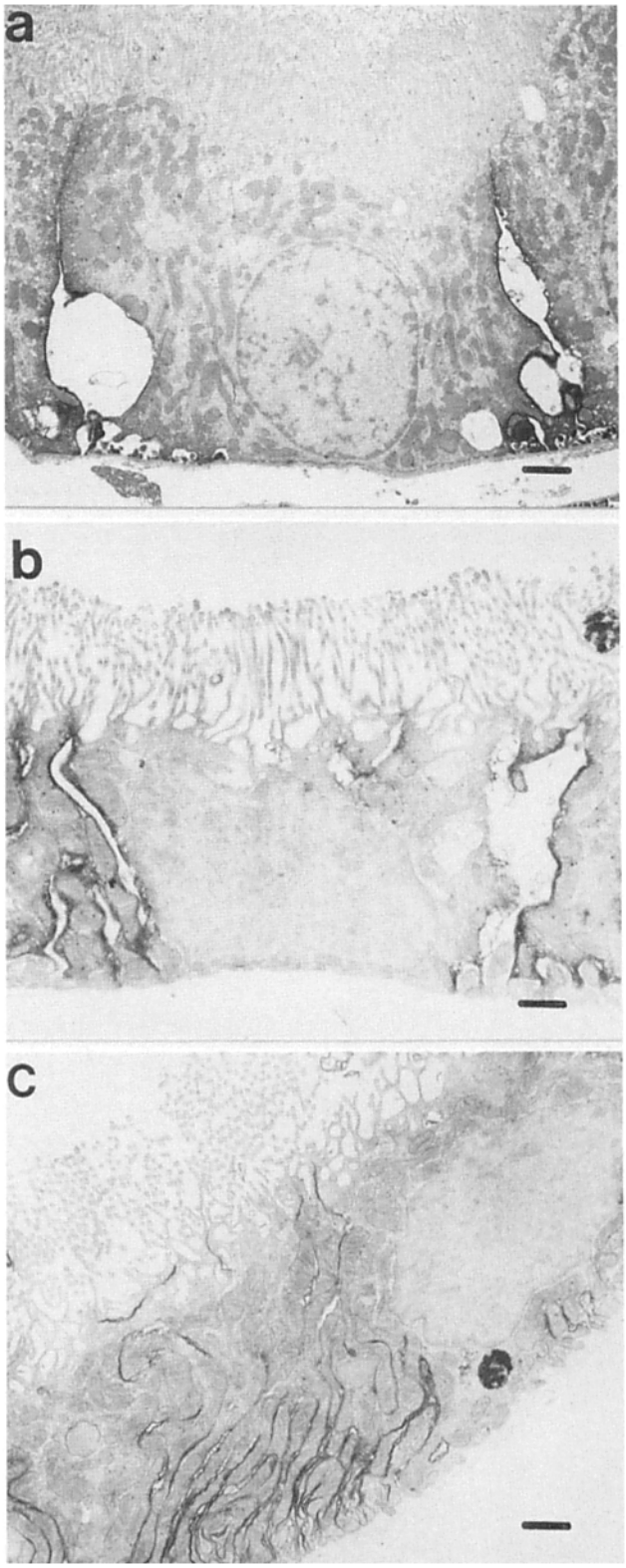

Figure 1. Na,K-ATPase, fodrin, and ankyrin are colocalized and polarized in rat kidney proximal tubule cells. Rat renal proximal tubules labeled with antibodies to the alpha subunit of Na,K-ATPase (*a*), fodrin (*b*), or ankyrin (*c*) are shown. Note that these proteins are colocalized at the lateral and basal infolded regions of the cell membrane and are excluded (except for patchy fodrin staining) from the apical brush border and regions where the plasmalemma and the basal lamina are opposed. Bar, 1.0 μM.

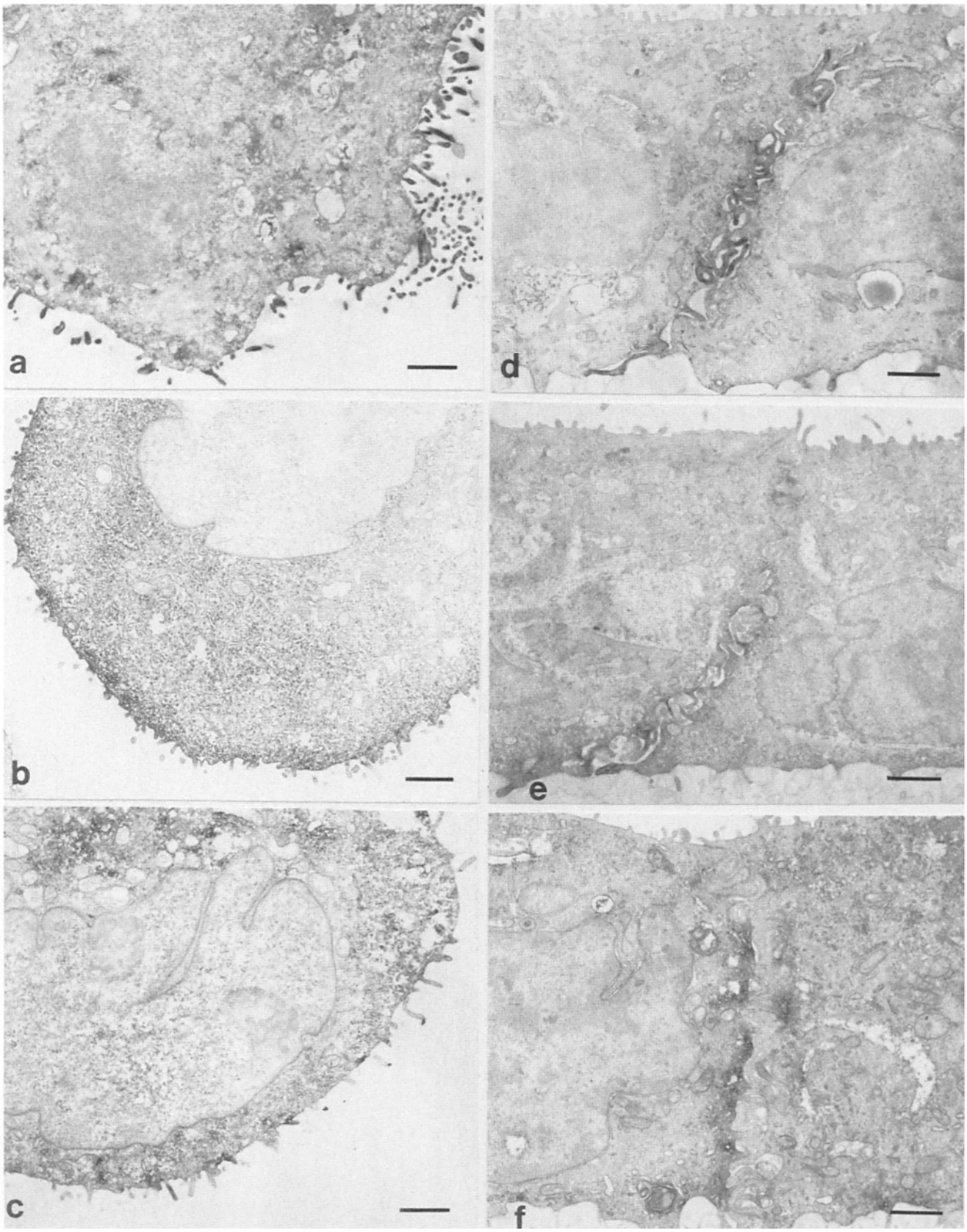

Figure 2. The cortical cytoskeleton is only polarized in confluent MDCK cells. MDCK cells grown in suspension culture (*a*, *b*, and *c*) or on a Millipore filter coated with laminin (*d*, *e*, and *f*) were examined by electron microscopy after immunoperoxidase labeling with antibodies to the alpha subunit of Na,K-ATPase (*a* and *d*), fodrin (*b* and *e*), or ankyrin (*c* and *f*). Note that the cells grown in suspension lack the polarized distribution of alpha Na,K-ATPase, fodrin, and ankyrin. A pattern similar to that seen in vivo, with colocalization of these proteins to the lateral and infolded regions of the cell membrane, is seen when the cells are grown on substrata coated with basement membrane components (cf. Fig. 1). Bar, 1.0 μM.

is observed for fodrin (Fig. 1 *b*), and for ankyrin (Fig. 1 *c*). Small amounts of reaction product were also observed with the fodrin antibody in the region of the apical membrane, consistent with the brush border characteristic of these cells. The pattern of fodrin, ankyrin, and Na,K-ATPase staining in the kidney tubule cells was nearly identical to that observed in the confluent monolayers of polarized MDCK cells (below).

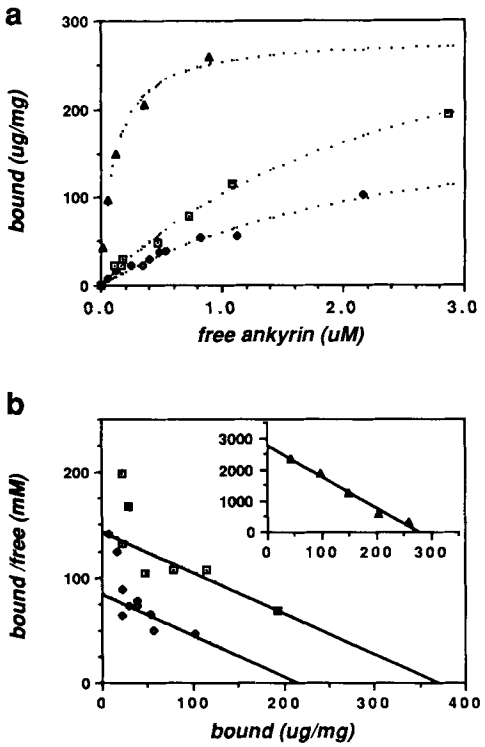

Figure 3. Ankyrin binds to Na,K-ATPase-enriched dog kidney membranes. The ability of ^{125}I-labeled erythrocyte ankyrin to bind either stripped human erythrocyte inside-out vesicles (sIOVs; ▲) or two different preparations of dog kidney membranes (□, ◆) was examined by cosedimentation assay. Nonspecific binding was determined as that amount of ankyrin that bound to an equivalent amount of erythrocyte resealed ghosts, and was deleted from the binding curves shown above. The magnitude of this correction ranged from 5 to 25% of the total counts sedimented with the membranes. (*a*) The specific binding isotherms for each membrane preparation demonstrated saturation. Nonlinear regression analysis assuming a single class of membrane ligand binding sites yielded the dotted curves shown. For the sIOVs, K_d was determined to be 0.11 ± 0.01 μM, with a B_{max} of 281 ± 9 μg/mg of membrane protein. This corresponds to a binding stoichiometry of one ankyrin molecule for every 3.2 ± 0.4 molecules of band 3. For the two kidney membrane preparations, K_d was determined to be 2.59 ± 0.41 and 2.57 ± 0.59 μM. B_{max} was determined to be 371 ± 36 and 215 ± 34 μg/ml for the two preparations shown. These values correspond roughly to one ankyrin for every 3.5 to 4.8 molecules of Na,K-ATPase in the membranes (see text). All errors are expressed as the standard error of the mean (SEM). (*b*) Scatchard analysis of the binding data; the curves presented are based on the values derived from the nonlinear regression analysis shown above. While there is a suggestion in this analysis that a trace number of binding sites with higher affinity for ankyrin may exist, the accuracy of the data, especially in this region of the binding curve, precludes such an assessment (see text).

Fodrin, Ankyrin, and Na,K-ATPase Are Codistributed Only in Confluent MDCK Cells

MDCK cells were grown either in suspension culture or to confluence on a laminin-coated millipore filter, and the distribution of fodrin, ankyrin, and Na,K-ATPase was determined by immunoelectron microscopy. The Na,K-ATPase and fodrin are codistributed at the basolateral surface of MDCK cells grown to confluence (Fig. 2, *d* and *e*), but, significantly, are excluded from those portions of the cell that are in contact with the laminin substrate. If the cells are grown in suspension culture (Fig. 2, *a* and *b*) or if the cells have not achieved confluence (data not shown), both fodrin and Na,K-ATPase appear to be randomly distributed (fodrin within the cell and Na,K-ATPase on the membrane). Ankyrin is also highly polarized in confluent MDCK cells, in a pattern similar to that of fodrin and Na,K-ATPase (Fig. 2 *f*). In nonconfluent MDCK cells, some of the ankyrin appears to be membrane associated, both at the plasma membrane and on intracellular membranes in a pattern similar to that for the Na,K-ATPase. The remainder of the ankyrin is distributed throughout the cytoplasm (Fig. 2 *c*). It is not known whether the ankyrin bound to internal membrane compartments represents material that has been recycled from the plasma membrane. Consistently, however, and unlike the case for the Na,K-ATPase, there always appears to be relatively more ankyrin on internal membranes than on the plasma membrane when these cells are in suspension.

Human Erythrocyte Ankyrin Binds to Dog Kidney Membranes Enriched in Na,K-ATPase

Since fodrin, ankyrin, and Na,K-ATPase were codistributed in the mature renal epithelial cell, it was of interest to determine the molecular basis for this observation. It is well-established that fodrin will bind to ankyrin and to nonerythroid analogues of ankyrin (e.g., Harris et al., 1986; and references therein), and that ankyrin will bind to the cytoplasmic domain of band 3 (Bennett and Stenbuck, 1980). While renal analogues of band 3 have been reported in the intercalated cells of the kidney (Drenckhahn et al., 1985), such analogues have not been detected in proximal tubule cells (Drenckhahn and Bennett, 1987; and data not shown). Therefore, kidney membranes highly enriched in Na,K-ATPase and devoid of peptides immunocrossreactive with human erythrocyte band 3 were prepared, and their ability to interact with human erythrocyte ankyrin was determined. In these membrane preparations, Na,K-ATPase accounted for >76% of the Coomassie Blue stainable protein present, as determined by densitometry of the stained SDS gels. The results of two binding experiments in which increasing amounts of ^{125}I labeled ankyrin was incubated for 3 h at room temperature with 20–40 μg of dog kidney membranes are shown in Fig. 3 *a*. The two preparations of kidney membranes shown in Fig. 3 *a* encompass the extremes of variability encountered in five different membrane preparations, with the exception of rare preparations that were devoid of binding activity. Also shown for comparison is the binding of ^{125}I-labeled ankyrin to stripped human erythrocyte inside-out vesicles (sIOV's). The binding constant (K_d) and membrane binding capacity (B_{max}) was determined for each experiment by nonlinear regression analysis (dotted curve). The binding data plotted according to Scatchard is also shown in Fig. 3 *b*. Nonspecific binding was determined as that amount of la-

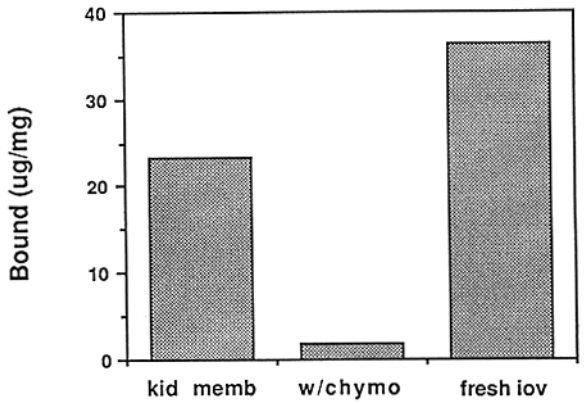

Figure 4. Proteolytic digestion of kidney membranes destroys their ankyrin binding capacity. Aliquots of Na,K-ATPase-enriched dog kidney membranes were treated with alpha-chymotrypsin at 0°C for 60 min, after which the protease was inhibited with 10 μM DFP, the membranes were washed, and then their ability to bind increasing amounts of ^{125}I-labeled ankyrin was measured. The results of one binding measurement are shown (*w/chymo*) and are compared with the binding to a comparable amount of undigested membranes (*kid memb*). This reduction in the amount of ankyrin bound is not due to residual protease activity acting on ankyrin, since the unbound ankyrin from the kidney membrane experiment bound strongly to sIOVs (*fresh iov*).

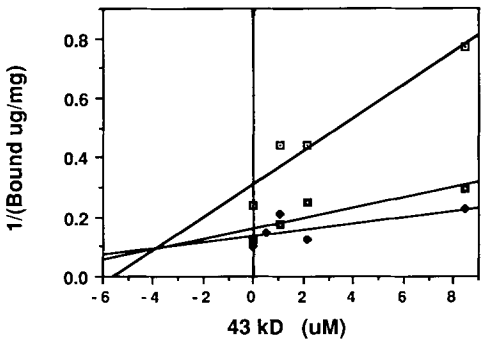

Figure 5. Inhibition of ankyrin binding to Na,K-ATPase by the cytoplasmic domain of erythrocyte band 3. The ability of the cytoplasmic 43-kD domain of erythrocyte band 3 to inhibit the binding of ankyrin to the Na,K-ATPase-enriched kidney membranes was investigated by incubating increasing amounts of the 43-kD fragment (an SDS-PAGE analysis of this fragment is shown in Fig. 6) with kidney membranes and with 0.1 (⊡), 0.2 (♦), or 0.3 (■) μM ankyrin. The kidney membranes used in these experiments had a binding capacity similar to those shown in the lower curve of Fig. 3. The data is plotted according to Dixon. Note that the 43-kD fragment inhibits ankyrin binding to Na,K-ATPase in a competitive fashion, with an estimated Ki of 3.5 ± 4.3 (SEM) μM.

beled ankyrin which bound to an equivalent amount of resealed erythrocyte ghosts, and is subtracted from the data points shown in Fig. 3. Generally, nonspecific binding accounted for 5–25% of the total counts precipitated with the membranes.

The sIOV's bound ankyrin with a K_d of 0.11 ± 0.01 (SEM) μM and B_{max} was 281 ± 9 (SEM) μg/mg membrane protein. These values of K_d are similar to those reported previously for the binding of ankyrin to sIOV's (Bennett and Stenbuck, 1980; Hargreaves et al., 1980), although we do not observe the small number of high-affinity (kD = 4.6 nM) sites noted by Bennett and Stenbuck (1980). The binding capacity of the sIOV's in this study (281 μg/mg) corresponds to one ankyrin bound for every 3.16 ± 0.42 (SEM) band 3 molecules in the membrane (given that 40 ± 10% of the protein in the sIOV's is band 3 as determined by densitometric scanning). This stoichiometry at saturation is very similar to that of the native red cell, and may indicate that tetramers of band 3 are only competent to bind a single molecule of ankyrin.

Ankyrin did not bind as strongly to the Na,K-ATPase-enriched kidney membranes, although the binding constant was quite reproducible from preparation to preparation. The K_d values determined by nonlinear regression analysis (dotted curves) for the two kidney membrane preparations shown in Fig. 3 *a* were 2.59 ± 0.41 (SEM) μM and 2.57 ± 0.59 (SEM) μM. In addition, there is a hint in the Scatchard analysis in both these experiments of a very small amount of a higher-affinity binding component (Fig. 3 *b*), an observation which, if real, would be of interest since these membranes appear to contain a second ankyrin binding protein present in trace amounts (see below). However, the Scatchard curve is difficult to evaluate at very low values of ligand bound, since the errors are greatest in this region and since any uncorrected background binding could skew the Scatchard analysis in the fashion shown in Fig. 3 *b*. Therefore, the best interpretation of these binding experiments is that only a single ankyrin binding site has been detected, and that if other site(s) of higher affinity exist (such as the band 3 analogue, which has been identified in a population of collecting duct cells [Drenckhahn et al., 1985], or the high molecular mass ankyrin binding peptide noted below), they must be of such low abundance that they are beyond the detection limits of these experiments.

The ankyrin binding capacity of the different Na,K-ATPase-enriched kidney membrane preparations was more variable than the K_d values, perhaps reflecting different degrees of purity, different states of the Na,K-ATPase, or the effects of variable proteolytic damage. The values of B_{max} determined for the two experiments shown in Fig. 3 *a* were 371 ± 36 (SEM) μg/mg and 215 ± 34 (SEM) μg/mg, corresponding to stoichiometries of one ankyrin bound for every 2.93 ± 0.58 (SEM) and 5.05 ± 1.18 molecules of Na,K-ATPase alpha-beta heterodimer.

To further confirm that the ankyrin was binding to a protein site on the dog kidney membranes, aliquots of membranes were treated with 1 μg/ml alpha-chymotrypsin for 60 min at 0°C in 20 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0, after which the chymotrypsin was inhibited with 10 μM DFP in the same buffer at pH 7.6, and the membranes washed four times. Most of the ankyrin binding capacity of these membranes was destroyed (Fig. 4). To exclude the possibility that the ankyrin was being damaged by residual protease activity accompanying the protease-treated membranes, the unbound ankyrin recovered from the supernatants of these experiments was used in a second binding assay with fresh sIOV membranes. In the presence of fresh (nonproteolyzed) sIOV's, normal ankyrin binding was observed (Fig. 4). Therefore, dog kidney membranes contain a protein receptor for ankyrin.

To explore the specificity of this receptor, the effect of the

cytoplasmic domain of erythrocyte band 3 on the binding of ankyrin to dog kidney membranes was examined. These results are shown in Fig. 5. The 43-kD band 3 fragment competitively inhibits the binding of erythrocyte ankyrin to the kidney membranes (Ki = 3.5 ± 4.3 [SEM] μM). Comparable amounts of BSA are without effect on ankyrin binding (data not shown). Thus, while band 3 analogues have not been identified in these Na,K-ATPase–enriched membranes, the interaction between ankyrin and the protein receptor appears to be specific and similar to the interaction between ankyrin and band 3 in the erythrocyte.

Ankyrin Binds to Solubilized Na,K-ATPase

To be assured that the ankyrin-binding capacity of the kidney membranes was due to Na,K-ATPase, the ability of solubilized ^{125}I-labeled Na,K-ATPase to bind to immobilized ankyrin was investigated. Kidney membranes were treated with $C_{12}E_8$ to solubilize the Na,K-ATPase, and the supernatant from a 200,000 *g* sedimentation was used for these binding experiments. Such preparations have been shown to retain full Na,K-ATPase activity (e.g., see Esmann, 1988). The material bound to ankyrin immobilized on CNBr–Sepharose is shown in Fig. 5, lane *1*. The results of an identical experiment using immobilized BSA is shown in Fig. 6, lane *2*. A negligible amount of Na,K-ATPase precipitates with the immobilized BSA compared to the amount of Na,K-ATPase that binds to the immobilized ankyrin (Fig. 6, lane *2*). Quantitatively, 17 times more ATPase bound to ankyrin compared to an equivalent amount of BSA under identical conditions.

Ankyrin Binds Directly to the Alpha Subunit of Na,K-ATPase

The colocalization and binding studies strongly suggested that Na,K-ATPase was the membrane receptor responsible for the attachment of ankyrin at the membrane cytoplasmic surface in MDCK and kidney epithelial cells. To directly examine the interaction between these two proteins, and to determine the subunit(s) of Na,K-ATPase involved, the binding of ^{125}I-labeled ankyrin to immobilized Na,K-ATPase was examined. These results are shown in Fig. 7. Ankyrin specifically binds to beta erythrocyte spectrin (Morrow et al., 1980) and to band 3 (Bennett and Stenbuck, 1980). Both of these interactions can be demonstrated in a blot of whole erythrocyte ghosts (lane *1*), although the binding of ankyrin to band 3 in these experiments is somewhat more difficult to discern, probably due to the diffuse nature of band 3 on the SDS gel. Ankyrin also binds strongly to the purified 43-kD fragment derived from band 3, as shown in lane *5*. In the erythrocyte ghost preparations, occasional binding to a 240-kD band is also observed (lane *1*), which does not appear to be alpha spectrin since purified preparations of spectrin (lane *4*) demonstrate ankyrin binding only to the beta subunit.

A similar high molecular mass (~240 kD) ankyrin-binding peptide is also present in the Na,K-ATPase–enriched dog kidney membranes (Fig. 7, lanes *2* and *3*, Fig. 8). This protein band is only discernable on silver stained gels of these membrane preparations (Fig. 8, *inset*). The nature of this large ankyrin-binding protein remains uncertain, although it appears to be tightly membrane associated since it is present in both KCl-stripped erythrocyte IOV's and in the Na,K-ATPase membrane preparations used here. While it appears to bind ankyrin avidly in these blotting assays, it is present in such low abundance relative to Na,K-ATPase that it cannot account for the large ankyrin binding capacity of the kidney membrane preparations. The relationship between the 240-kD ankyrin binding peptide of erythrocytes, and the one in kidney membranes is unknown.

The most abundant ankyrin binding protein in these preparations is at 95–96 kD, which corresponds precisely to the alpha subunit of Na,K-ATPase. No comparable binding to the beta subunit of Na,K-ATPase at 55–60 kD is detected. To confirm that the alpha subunit of Na,K-ATPase was responsible for this binding, two additional experiments were performed. Advantage was taken of the anomalous behavior of Na,K-ATPase on SDS-PAGE after it is boiled in the presence of reducing agents. After incubation in SDS-solubilizing buffer (Laemmli, 1970) with 5% 2-mercaptoethanol at 37°C for 20 min, Na,K-ATPase migrates as two discrete bands on SDS-PAGE (Fig. 7 *a*, lane *2*), corresponding to the alpha and beta subunits. However, if an identical sample is boiled in the same SDS solubilizing buffer, a new band migrating near 140 kD is generated (Hiatt et al., 1984) (Fig. 7 *a*, lane *3*). Immunoblotting experiments confirm that this new band contains the alpha subunit of Na,K-ATPase (data not shown). It is probable that this new band represents a cross-linked alpha–beta heterodimer (Nelson and Veshnock, 1987*b*). This new band, containing the alpha subunit of Na,K-ATPase, also binds to ankyrin (Fig. 7 *b*, lane *3*). To investigate the possibility that a contaminating protein with the same molecular mass as alpha Na,K-ATPase was responsible for the ankyrin binding, the kidney membrane preparations were analyzed by two-dimensional isoelectric focusing and SDS-PAGE, followed by silver staining (Fig. 7 *c*). By this analysis, a single peptide band (alpha Na,K-ATPase) with an isoelectric point between ~5.8 and 5.2 accounted for

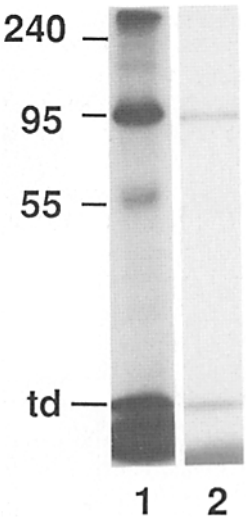

Figure 6. Solubilized Na,K-ATPase binds to immobilized ankyrin. Na,K-ATPase–enriched kidney membranes were labeled with ^{125}I using the Bolton-Hunter reagent, and then the Na-K-ATPase was solubilized in $C_{12}E_8$. The 200,000-*g* supernatant from the solubilization was then incubated for 3 h at 0°C with ankyrin (lane *1*) or an equivalent amount of BSA (lane *2*) which had been immobilized on CNBr-activated Sepharose. The samples were then sedimented and washed, and the bound material analyzed by SDS-PAGE and autoradiography. The autoradiograms are shown, with the apparent molecular masses of a series of standards (not shown) marked. Note the prominent and specific binding of Na,K-ATPase to the immobilized ankyrin. The ratio of ankyrin/BSA binding of Na,K-ATPase in these experiments ranged from 12:1 to 24:1. Also note that there is only a minor band in the region of 240 kD, possibly corresponding to the band strongly labeled on the nitrocellulose transfers shown in Fig. 7.

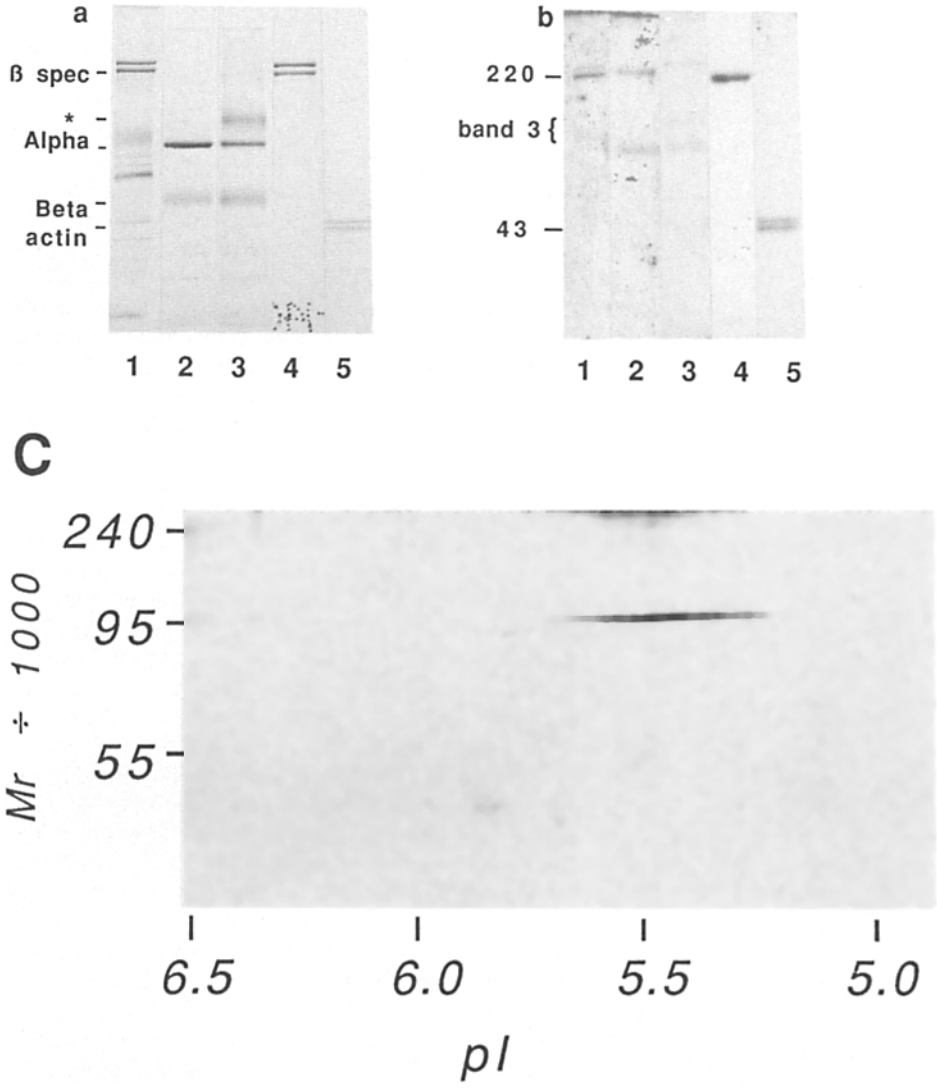

Figure 7. Ankyrin binds to the alpha-Na,K-ATPase subunit. The ability of various erythrocyte and kidney proteins to bind ankyrin after electrophoretic transfer to nitrocellulose membranes was examined by incubation of the transferred proteins with ^{125}I-labeled ankyrin. (*a*) Coomassie Blue–stained 5–15% SDS polyacrylamide gel identical to the one used for electrophoretic transfer to the nitrocellulose. Lane *1*, human erythrocyte ghosts containing 1 mg/ml BSA and cytochrome C as additional molecular mass standards. Lane *2*, Na,K-ATPase–enriched dog kidney membranes, solubilized at 37°C; note the alpha-ATPase subunit at 96 kD, and the beta-ATPase subunit at 55–50 kD. Lane *3*, the same Na,K-ATPase–enriched dog kidney membrane preparation shown in lane *2* after solubilization by boiling for 3 min; note the additional band (*) generated near 140 kD. Lane *4*, purified human erythrocyte spectrin. Lane *5*, purified cytoplasmic domain of band 3, containing peptides at 43–41 kD. Some of the prominent bands are identified in the figure. (*b*) Autoradiogram of a nitrocellulose transfer of a gel identical to the one shown in *a*, after incubation with radiolabeled ankyrin. Lane *1*, note the binding of ankyrin to beta spectrin, a diffuse and faint activity over band 3, and a weak band at ~240 kD. This binding at 240 kD is unlikely to be due to alpha spectrin, since purified preparations of spectrin do not demonstrate such activity (lane *4*). The binding of ankyrin to band 3 is most apparent in lane *5*, where binding to the purified cytoplasmic domain of band 3 is demonstrated. Lanes *2* and *3*, in the kidney membranes, the alpha subunit of Na,K-ATPase binds the ankyrin strongly, as does the new band arising in the boiled membrane preparations (marked by *), which contains alpha Na,K-ATPase (see text). In addition, there is binding to a band at ~240 kD, which corresponds to a faint protein band that is visible only after silver staining of the gel (see Fig. 8). (*c*) Two-dimensional IEF/SDS-PAGE analysis of the kidney membrane preparation. Alpha Na,K-ATPase focuses as a band centered near a pI of 5.5. The only contaminant of this molecular mass that can be identified is a vanishingly faint band near the basic end of the gel. The faint 240-kD peptide appears to also focus near pI 5.5, at a position slightly more basic than the center of the alpha Na,K-ATPase band. The SDS gel is 10–15% acrylamide, silver stained.

almost all of the detectable protein at 95–96 kD. Taken together, these data strongly indicate that the alpha subunit of Na,K-ATPase is the ankyrin binding species in these experiments.

Finally, it was of importance to determine whether the binding of ankyrin to the Na,K-ATPase in these blot assays was also inhibitable by erythrocyte band 3. Therefore, the binding experiments were repeated with ^{125}I-labeled ankyrin in the presence of increasing amounts of human erythrocyte band 3 which had been partially solubilized by Tween 20 from erythrocyte membranes stripped of all peripheral protein (Fig. 8). A densitometry scan of a Coomassie Blue–stained gel identical to the one that was used for the transfer to nitrocellulose is shown in Fig. 8 *A*. The gel itself is also shown (*inset*), along with a silver stain of the same gel. The positions of the alpha and beta subunits of Na,K-ATPase and minor protein contaminants are evident. In the absence of added band 3, there was strong binding of ^{125}I-labeled ankyrin to the 240-kD peptide and to alpha Na,K-ATPase at 95–96 kD (Fig. 8 *B*, scan *1*). With the addition of 119 μg/ml (scan *2*) and 238 μg/ml (scan *3*) of band 3, there was a progressive and complete inhibition of ankyrin binding to both the 240- and 95-kD bands. In these experiments, care was taken to avoid any agitation of the blots that could artifactually strip them of ankyrin that was bound nonspecifically to the blot and specifically to the partially solubilized band 3.

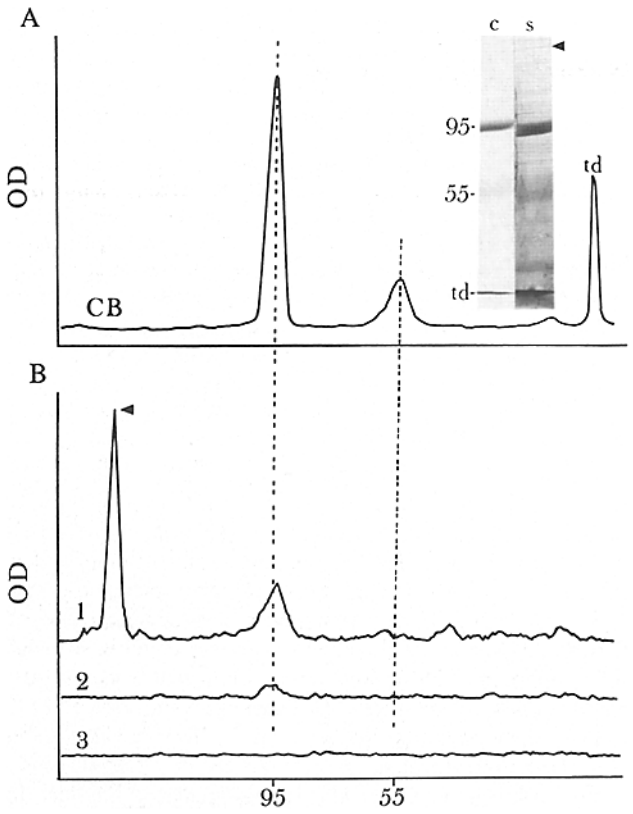

Figure 8. Ankyrin binding to alpha Na,K-ATPase is inhibited by band 3. (*A*) Densitometric scan of the Coomassie Blue-(*CB*) stained gel analysis of the kidney membranes used for these experiments. The positions of the alpha (~95 kD) and beta (~55 kD) subunits are indicated, as well as the position of the dye front (*td*). The gel itself stained by Coomassie Blue (lane *c*) and by silver (lane *s*) is also shown (*inset*). The arrowhead marks the faint band at 240 kD. (*B*) Densitometric scans of the autoradiograms resulting from the incubation of ^{125}I-labeled ankyrin with nitrocellulose transfers of the gel shown in the *inset* in *A*, in the presence of increasing amounts of band 3 partially solubilized from stripped erythrocyte membranes. Tracing *1*, no added band 3; tracing *2*, 119 μg/ml band 3; tracing *3*, 238 μg/ml band 3. Note the complete inhibition of ankyrin binding to both the 240-kD band and the alpha subunit of Na,K-ATPase. Separate experiments (not shown) established that the ankyrin was binding to the band 3 added as an inhibitor in these experiments and was not being damaged or proteolyzed.

Discussion

The results reported here indicate that the alpha subunit of Na,K-ATPase interacts with ankyrin, and that this interaction is probably responsible for the codistribution of these proteins with fodrin at the basolateral infoldings in both mature renal proximal tubule epithelial cells and in MDCK cells grown to confluence. The interaction between ankyrin and alpha Na,K-ATPase appears to involve the same region of ankyrin that is responsible for its interaction with band 3 in the erythrocyte, since the 43-kD cytoplasmic domain of band 3 is an effective inhibitor of the ankyrin and Na,K-ATPase interaction. Since alpha Na,K-ATPase and band 3 share no overall sequence homology (Kopito and Lodish, 1985; Shull et al., 1985; Schneider et al., 1985) and do not crossreact immunologically (unpublished observations), it is clear that ankyrin and proteins related to ankyrin may constitute a general mechanism linking different classes of integral membrane proteins to the spectrin (fodrin)-actin-based cortical cytoskeleton. Integral membrane proteins other than band 3 and Na,K-ATPase which are likely candidates for such linkage to a spectrin-actin cytoskeleton would include the glutamate transporter of the postsynaptic density (Lynch and Baudry, 1984), several lymphocyte receptors (e.g., see Nelson et al., 1983), and perhaps other membrane proteins such as those involved in vesicle secretion (Perrin et al., 1987). Recently, an interaction between the sodium channel and ankyrin has also been demonstrated (Srinivasan et al., 1988).

Although no overall sequence homology exists between the cytoplasmic domain of band 3 and alpha Na,K-ATPase, the fact that both proteins compete for ankyrin binding suggests that they must at least share a related ankyrin-binding region. Recently, the cDNA encoding a nonerythroid analogue of band 3 has been isolated and sequenced (Demuth et al., 1987). Although the overall degree of sequence identity in the cytoplasmic domain between the nonerythroid and erythroid band 3 was only 35%, these workers identified a highly conserved region in this domain spanning residues 157–177, which they proposed to be the ankyrin-binding region (Fig. 9). This assignment is also consistent with earlier observations attributing ankyrin binding to this region of band 3 (Low et al., 1984). A search for regions in the cytoplasmic domain of alpha Na,K-ATPase homologous to this putative ankyrin-binding sequence identified the alignment shown in Fig. 9. If allowance is made for conservative amino acid replacement, residues 646–665 of alpha Na,K-ATPase align with residues 157–177 of nonerythroid band 3 with a 63.2% degree of similarity. When compared to the putative ankyrin-binding sequence of human erythrocyte band 3, the degree of similarity is 57.9%. This level of homology approximates that noted in the actin-binding domain between otherwise unrelated proteins (Petrucci et al., 1988; McCaffery and DeGennaro, 1986), and suggests that the ankyrin-binding domain of Na,K-ATPase may encompass these residues.

While the results presented here agree qualitatively with those previously reported by Nelson and Veshnock (1987*b*), we consistently find that the binding of erythrocyte ankyrin to Na,K-ATPase is not as strong as reported in that study (K_d = 2.6 μM vs. 0.002 μM). In addition, whereas the previous study reported only one ankyrin bound for every 17–23 molecules of Na,K-ATPase heterodimer, we find a binding stoichiometry of 1:4, nearly identical to that for the binding of ankyrin to band 3 in erythrocyte membranes. We do not fully understand the reasons for this apparent discrepancy, although a comparison of the binding curve presented in that study with those presented here suggests that the previous studies may not have been carried to saturation. A contribution from a high affinity but minor component (such as the 240-kD peptide noted here) would also be inappropriately weighted in a Scatchard analysis. This would not be a problem with the regression analysis used here. Alternatively, it is also possible that the binding affinity of ankyrin for Na,K-ATPase is posttranslationally regulated (see the discussion below), and that the differences in the measured binding affinities between the two studies reflect different "states" of the Na,K-ATPase.

Beyond the fact that purified and solubilized Na,K-ATPase

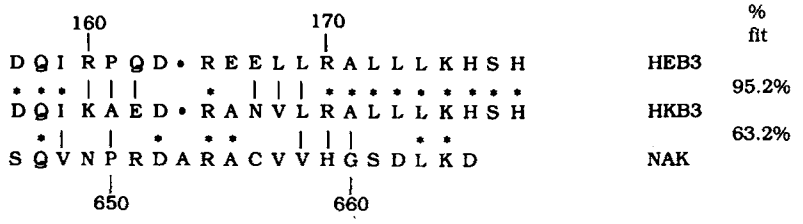

Figure 9. Comparison of amino acid sequence of Na,K-ATPase with the putative ankyrin binding region of erythroid and nonerythroid band 3. The program BESTFIT was used to compare the putative ankyrin binding sequence of both human erythroid band 3 (*HEB3*) (Kaul et al., 1983) and human nonerythroid band 3 (*HKB3*) (Demuth et al., 1986) with sheep Na,K-ATPase (*NAK*) (Shull et al., 1985). The region of best alignment for both band 3 sequences is shown; both identified residues 646–665 of the Na,K-ATPase as the most similar region. The degree of similarity was 63.2% for the nonerythroid band 3, and 57.9% for erythroid band 3, both with a one-residue gap. The degree of similarity between the two band 3 sequences was 95.2%. Identical residues are marked by *; similar residues by |. (BESTFIT is a program supplied by the University of Wisconsin Genetics Computer Group, Madison, WI.)

binds specifically to immobilized ankyrin, it should be emphasized that for reasons of simple stoichiometry, it is unlikely that the 240-kD protein band could account for the bulk of the ankyrin that binds to kidney membranes. Between 75 and 90% of the protein in these membranes is Na,K-ATPase, as determined by both Coomassie Blue and silver staining techniques. The 240-kD protein accounts for <5% of the protein. Therefore, each molecule of the 240-kD protein would have to bind over five molecules of ankyrin to account for the capacity of these membranes. By comparison, each molecule of band 3 in erythrocyte membranes binds on the average just 0.25 molecules of ankyrin. While it is always possible that the 240-kD protein does not stain well using these techniques, a comparison of the protein content of the kidney membranes as determined by protein (Lowry et al., 1951) analysis with the content of Na,K-ATPase by immunoblotting (Kashgarian et al., 1985), or by radiolabeling (this manuscript), gives no indication for the existence of a large pool of heretofore unrecognized protein. This conclusion is also supported by earlier studies characterizing these membranes (Jorgensen, 1982).

Even though the precise role that the spectrin-based cortical cytoskeleton plays in the biology of the renal cell remains uncertain, the interaction of ankyrin with Na,K-ATPase has many implications. The colocalization of spectrin and ankyrin at the basolateral domain may indicate that these cytoskeletal proteins are simply required for the maintenance of the polarized distribution of the Na,K-ATPase, as has recently been proposed (Drenckhahn and Bennett, 1987; Nelson and Veshnock, 1987*b*). However, it is also possible that the interaction between spectrin, ankyrin, and Na,K-ATPase may play a more active role in guiding the topographic assembly of this integral membrane protein. For example, after cell contact in MDCK cells, even in the presence of protein synthesis inhibitors, Na,K-ATPase spontaneously polarizes (Nelson and Veshnock, 1987*a*). If cell contact locally activates the coupling of Na,K-ATPase to ankyrin, or the coupling of ankyrin to spectrin, then one could envision a mechanism whereby this local activation would result in the formation of a topographically distinct cytoskeletal array that might entrap additional Na,K-ATPase molecules as they diffused into the region, or capture Na,K-ATPase containing vesicles "targetted" to the basolateral domain. With regard to this latter hypothesis, it is interesting to note that anti-alpha fodrin antibodies block secretion in chromaffin cells (Perrin et al., 1987).

Although the results reported here might suggest that many classes of integral membrane proteins use ankyrin mediated linkages to the cortical cytoskeleton, it is unlikely that this will be universally true, since ankyrin independent linkages of spectrin to the membrane also appear to exist. Protein 4.1 mediates the attachment of spectrin to integral membrane proteins in the erythrocyte (reviewed in Morrow and Anderson, 1986), and avian intestinal brush border spectrin does not interact with either ankyrin (Howe et al., 1985) or protein 4.1 (Coleman et al., 1987), but is capable of binding to erythrocyte membrane vesicles (Howe et al., 1985). Ankyrin analogues also do not appear to mediate the association of the acetylcholine receptor with an isoform of beta spectrin at the skeletal muscle motor endplate (Bloch and Morrow, 1989), and a direct association between fodrin and an integral lymphocyte glycoprotein (gp180) has been reported (Bourguignon et al., 1985).

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