Distinguishing Roles of the Membrane-Cytoskeleton and Cadherin Mediated Cell–Cell Adhesion in Generating Different Na^+,K^+-ATPase Distributions in Polarized Epithelia

James A. Marrs, Eugene W. Napolitano,* Cynthia Murphy-Erdosh,* Robert W. Mays, Louis F. Reichardt,* and W. James Nelson

Department of Molecular and Cellular Physiology, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, California 94305-5426; and * Department of Physiology, Howard Hughes Medical Institute, University of California at San Francisco, California 94143-0724

Abstract. In simple epithelia, the distribution of ion transporting proteins between the apical or basal-lateral domains of the plasma membrane is important for determining directions of vectorial ion transport across the epithelium. In the choroid plexus, Na^+,K^+-ATPase is localized to the apical plasma membrane domain where it regulates sodium secretion and production of cerebrospinal fluid; in contrast, Na^+,K^+-ATPase is localized to the basal-lateral membrane of cells in the kidney nephron where it regulates ion and solute reabsorption. The mechanisms involved in restricting Na^+,K^+-ATPase distribution to different membrane domains in these simple epithelia are poorly understood. Previous studies have indicated a role for E-cadherin mediated cell–cell adhesion and membrane-cytoskeleton (ankyrin and fodrin) assembly in regulating Na^+,K^+-ATPase distribution in absorptive kidney epithelial cells. Confocal immunofluorescence microscopy reveals that in chicken and rat choroid plexus epithelium, fodrin, and ankyrin colocalize with Na^+,K^+-ATPase at the apical plasma membrane, but fodrin, ankyrin, and adducin also localize at the lateral plasma membrane where Na^+,K^+-ATPase is absent. Biochemical analysis shows that fodrin, ankyrin, and Na^+,K^+-ATPase are relatively resistant to extraction from cells in buffers containing Triton X-100. The fractions of Na^+,K^+-ATPase, fodrin, and ankyrin that are extracted from cells cosediment in sucrose gradients at ~10.5 S. Further separation of the 10.5 S peak of proteins by electrophoresis in nondenaturing polyacrylamide gels revealed that fodrin, ankyrin, and Na^+,K^+-ATPase comigrate, indicating that these proteins are in a high molecular weight complex similar to that found previously in kidney epithelial cells. In contrast, the anion exchanger (AE2), a marker protein of the basal-lateral plasma membrane in the choroid plexus, did not cosediment in sucrose gradients or comigrate in nondenaturing polyacrylamide gels with the complex of Na^+,K^+-ATPase, ankyrin, and fodrin. Ca^{++}-dependent cell adhesion molecules (cadherins) were detected at lateral membranes of the choroid plexus epithelium and colocalized with a distinct fraction of ankyrin, fodrin, and adducin. Cadherins did not colocalize with Na^+,K^+-ATPase and were absent from the apical membrane. The fraction of cadherins that was extracted with buffers containing Triton X-100 cosedimented with ankyrin and fodrin in sucrose gradients and comigrated in nondenaturing gels with ankyrin and fodrin in a high molecular weight complex. Since a previous study showed that E-cadherin is an instructive inducer of Na^+,K^+-ATPase distribution, we examined protein distributions in fibroblasts transfected with B-cadherin, a prominent cadherin expressed in the choroid plexus epithelium. The results show that Na^+,K^+-ATPase and fodrin become concentrated at cell–cell contracts in cells expressing E-cadherin, but not in cells expressing B-cadherin, indicating differences in the potential of these cadherins to induce Na^+,K^+-ATPase accumulation at cell–cell contacts. These results provide new insights into the roles of the membrane-cytoskeleton and cadherins in generating different distributions of the same protein in simple epithelial cells.

Please address all correspondence to W. James Nelson, Department of Molecular and Cellular Physiology, Beckman Center, B121, Stanford University School of Medicine, Stanford, CA 94305-5426.

SIMPLE, transporting epithelia line the cavities of many animal tissues and perform cell-type specific functions of vectorial secretion or absorption. These cells exhibit

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a polarized distribution of ion transporting proteins between two structurally and functionally distinct plasma membrane domains, termed apical and basal-lateral (Mostov et al., 1992; Nelson, 1992; Rodriguez-Boulan and Powell, 1992). Sodium, potassium-adenosine triphosphatase (Na^+,K^+-ATPase)[1] plays a critical role in the function of both secretory and absorptive epithelia. In the choroid plexus epithelium, a secretory epithelium, Na^+,K^+-ATpase is restricted to the apical plasma membrane that faces the lumen of the brain ventricles (Wright, 1972; Masuzawa et al., 1984; Seigel et al., 1984; Masuzawa et al., 1985; Ernst et al., 1986; Spector and Johanson, 1989); this distribution results in net sodium secretion form the blood supply into the ventricles, and the secretion of cerebrospinal fluid. This function differs from that in kidney, and absorptive epithelia, in which Na^+,K^+-ATPase is localized to the basal-lateral plasma membrane and regulates sodium and water reabsorption from the lumen of the nephron to the blood supply (Almers and Stirling, 1984). An intriguing question is how the same protein, Na^+,K^+-ATPase, becomes localized to opposite membrane domains in different epithelial cells. An understanding of the mechanisms involved in this process may also provide new insight into several disease states of absorptive renal epithelial cells (ischemia, polycystic kidney disease) in which Na^+,K^+-ATPase has an abnormal distribution in the apical membrane domain (Molitoris, 1991; Wilson et al., 1991; Avner et al., 1992).

Mechanisms for generating polarized protein distributions in epithelial cells are being extensively studied. Particular emphasis has been placed on understanding how direct targeting of proteins from the Golgi complex to specific plasma membrane domains occurs (Mostov et al., 1992; Nelson, 1992; Rodriguez-Boulan and Powell, 1992). Recent studies of mechanisms involved in regulating the distribution of Na^+,K^+-ATPase in kidney epithelial cells in vitro indicate that cell-cell adhesion and subsequent assembly of the membrane-cytoskeleton also play important roles in restricting the distribution of Na^+,K^+-ATPase to the (basal-) lateral membrane of those cells (McNeill et al., 1990; Nelson et al., 1990*a*).

Specific cell-cell contacts in epithelial cells are mediated by a superfamily of Ca^{++}-dependent cell adhesion proteins, termed cadherins (Geiger and Ayalon, 1992; Kemler, 1992). In renal epithelial cells, cadherins have been shown to play a critical role in regulating membrane-cytoskeleton assembly and the induction of epithelial cell polarity. In MDCK cells, E-cadherin mediated cell-cell contacts coincide with assembly of the membrane-cytoskeleton and localization of Na^+,K^+-ATPase on the forming lateral plasma membrane domain (Nelson and Veshnock, 1986, 1987*b*; Nelson et al., 1990*b*). In addition, E-cadherin has been shown to act directly as an instructive inducer of Na^+,K^+-ATPase and fodrin localization to cell-cell contacts (lateral membrane domain) in E-cadherin-transfected fibroblasts (McNeill et al., 1990). It is not known whether these properties are shared by other members of the cadherin superfamily.

The membrane-cytoskeleton is composed of fodrin (the nonerythroid isoform of spectrin), ankyrin, actin, and associating proteins (Bennett, 1990). Erythroid adducin forms a ternary complex with spectrin and actin, and may regulate membrane-cytoskeleton assembly (Gardner and Bennett, 1987; Mische et al., 1987); the function of adducin in nonerythroid cells may be analogous (Bennett et al., 1988; Coleman et al., 1989; Kaiser et al., 1989).

The membrane-cytoskeleton is linked to several integral membrane proteins (Bennett, 1990). The first major binding site for ankyrin and the membrane-cytoskeleton was identified as the anion exchanger (AEl or band 3) in the erythrocyte (Tyler et al., 1979; Bennett and Stenbuck, 1980; Hargreaves et al., 1980). Other isoforms of the anion exchanger have been identified which have high sequence identity to AEl (Kopito, 1990); AE2 is expressed on the basal-lateral plasma membrane of the choroid plexus epithelium (Lindsey et al., 1990), and in parietal cells of the stomach epithelium (Kudrycki et al., 1990). At present it is not known whether AE2 binds to ankyrin and the membrane-cytoskeleton. Direct binding studies with purified proteins have also demonstrated that ankyrin binds Na^+,K^+-ATPase with high affinity (Nelson and Veshnock, 1987*a*; Morrow et al., 1989; Davis and Bennett, 1990), and a complex of these proteins with fodrin has been isolated from renal epithelial cells (Koob et al., 1987; Nelson and Hammerton, 1989). Assembly of Na^+,K^+-ATPase into the membrane-cytoskeleton may stabilize and retain this protein in the basal-lateral plasma membrane domain (Hammerton et al., 1991). These studies have led to a model in which E-cadherin mediated cell-cell adhesion leads to localized membrane-cytoskeleton assembly at cell-cell contacts, resulting in stabilization and subsequent accumulation of Na^+,K^+-ATPase to these sites (Nelson et al., 1990*a*).

Here, we demonstrate that in the apical membrane domain of the choroid plexus, Na^+,K^+-ATPase, colocalizes with components of the membrane-cytoskeleton (ankyrin and fodrin), which can be isolated in a multi-protein complex similar to that demonstrated previously in MDCK cells (Nelson and Hammerton, 1989). We also find that the membrane-cytoskeleton forms a complex with cadherins which are located in the lateral membrane domain of the choroid plexus epithelium, which, unlike lateral membranes in absorptive epithelial cells, lacks Na^+,K^+-ATPase. Significantly, studies of protein distributions in fibroblasts transfected with E- or B-cadherin show that Na^+,K^+-ATPase and fodrin become concentrated at cell-cell contacts in cells expressing E-cadherin, as previously described (McNeill et al., 1990), but not in cells expressing B-cadherin. These studies indicate similarities in the role of the membrane-cytoskeleton in stabilizing Na^+, K^+-ATPase in a specific membrane domain in secretory and absorptive epithelia, but indicate differences in the potential of the cadherins expressed in these cells to influence Na^+,K^+-ATPase distribution.

Materials and Methods

Tissues and Cells

Choroid plexus was freshly dissected from the lateral ventricles of E17-E20 chicken brains and rat choroid plexus was dissected from P7-P14 pups; tissue was removed within 3-5 min of animal sacrifice. MDCK cells have been described previously (Nelson and Veshnock, 1986). E-cadherin transfected and control L cells were provided by Dr. Rolf Kemler and have been described previously (Ozawa et al., 1989). L cells were also transfected with chicken B-cadherin cDNA regulated by the β-actin promoter and will be

1. *Abbreviation used in this paper*: Na^+,K^+-ATPase, sodium, potassium-adenosine triphosphatase.

described in detail later (Murphy-Erdosh, C., E. W. Napolitano, and L. F. Reichardt, manuscript in preparation).

Electron Microscopy

Freshly dissected choroid plexus from chick embryos or rat pups were fixed in 2% glutaraldehyde (all electron microscopy chemicals are from Polysciences, Warrington, PA) in phosphate buffer for 1.5 h, and then rinsed two times in phosphate buffer at room temperature. The tissue was postfixed in 1% OsO_4 for 1 h at room temperature, and then rinsed two times in distilled water. Samples were then stained en block in 0.25% uranyl acetate overnight, and then rinsed two times in distilled water. The tissue was dehydrated through an ethanol series (25-100%), followed by treatment with 100% propylene oxide two times for 5 min each. Samples were then infiltrated with Poly/bed 812 by a series of 3:1 (1 h), 1:1 (overnight), 1:3 (3 h) of propylene oxide: Poly/bed 812, followed by 100% Poly/bed 812 for 4 h. Samples were embedded in fresh Poly/bed 812 overnight at 65°C. Thin sections were viewed using a Phillips 201 electron microscope.

Triton X-100 Extraction and Sucrose Density Gradient Centrifugation

Tissues were washed briefly in PBS, and then extracted with isotonic buffer containing Triton X-100 (0.5% Triton X-100 in 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.1 mM DTT and 0.5 mM PMSF) for 10-15 min at 4°C. The choroid plexus epithelium is found on the surface of this tissue, providing complete access to detergent solution during extraction. Samples were centrifuged at 48,000 *g* for 15 min to separate the Triton X-100 soluble and insoluble fractions. Linear sucrose gradients (5-20%) were overlaid with the Triton X-100 soluble fraction of chicken choroid plexus and centrifuged in a SW60Ti rotor (Beckman Instrs. Inc., Fullerton, CA) at 60,000 rpm for 5 h at 4°C (Nelson et al., 1990). Gradients were fractionated from bottom to top into 18 fractions.

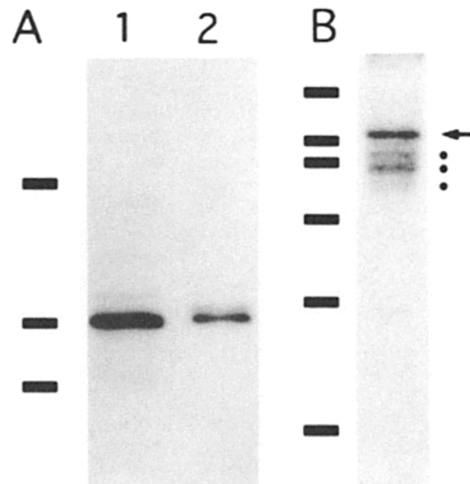
Gel Electrophoresis and Immunoblotting

For SDS-PAGE, equal proportions by volume of protein samples were solubilized in SDS-PAGE sample buffer and separated in 7.5% polyacrylamide SDS-gels; proteins were transferred to nitrocellulose for immunoblotting as previously described (Nelson and Veshnock, 1986), except that a $[^{125}I]$ donkey anti-rabbit secondary antibody (Amersham Corp., Arlington Heights, IL) was used to detect immune complexes. Relative amounts of protein detected by autoradiography were quantified by scanning densitometry (Hoefer Sci. Instr., San Francisco, CA, model GS300). For native gels, non-denaturing gel sample buffer was added to sucrose gradient fractions and samples were loaded on 2-4% polyacrylamide gradient native gels: these gels were electrophoresed at 50 V for 60 h at 4°C, as described previously (Nelson and Veshnock, 1987*a*). Proteins were transferred to nitrocellulose for immunoblot analysis as described above.

Antibodies

Antisera to the α- and β-subunits of Na^+,K^+-ATPase from dog kidney (Nelson and Hammerton, 1989), E-cadherin extracellular domain from dog kidney (Shore and Nelson, 1991), α-fodrin from calf lens, and human erythrocyte adducin (provided by Kathleen Siemers, Stanford University) were raised in rabbits. Rabbit polyclonal antiserum raised to chicken erythrocyte ankyrin was provided by Dr. Elizabeth Repasky (Roswell Park Cancer Center). Anti-peptide antiserum which recognizes the anion exchanger isoforms AE1 and AE2 was provided by Dr. Ron Kopito (Stanford University). Na^+,K^+-ATPase α- and β-subunit isoform specific rabbit antisera were provided by Dr. Robert Levenson (Yale University). Rabbit antiserum to N-cadherin was provided by Dr. Masatoshi Takeichi (Kyoto University). The monoclonal antibody DECMA-1 was provided by Dr. Rolf Kemler (Max Planck Institut fur Immunbiologie). The monoclonal antibody 5A6 was raised to B-cadherin (Murphy-Erdosh, C., E. W. Napolitano, and L. F. Reichardt, manuscript in preparation).

A pan-cadherin rabbit antiserum was raised to a fusion protein of glutathione-S-transferase and the cytoplasmic domain of mouse E-cadherin; the antiserum recognizes different cadherins including B-cadherin expressed in the choroid plexus (see below). The fusion protein was generated by PCR on a cDNA encoding mouse E-cadherin (provided by Dr. Rolf Kemler, Max-Planck-Institut fur Immunbiologie) using 2 primers: 5'-CGG GAT CCA GAA CGG TGG TCA AAG AGC CC-3', and 5'-CGG AAT TCC CAT GGT GCC ACA CGG GGG AG-3'. The PCR product was digested with EcoRI and BamHI and ligated into pGEX-2T (Pharmacia LKB Biotechnology, Piscataway, NJ). This construct encoded a glutathione-S-transferase fusion protein with the complete cytoplasmic domain of E-cadherin. Fusion protein was expressed in *E. coli* strain AB1899 and purified using glutathione agarose (Sigma Chem. Co., St. Louis, MO). The fusion protein was excised from preparative SDS gels, electroeluted, and injected into New Zealand White rabbits with Freund's adjuvant.



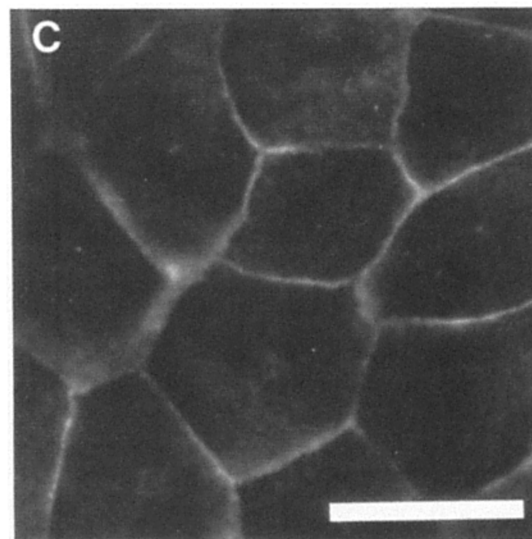


Figure 1. Characterization of antiserum raised to the COOH-terminal, cytoplasmic domain of E-cadherin. (*A*) Immunoblot analysis of MDCK extract (lane *1*) and chick choroid plexus extract (lane *2*) demonstrates reactivity of the antiserum with E- and B-cadherin, respectively. Molecular weight markers: 205, 116, and 97 kD. (*B*) Immunoprecipitation of E-cadherin from $[^{35}S]$methionine-labeled protein extract of MDCK cells (see Materials and Methods). Results show a characteristic and diagnostic pattern of coimmunoprecipitated proteins identified as catenins (α, β, and γ: 102, 94, and 83 kD, respectively). Molecular weight markers: 205, 116, 97, 66, 45, and 29 kD. Arrow indicates the immunoprecipitated E-cadherin, and bullets indicate the three coimmunoprecipitated catenins. (*C*) Immunofluorescence staining of an MDCK cell monolayer using anti-cadherin cytoplasmic domain antiserum (en face view) demonstrates characteristic cell-cell contact staining. Bar, 50 μm.

This E-cadherin cytoplasmic domain antiserum recognizes different cadherins from many sources including E-cadherin from MDCK cells (Fig. 1 *A*, lane *1*), and N-cadherin from chick and mouse brain (data not shown). We also analyzed protein extracts to determine cadherin expression in the choroid plexus. Two protein bands were recognized (Fig. 1 *A*, lane *2*; see below). As a direct demonstration that this antiserum specifically reacts with cadherins, E-cadherin was immunoprecipitated from MDCK cells with an antiserum specific for the extracellular domain of renal E-cadherin (Shore and Nelson, 1991); an immunoblot was then probed with the antiserum against the cytoplasmic domain of E-cadherin, and the immunoprecipitated E-cadherin was recognized (Siemers, K., unpublished data). This antiserum also coimmunoprecipitated α-, β-, and γ-catenins with cadherin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989) from MDCK cells (Fig. 1 *B*), chick choroid plexus, intestine, and brain (data not shown). The antiserum also produced a characteristic lateral plasma membrane staining pattern of E-cadherin in confluent monolayers of MDCK cells (Fig. 1 *C*).

Immunofluorescence Microscopy

Freshly dissected choroid plexus was fixed and permeabilized in 80% methanol and 20% DMSF overnight at −20°C. Tissue was washed in PBS for 1 h at room temperature. Tissue was then blocked with 20% goat serum in PBS for 30 min. Primary antibodies were diluted 1:100 in PBS with 20% goat serum and incubated for 3 h at room temperature. Washing was performed in PBS for 60 min. Secondary antibody (goat anti-rabbit conjugated with rhodamine, Boehringer Mannheim, Indianapolis, IN) was diluted 1:150 in PBS and incubated on tissue for 30 min. Washing was performed as for the primary antibody. Stained tissue was fixed with 2% paraformaldehyde in PBS for 10 min at 4°C, and then washed two times with PBS. Tissue was mounted on slides and viewed using a customized DIC system adapted on a laser scanning confocal microscope on a Zeiss IM35 microscope (Carl Zeiss, Oberkochen, Germany; for details see Hammerton et al., 1991; McNeill et al., 1993).

MDCK cells, L cells, and cadherin transfected L cells were grown on coverslips and stained for cadherin, Na^+,K^+-ATPase, and fodrin. Cells were rinsed briefly with PBS, and then fixed with 100% methanol at −80°C for 10 min. Cells were again rinsed with PBS. For E- and B-cadherin staining of fibroblasts, cells were fixed with 3% paraformaldehyde in PBS. Cells were blocked with 5% goat serum and 0.2% BSA in PBS for 20 min. Primary antibodies were diluted 1:200 in PBS with 0.2% BSA and incubated with sections for 60–90 min. Washing was performed in PBS with 0.2% BSA for 20 min. Secondary antibody (rhodamine conjugated, Boehringer Mannheim) was diluted 1:150 in PBS and 0.2% BSA and incubated for 20 min. Slides were viewed using an Axiophot microscope equipped with epifluorescence (Carl Zeiss Instrs., Thornwood, NY), and photographed using Kodak technical pan film (Eastman Kodak, Rochester, NY).

Immunoprecipitation

MDCK cells were metabolically labeled using [^{35}S]methionine (New England Nuclear DuPont, Wilmington, DE) for 4 h (Nelson and Veshnock, 1986). Triton X-100 extracts were precleared using preimmune antiserum and *Staphylococcus aureus* cells (Calbiochem, La Jolla, CA). Primary antibody (anti-E-cadherin cytoplasmic domain) was incubated with the precleared extract. Immune complexes were precipitated using protein A-agarose beads (Pharmacia LKB Biotechnology). Precipitates were washed using stringent conditions as described previously (Shore and Nelson, 1991).

Results

Ultrastructure of the Choroid Plexus

In the choroid plexus, a monolayer of epithelial cells separates the choroid blood supply from the brain ventricles (Fig. 2). In general, for both chick (Fig. 2 *A*) and rat choroid plexus (Fig. 2 *C*), the basal surface of the cells contacts a basement membrane that is adjacent to blood vessels and the stroma (extracellular matrix and fibroblasts). The apical membrane is directly exposed to the cerebrospinal fluid within the brain ventricles. Characteristically, the apical surface has numerous, swollen microvilli. A brush border does not form. Numerous cilia are present at the apical membrane, especially in the chick cells. Electron dense structures present at the apex of the lateral plasma membranes are characteristic of an apical junctional complex containing *zonula occludens* and *zonula adherens* (Fig. 2, *B* and *D*). Desmosomes (*maculae adherens*) were not observed, nor were they detected by immunofluorescence using antiserum specific for desmoplakin (data not shown).

Some differences were observed between the rat and chick choroid plexus (Fig. 2, *A* and *C*). The rat choroid plexus is comprised of cuboidal cells, whereas the cells are columnar in the chick. Consequently, the lateral domain of the rat choroid plexus cells is short relative to that of the chick cells. Also, the rat choroid plexus epithelial cells have a more elaborate, dome-shaped apical membrane domain than that in the chick.

Colocalization of Na^+,K^+-ATPase, Fodrin, and Ankyrin in Choroid Plexus Epithelium

The subcellular distribution of Na^+,K^+-ATPase, fodrin, and ankyrin in rat and chicken choroid plexus were compared by confocal laser scanning immunofluorescence microscopy. In rat choroid plexus, α-subunit (Fig. 3, *a* and *b*) and β-subunit (data not shown) of Na^+,K^+-ATPase localized to the apical plasma membrane, as previously observed for rat and other species (Masuzawa et al., 1984, 1985; Seigel et al., 1984; Ernst et al., 1986). Na^+,K^+-ATPase staining was similar if tissues were either fixed before permeabilization or first extracted with an isotonic buffer containing Triton X-100, and then fixed (data with shown). We did not detect staining of Na^+,K^+-ATPase at the lateral or basal membranes. Immunoblotting of whole cell extracts (Marrs, J. A., unpublished observations) and an in situ hybridization study (Watts et al., 1991) showed that the $\alpha 1$ and $\beta 1$ isoforms of Na^+,K^+-ATPase are expressed in the choroid plexus epithelium. These are the same isoforms that are expressed in the kidney (Sweadner, 1989); an additional β subunit isoform ($\beta 2$) may also be expressed in the choroid plexus (Watts et al., 1991).

Immunofluorescence showed that α-fodrin was colocalized with Na^+,K^+-ATPase at the apical plasma membrane of rat choroid plexus epithelial cells (Fig. 3, *c* and *d*), and also was found at the lateral plasma membrane. A very low level of cytoplasmic staining was observed with α-fodrin antiserum that included a distinct spot of fluorescence of unknown origin above the nucleus of most cells. There was little or no staining of the basal plasma membrane. Ankyrin antisera available for this study did not react with ankyrin in sections or whole mounts of the choroid plexus epithelial cells (data not shown). Adducin was detected at the lateral plasma membrane of the rat choroid plexus epithelial cells (Fig. 3, *e* and *f*).

To determine whether or not these distributions of membrane-cytoskeletal proteins were unique to mammalian species, we examined avian choroid plexus. In the chick choroid plexus, α-subunit (Fig. 4, *a* and *b*) and β-subunit (data not shown) of Na^+,K^+-ATPase were restricted to the apical plasma membrane of the epithelial cells. As in rat choroid plexus, Na^+,K^+-ATPase staining was retained if the tissue was first extracted with an isotonic buffer containing Triton X-100, and then fixed and processed for immunofluorescence (data not shown; see below). Fodrin staining (Fig. 4, *c* and

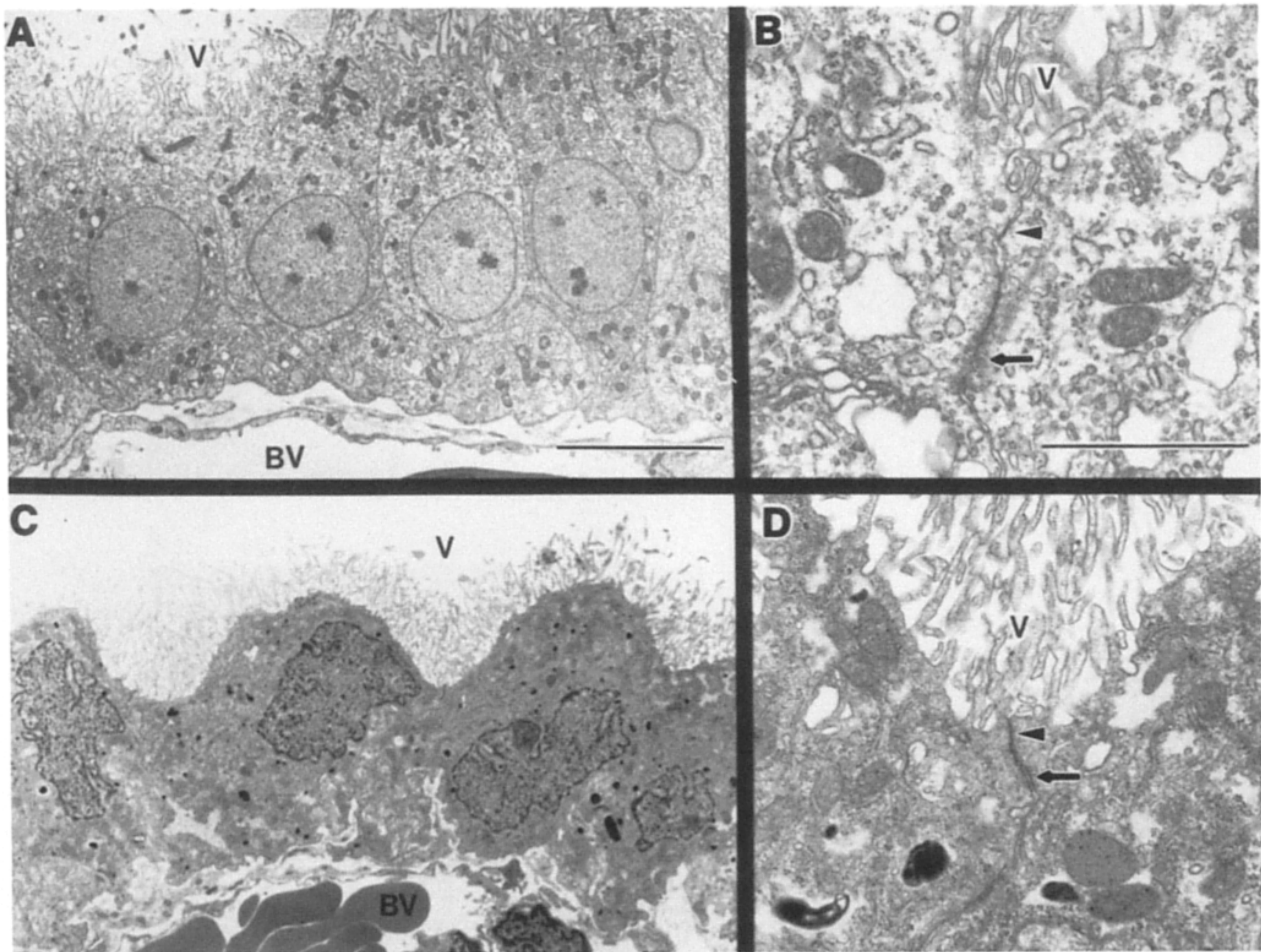


Figure 2. Ultrastructure of the choroid plexus. In choroid plexus, the epithelial cell layer separates the brain ventricle (*V*) from the blood supply. The blood supply is found within the stroma (or the choroid) which contains blood vessels (*BV*) and extracellular matrix. (*A*) The chick choroid plexus epithelium has a columnar morphology and numerous cilia on the apical surface. (*B*) Higher magnification of the chick choroid plexus epithelial cell apical-lateral junctional domain shows the tight and adherens junctions. (*C*) The rat choroid plexus epithelium has a more cuboidal morphology and a more extensive, dome-shaped apical domain, in contrast to that of the chick (*A*). (*D*) Higher magnification view of the rat choroid plexus epithelial cell junctional complex. Arrowheads indicate the tight junction, and arrows indicate the adherens junction. Bars: (*A* and *C*) 5 μm; (*B* and *D*) 1 μm.

d) was also localized to the apical membrane in a distribution overlapping that of Na^+,K^+-ATPase; in addition, we detected staining of α-fodrin at the lateral membranes. The basal plasma membrane did not exhibit α-fodrin staining. Note that the staining intensity of α-fodrin is significantly greater on the apical membrane than at the lateral membranes, especially when it is taken into account that the lateral membrane staining is contributed by staining from two closely apposed membranes. Immunofluorescence staining with ankyrin antibodies resulted in labeling of both the apical and lateral membranes similar to that of α-fodrin (Fig. 4, *e* and *f*); we speculate that our ankyrin antibody recognizes the avian isoform of ankyrin, but not the rat isoform. Adducin was localized to the lateral, but not apical or basal membranes in the chick choroid plexus epithelium (Fig. 4, *g* and *h*).

Differential Extraction of Membrane and Membrane-Cytoskeletal Proteins from the Choroid Plexus

To examine the association of Na^+,K^+-ATPase with the membrane-cytoskeleton, we analyzed the relative extractability of Na^+, K^+-ATPase and membrane-cytoskeletal proteins from chick choroid plexus in buffers containing Triton X-100. The chick choroid plexus was chosen for further study since all antibodies detected specific proteins expressed in this species. The epithelium lies on the surface of the choroid plexus, providing complete access to the detergent solution during extraction. Since the majority of Na^+,K^+-ATPase, fodrin, ankyrin, cadherin, and AE2 are found in the epithelial cells by immunofluorescent staining and very low staining was seen in non-epithelial cells (Figs.

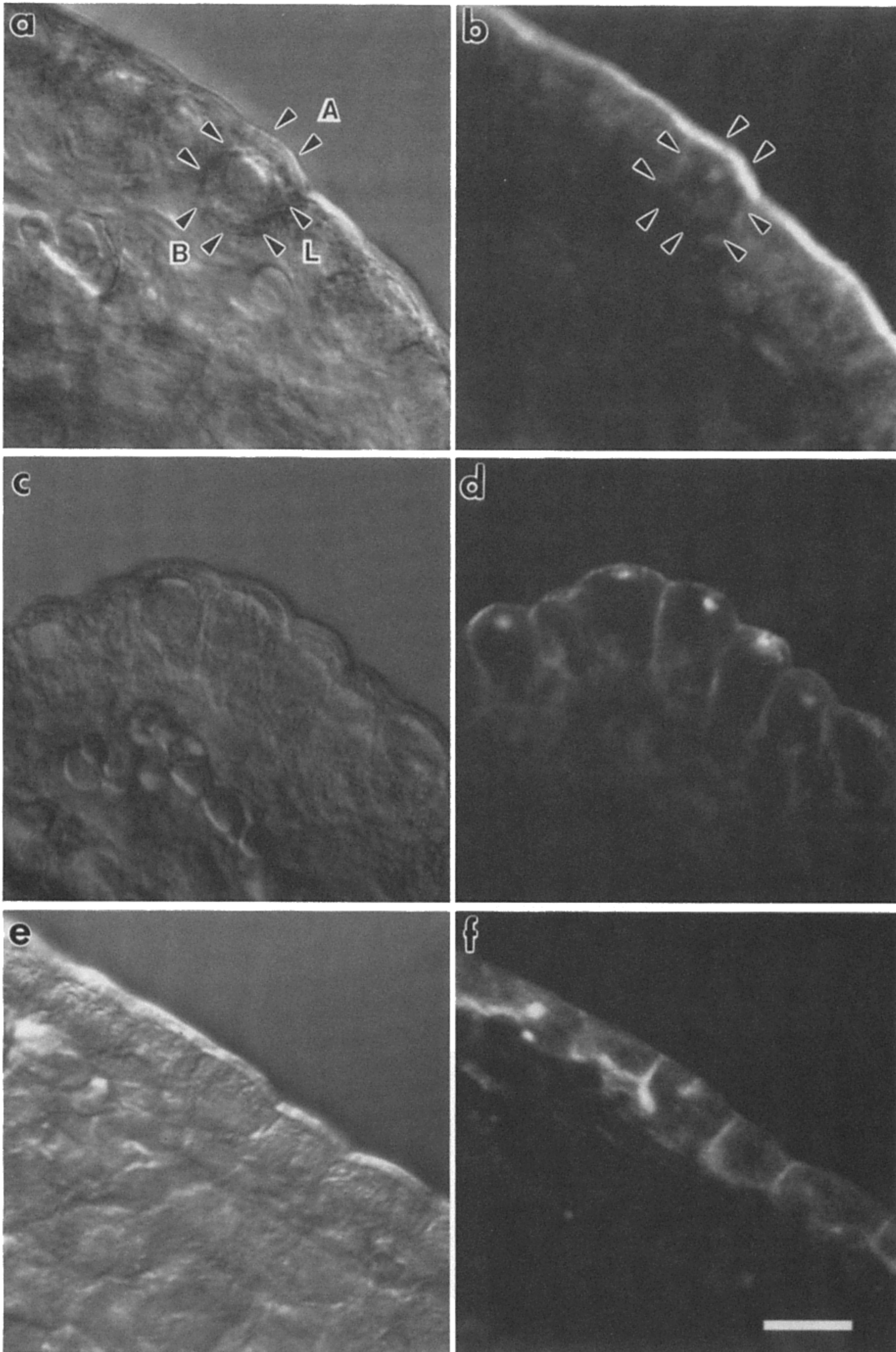


Figure 3. Distribution of Na^+,K^+-ATPase, fodrin, and adducin by laser scanning immunofluorescence confocal microscopy of rat choroid plexus epithelium. Choroid plexus was stained with antisera to Na^+,K^+-ATPase (*b*), fodrin (*d*), and adducin (*f*). Corresponding DIC images are shown (*a*, *c*, and *e*). Arrowheads delimit the boundary of an epithelial cell, marking the apical (*A*), lateral (*L*), and basal (*B*) membrane domains. Bar, 10 μm.

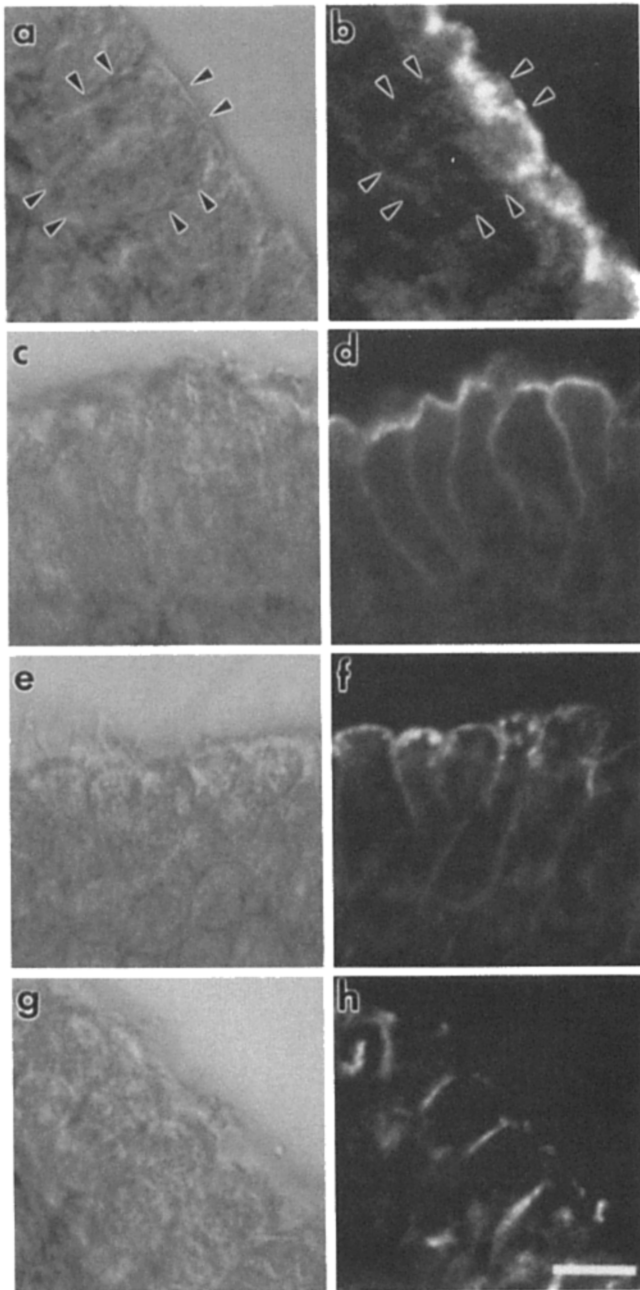


Figure 4. Distribution of Na^+,K^+-ATPase, fodrin, ankyrin, and adducin by laser scanning immunofluorescence confocal microscopy of chicken choroid plexus epithelium. Choroid plexus was stained with antisera to Na^+,K^+-ATPase (*b*), fodrin (*d*), ankyrin (*f*), and adducin (*h*). Corresponding DIC images are shown (*a*, *c*, *e*, and *g*). Arrowheads delimit the boundary of an epithelial cell, marking the apical, lateral, and basal membrane domains. Bar, 10 μm.

3 and 4 and see below), non-epithelial cells probably contribute only a small fraction of the total amounts of these proteins in the analysis. After extraction, soluble and insoluble fractions were partitioned by centrifugation. Equal proportions by volume of each fraction were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against membrane and membrane-cytoskeletal proteins. The relative amounts of each protein in the Triton X-100 soluble

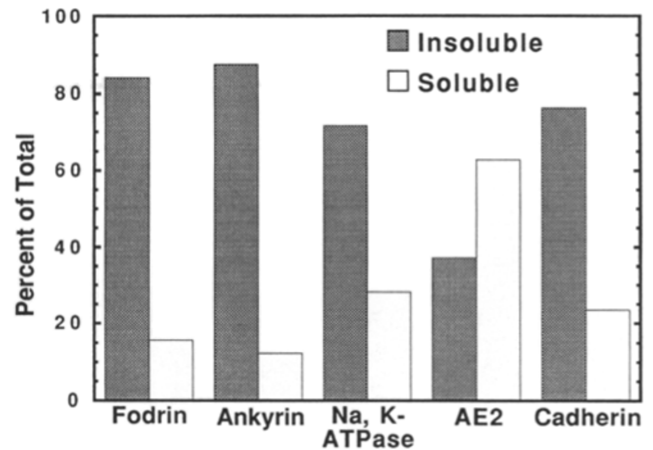


Figure 5. Triton X-100 solubility of membrane and membrane-cytoskeletal proteins in the chick choroid plexus. Choroid plexus was dissected and extracted with 0.5% Triton X-100 in isotonic buffer. Soluble and insoluble fractions were separated by centrifugation and analyzed by immunoblotting with specific antisera raised to Na^+,K^+-ATPase, fodrin, ankyrin, and cadherin cytoplasmic domain (see Materials and Methods). Autoradiograms were quantified by scanning densitometry. These data are the product of one experiment, but the experiment was repeated three times and was reproducible. Proportions of the different proteins found in the two fractions are shown as a percentage of the total of both fractions.

and insoluble fractions were determined by scanning densitometry of the resulting autoradiograms (Fig. 5). Results showed that 84% of fodrin and 87% ankyrin were resistant to extraction in buffers containing Triton X-100. Seventy two percent of Na^+,K^+-ATPase was also resistant to extraction from the cells under these conditions.

Previous studies have demonstrated that an isoform of the anion exchanger is expressed in the choroid plexus epithelium, termed AE2 (Lindsey et al., 1990). AE2 was localized to the basal and lateral plasma membrane domains in both the rat (Lindsey et al., 1990) and chick choroid plexus epithelial cells (Fig. 6). In contrast to Na^+,K^+-ATPase, we found that 37% of AE2 was insoluble in buffers containing Triton X-100 in the choroid plexus (Fig. 5).

Sucrose Density Gradient Analysis of Membrane and Membrane-Cytoskeletal Proteins Demonstrates a Multi-Protein Complex

That a significant proportion of Na^+,K^+-ATPase, fodrin, and ankyrin are resistant to extraction from the choroid plexus suggests that these proteins form a membrane-cytoskeletal complex similar to that described in MDCK cells. Because it is difficult to dissociate native complexes from the detergent-insoluble membrane-cytoskeletons (the insoluble fraction can be dissociated in buffers containing high salt, urea, or SDS which do not preserve membrane-cytoskeletal protein–protein interactions; see Nelson and Hammerton, 1989), we looked for Triton X-100 soluble protein complexes. This approach is supported by our previous observation that the Triton X-100 soluble fraction from MDCK cells contains a higher molecular weight complex of fodrin tetramers, ankyrin with either Na^+,K^+-ATPase or

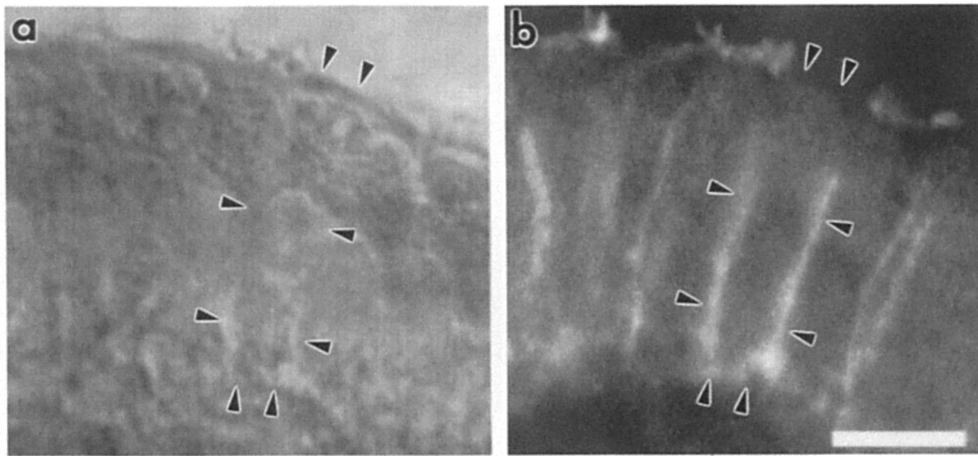


Figure 6. Distribution of AE2 by laser scanning confocal immunofluorescence microscopy in the chicken choroid plexus. (*a*) DIC image of choroid plexus epithelium, and (*b*) anti-AE2 staining showing the basal-lateral plasma membrane distribution of the protein. Arrowheads delimit the boundary of an epithelial cell, marking the apical, lateral, and basal membrane domains. Bar, 10 μm.

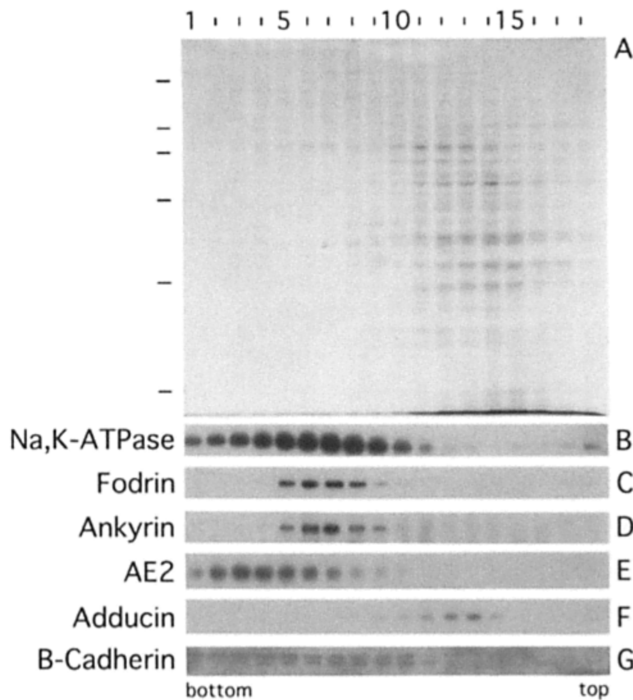


Figure 7. Sucrose density gradient analysis of membrane and membrane-cytoskeletal proteins extracted from chick choroid plexus. Sucrose (5–20%) density gradients were fractionated (18 fractions; fraction 1 is the bottom of the gradient and fraction 18 is the top of the gradient), and fractions were separated in SDS-polyacrylamide gels. Gels were stained with Coomassie blue (*A*), or immunoblotted with specific antisera raised to: (*B*) Na$^+$,K$^+$-ATPase; (*C*) fodrin; (*D*) ankyrin; (*E*) AE2; (*F*) adducin; and (*G*) cadherin. Two protein bands were recognized by the cadherin antiserum. One of these (faster migrating) bands had the same relative electrophoretic mobility as B-cadherin (M_r = 120 kD). The other band may represent a precursor to B-cadherin or another cadherin species. This 120-kD cadherin was used for the quantification in Fig. 8 (see text). Sucrose gradient analysis was performed over five times each for chicken and bovine (data not shown) tissues, and were very reproducible. Data from one representative gradient are shown so the sedimentation profiles for different proteins are directly comparable. SDS-PAGE molecular weight markers: myosin, 205 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; BSA, 66 kD; ovalbumin, 45 kD; and carbonic anhydrase, 29 kD. S value marker proteins: apoferritin (17.2 S), peak fraction 2; catalase (11.35 S), peak fraction 6; aldolase (7.35 S), peak fraction 12; BSA (4.6 S), peak fraction 15.

E-cadherin (Nelson and Hammerton, 1989; Nelson et al., 1990). The sedimentation profile and electrophoretic mobility on nondenaturing polyacrylamide gels of these protein complexes were distinctly different from those of individual, purified proteins, but were similar to the properties of complexes of ankyrin, fodrin, and Na$^+$,K$^+$-ATPase reconstituted in vitro (Nelson and Veshnock, 1987*a*; Nelson and Hammerton, 1989; Nelson et al., 1990).

To directly assess whether solubilized Na$^+$,K$^+$-ATPase, fodrin, and ankyrin are in a protein complex, we separated the Triton X-100 soluble fraction from the choroid plexus on 5–20% linear sucrose gradients. The sedimentation profile of individual proteins in the sucrose gradients was determined by SDS-PAGE of each fraction of the gradient followed by immunoblotting with specific antisera (Fig. 7).

The Coomassie blue-stained gel of the fractions from sucrose gradients (Fig. 7 *A*) shows that the majority of proteins extracted from the chick choroid plexus sediment in fractions near the top of the gradient (fraction numbers >11). Immunoblotting demonstrated that Na$^+$,K$^+$-ATPase sedimented in a peak between fractions 5 and 8 (Figs. 7 *B* and 8 *A*). A shoulder on the peak of Na$^+$,K$^+$-ATPase sedimented in the more dense fractions; this may represent protein not assembled with the membrane-cytoskeleton since we have observed that purified Na$^+$,K$^+$-ATPase may sediment in this region of the gradient (data not shown). By comparison with the sedimentation profiles of standard proteins of known S values, we estimated that the peak of Na$^+$,K$^+$-ATPase sedimented at ∼10.5 S. Previous studies also showed that purified Na$^+$,K$^+$-ATPase has a distinctly different sedimentation profile (Nelson and Veshnock, 1987*a*; Nelson and Hammerton, 1989).

Analysis of the sedimentation profiles of fodrin and ankyrin also revealed single, sharp peaks of both proteins between fractions 5–8 (∼10.5 S) that overlapped the peak of Na$^+$,K$^+$-ATPase (Fig. 7, *C* and *D* and Fig. 8 *B*). In contrast with the sedimentation of these protein complexes, purified spectrin and ankyrin sediment at ∼9.5 S and ∼7.5 S, respectively (Nelson and Hammerton, 1989).

We investigated whether other membrane and membrane-cytoskeletal proteins cosediment with the Triton X-100 soluble fraction of fodrin and ankyrin from the choroid plexus. We compared the sedimentation profiles of AE2, ankyrin, and fodrin in the sucrose gradients. By analogy to AE1, AE2 may be bound to ankyrin and the membrane-cytoskeleton (see Introduction). AE2 sedimented as a single, sharp peak

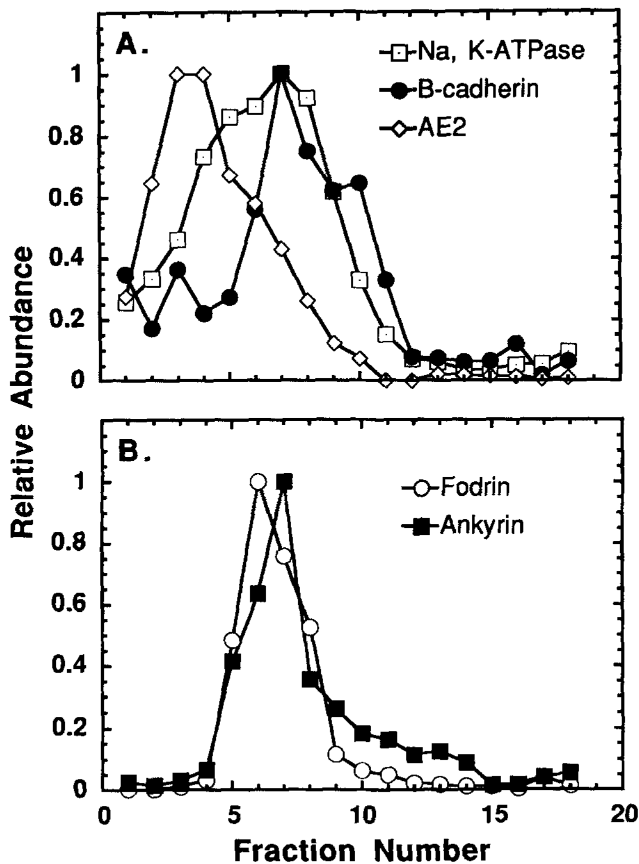


Figure 8. Sucrose gradient sedimentation profiles of membrane and membrane-cytoskeletal proteins extracted from chick choroid plexus. Immunoblots presented in Fig.7 were quantitated by scanning densitometry. The relative abundance of each protein in each fraction (arbitrary units) is plotted against the fraction number (18 fractions; fraction 1 is the bottom of the gradient and fraction 18 is the top of the gradient). (*A*) Na^+,K^+-ATPase, cadherin, and AE2 sedimentation profiles. (*B*) The distribution of fodrin and ankyrin in sucrose gradients. Fodrin, ankyrin, Na^+,K^+-ATPase, and cadherin cosediment at ~10.5 S; a second peak (~8.5 S) of cadherin may represent a fraction of cadherin that was not assembled with fodrin and ankyrin (see text for discussion). The major peak distributions of AE2 and adducin (not shown) did not cosediment with the major peaks of ankyrin and fodrin. Sucrose gradient analysis was performed over five times each for chicken and bovine (data not shown) tissues, and were very reproducible. Data from one representative gradient are shown so the sedimentation profiles for different proteins are directly comparable.

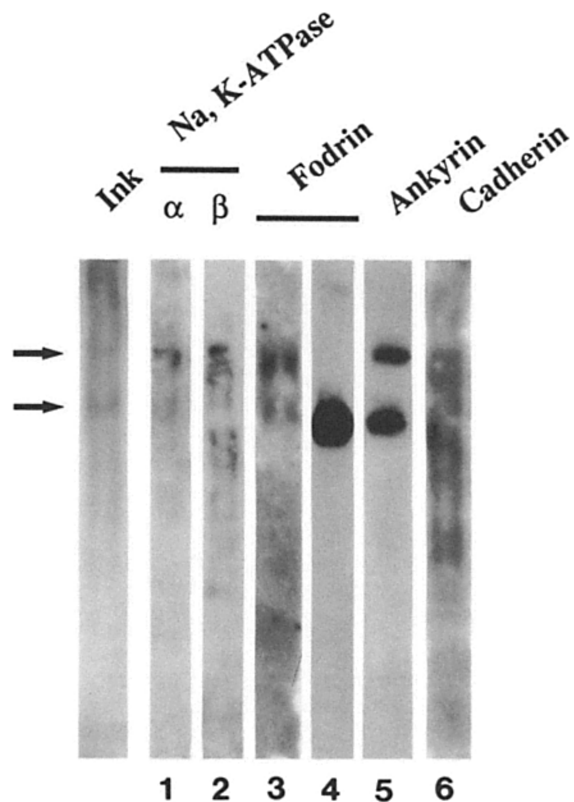


Figure 9. Nondenaturing polyacrylamide gel analysis of protein complexes containing Na^+,K^+-ATPase, fodrin, ankyrin, and cadherin. Peak sucrose gradient fractions of Na^+,K^+-ATPase, fodrin, ankyrin, and cadherin (fractions 7 and 8; see Fig. 7) were separated in 2–4% polyacrylamide nondenaturing gels and transferred to nitrocellulose (see Materials and Methods). India ink stained filters (*ink*) revealed two protein bands which comigrated with complexes of fodrin, ankyrin, and integral membrane proteins from MDCK cells (*arrows* indicate the migration of the two complexes observed by India ink staining). Immunoblotting was performed on identical transfers using specific antisera against Na^+,K^+-ATPase α- (lane *1*) and β- (lane *2*) subunits, fodrin (lanes *3* and *4*), ankyrin (lane *5*), and cadherin (lane *6*).

of protein between fractions 2 and 5 (~15 S); we did not detect a peak of AE2 that cosedimented with ankyrin and fodrin, although there was a shoulder on the AE2 sedimentation profile that coincided with the peaks of ankyrin and fodrin (Figs. 7 *E* and 8 *A*).

Adducin, a protein which forms a ternary complex with spectrin and actin and regulates membrane-cytoskeletal assembly in erythroid and nonerythroid cells (Gardner and Bennett, 1987; Mische et al., 1987; Bennett et al., 1988), colocalized with fodrin and ankyrin at the lateral membrane of the chicken choroid plexus (see Fig. 4 *C*). The sedimentation profile of extracted adducin revealed a single peak of protein between fractions 12 and 13 (~7 S) that was well separated from the fractions containing Na^+,K^+-ATPase, ankyrin, and fodrin (Fig. 7 *F*). Analysis of the sedimentation profile of adducin extracted from MDCK cells also revealed a similar difference in the sedimentation profiles of adducin and ankyrin/fodrin (Nelson, W. J., unpublished results). At present we do not know whether the conditions used to extract protein complexes resulted in complete dissociation of adducin, or whether there is a pool of unassembled adducin.

Nondenaturing Polyacrylamide Gel Electrophoresis of the 10.5 S Sucrose Gradient Fraction Demonstrates the Presence of a Complex of Na^+,K^+-ATPase, Fodrin, and Ankyrin

To further examine the association of membrane-cytoskeletal proteins and Na^+,K^+-ATPase in the choroid plexus, we separated the sucrose gradient fractions containing fodrin, ankyrin, and Na^+,K^+-ATPase in nondenaturing polyacrylamide gels. Proteins were transferred to nitrocellulose. Two major protein bands were detected by staining with India ink (Fig. 9); the relative migration of these protein bands in the non-denaturing gel is closely similar to two protein complexes extracted from MDCK cells and separated by identical procedures (see below). Individual proteins were identified by immunoblotting; differences in antibody binding

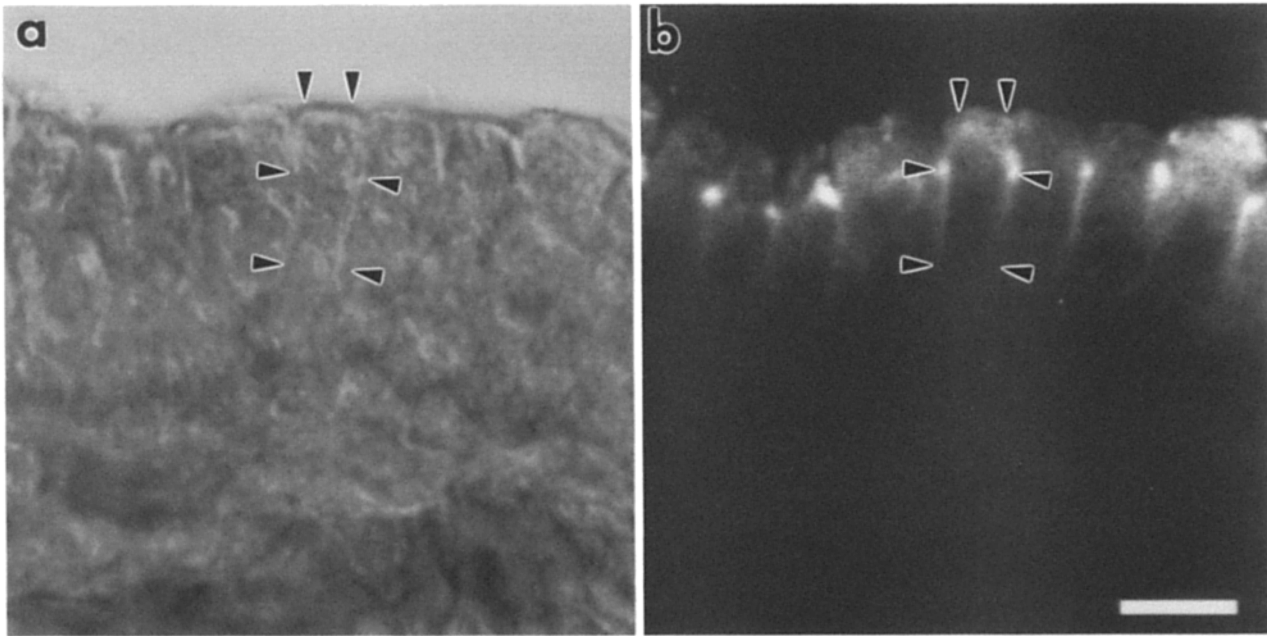


Figure 10. Subcellular distribution of cadherin in choroid plexus epithelium. Choroid plexus was stained with antiserum raised to the COOH-terminal, cytoplasmic domain of E-cadherin which recognized B-cadherin. Using laser scanning immunofluorescence microscopy, cadherin staining was observed at regions of cell–cell contact in chick (*b*) choroid plexus epithelial cells. Note the bright spot of fluorescence at the apico-lateral junction (presumably adherens junction). (*a*) Corresponding DIC image of fluorescence image in *b*. Arrowheads delimit the boundary of an epithelial cell, marking the apical and lateral membrane domains. Bar, 10 μm.

were not due to different protein loadings but represent different antibody affinities. When immunoblots were probed with antibodies to AE2, no protein bands were detected (data not shown), confirming that AE2 was separate from the ankyrin/fodrin complex.

Replicate immunoblots were probed with antisera specific for either the α- or β-subunit of Na^+,K^+-ATPase. Results showed that the slower migrating of the two protein bands reacted with the antibodies (Fig. 9, lanes *1* and *2*). Analysis of immunoblots probed with antisera specific for either fodrin or ankyrin revealed that both protein bands reacted (Fig. 9, lanes *3* and *5*). In some experiments, we detected fodrin immunoreactivity in only the faster migrating of the two protein bands (Fig. 9, lane *4*); the reason for this difference is not known, but may be due to lability of the ankyrin/fodrin interaction under these extraction and separation conditions.

Together, these studies indicate that Na^+,K^+-ATPase, ankyrin, and fodrin are present in a membrane-cytoskeletal complex. It is noteworthy that previous studies showed that purified Na^+,K^+-ATPase, ankyrin, and fodrin had significantly different electrophoretic mobilities than this membrane-cytoskeleton complex in nondenaturing polyacrylamide gels under identical separation conditions (Nelson and Veshnock, 1987*a*; Nelson and Hammerton, 1989).

Expression of Cadherins in the Choroid Plexus Epithelium and Association with the Membrane-Cytoskeleton

The above results provide evidence that Na^+,K^+-ATPase is in a membrane-cytoskeletal protein complex with ankyrin and fodrin in the choroid plexus epithelium, and that the characteristics of this complex are closely similar to those described for a membrane-cytoskeletal complex of these proteins in renal epithelial cells. Previous studies in MDCK cells showed that membrane-cytoskeleton assembly and Na^+,K^+-ATPase localization were regulated by E-cadherin-mediated cell–cell contacts. Therefore, we sought to investigate cadherin expression and distribution in the choroid plexus epithelium to determine the role of cadherins in the organization of the membrane-cytoskeleton and Na^+,K^+-ATPase.

The complement of cadherins expressed in the choroid plexus has not been investigated in detail. We raised a polyclonal antiserum against the COOH-terminal cytoplasmic domain of E-cadherin, which has extensive identity with nearly all classical cadherins (Geiger and Ayalon, 1992; Kemler, 1992). This antiserum recognizes different cadherins from many sources (see Fig. 1 and Materials and Methods). Using the antiserum against the cytoplasmic domain of E-cadherin, we analyzed protein extracts to determine cadherin expression and distribution in the choroid plexus. Two protein bands were recognized (Fig. 1 *A*, lane *2*; Fig. 7 *G*). Neither of these protein bands reacted with antisera specific for either E- or N-cadherin (data not shown). One of the reactive protein bands had the same electrophoretic mobility as B-cadherin (M_r = 120 kD), a member of the cadherin superfamily that was shown recently to be expressed at high levels in the chick choroid plexus (Napolitano et al., 1991; Sorkin et al., 1991). The other protein band may represent a precursor of the mature B-cadherin, or another cadherin species. To demonstrate directly that the antiserum against the cytoplasmic domain of E-cadherin recognized B-cadherin, we probed immunoblots of proteins extracted from B-cadherin transfected fibroblasts; the results showed that the antiserum reacted with B-cadherin (data not shown). In addition, this antiserum localized cadherin molecules to the lateral plasma membrane in

choroid plexus epithelial cells (see below), similar to the distribution of B-cadherin revealed by a B-cadherin specific monoclonal antibody (Murphy-Erdosh, C., E. W. Napolitano, and L. F. Reichardt, manuscript in preparation). Taken together these results suggest that the predominant cadherin expressed in the choroid plexus epithelium is B-cadherin, and that another cadherin, which is neither E- or N-cadherin, may also be present.

Immunofluorescence analysis of chick choroid plexus using the antiserum against the cytoplasmic domain of E-cadherin demonstrated that cadherin localized to lateral plasma membranes (Fig. 10), and that it colocalized with the membrane-cytoskeleton (Figs. 3, *c* and *d* and 4, *c* and *d*). Although cadherin staining was detected along the length of the lateral membrane, a greater intensity of cadherin staining was detected at the apex of the lateral membrane in the region of the zonula adherens (compare with Fig. 2), similar to the distribution of cadherins in other epithelial cells (Boller et al., 1985). Seventy six percent of cadherins were resistant to extraction from chick choroid plexus in buffers containing Triton X-100 (Fig. 5). This result is consistent with the possibility that cadherins expressed in the choroid plexus epithelium, like E-cadherin, may associate with the membrane-cytoskeleton.

We analyzed the sedimentation profile of the Triton X-100 soluble pool of cadherins in sucrose gradients (Fig. 7 *G*). Two protein bands were detected by SDS-PAGE of sucrose gradient fractions (see also Fig. 1). A broad distribution of B-cadherin (M_r = 120 kD, faster migrating band in Fig. 7 *G*) between fractions 5 and 11 was observed, although the peak fraction (#7; ~10.5 S) cosedimented with the peak of ankyrin (Figs. 7 *G* and 8 *A*). The quantification of the 120-kD protein band is shown in Fig. 8 *A*. Analysis of cadherins from fraction 7 of the sucrose gradient in nondenaturing polyacrylamide gels revealed a more complex pattern of protein bands than those detected with Na^+,K^+-ATPase, ankyrin or fodrin antibodies; one of the reactive bands comigrated with the slow migrating protein band which contains fodrin, ankyrin, and Na^+,K^+-ATPase (Fig. 9, lane *6*).

Different Abilities of B- and E-Cadherin to Induce the Localization of Na^+,K^+-ATPase and Fodrin in Transfected Fibroblasts

Previous experiments showed that expression of E-cadherin in transfected fibroblasts induces the localization of Na^+, K^+-ATPase and fodrin to regions of cell–cell contact (McNeill et al., 1990). We used the same assay to examine whether B-cadherin also regulates Na^+,K^+-ATPase distribution. E-cadherin transfected cells showed colocalization of Na^+,K^+-ATPase and fodrin with E-cadherin at regions of cell–cell contact (Fig. 11 *A a*, *b*, and *c*), as previously observed (McNeill et al., 1990). B-cadherin was detected at regions of cell–cell contact in the B-cadherin transfected fibroblasts (Fig. 11 *A d*). In contrast to the E-cadherin transfected cells, however, Na^+,K^+-ATPase and fodrin did not colocalize with B-cadherin, even in densely seeded cell cultures (Fig. 11 *A e* and *f*); both proteins were diffusely distributed similar to that seen in untransfected fibroblasts (Fig. 11 *A g–i*).

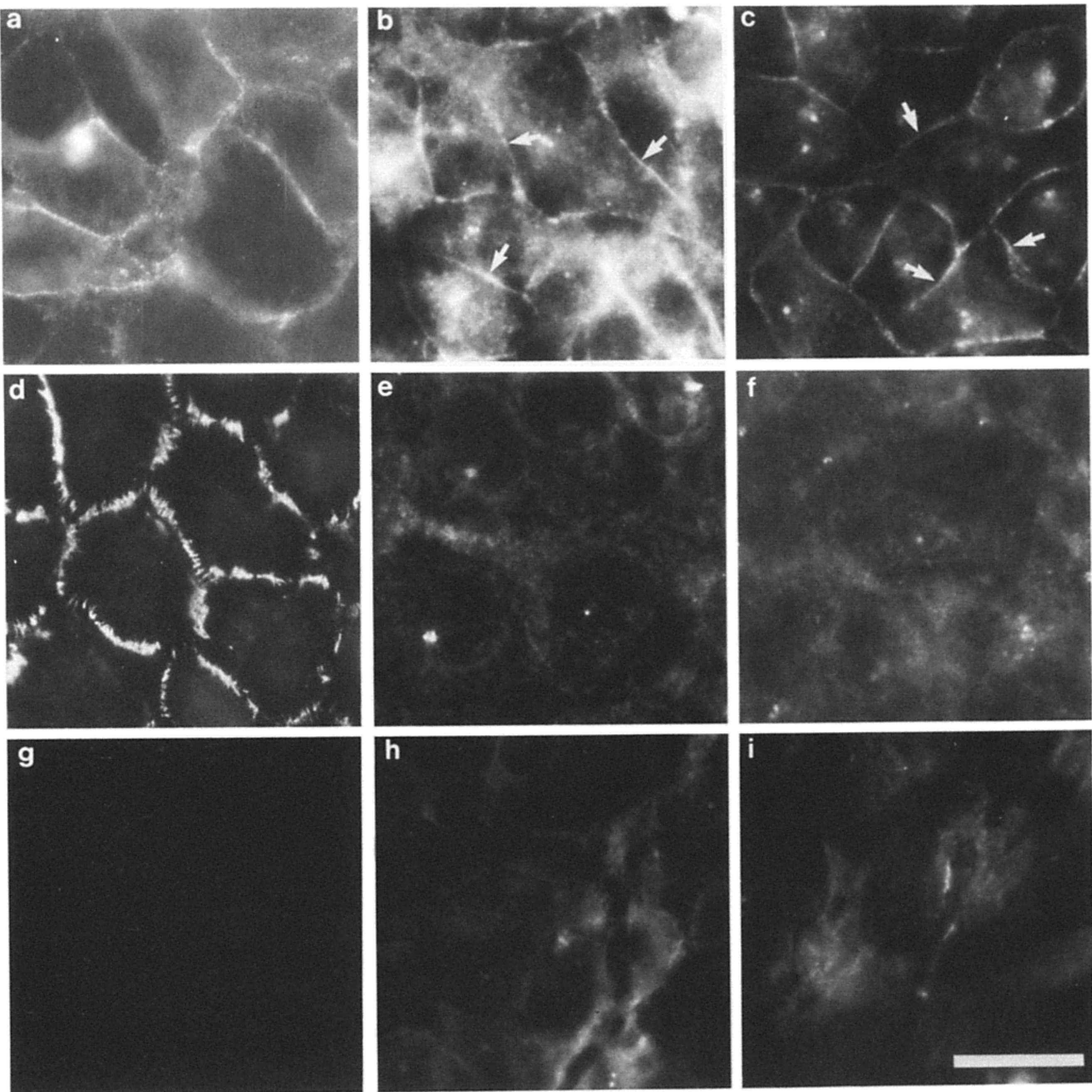
It should be noted that the different clones of L cells were not selected by morphological criteria, but were randomly taken from clones of cells resistant to G418. In addition, we have not detected accumulation of Na^+,K^+-ATPase at lateral membranes (cell–cell contacts) of several L cell clones, not expressing exogenous cadherin, beyond that expected for a randomly dispersed cell surface protein on two closely apposed membranes in these superconfluent cultures (for example, see Fig. 11 *A e*). In previous studies, we found that the distributions of the major histocompatability antigen and cell surface proteins detected with FITC-conjugated WGA were unaffected by expression of cadherin (McNeill et al., 1990), indicating that effects of cadherin expression on Na^+,K^+-ATPase distribution are specific. Finally, accumulation of Na^+,K^+-ATPase to lateral membranes of L cells did not occur if extracellular Ca^{++} was removed from the medium, thereby disrupting cadherin-mediated cell adhesion, or if the cytoplasmic binding site for cytoskeletal (catenins) on E-cadherin was deleted (McNeill et al., 1990).

The steady state levels of cadherin expression were determined in the transfected fibroblasts to examine whether differences in E- and B-cadherin induction of Na^+,K^+-ATPase redistribution were due to lower levels of B-cadherin expression. Rather than compare levels of E- and B-cadherin with specific antibodies which could recognize the proteins with different efficiencies, we used our antibody raised against the E-cadherin cytoplasmic domain which is 88% identical to B-cadherin (Fig. 11 *B*); we expect that this antibody would recognize E- and B-cadherin with similar affinities. Immunoblots using antiserum against the COOH-terminal cytoplasmic domain of E-cadherin showed that B-cadherin expression was 85% of the E-cadherin expression in the transfected fibroblast lines, and that no cadherin was detected in the untransfected fibroblasts. In addition, we detected similar levels of catenins coimmunoprecipitated with E- and B-cadherin from transfected fibroblasts (data not shown). These results show that the different functional property of E- and B-cadherin to induce Na^+,K^+-ATPase redistribution is not due to differences in either levels of expression of cadherins, or affinity of the two cadherins for catenins, and therefore may represent an intrinsic difference in the properties of each cadherin.

Discussion

The choroid plexus epithelium plays a central role in the secretion of cerebrospinal fluid by vectorial transport of ions and solutes from the blood supply to the brain ventricles. In contrast, absorptive epithelia that are found in the kidney nephron transport ions and solutes in the opposite direction, from the lumen of the tubule to the blood supply. To perform these opposing vectorial transport functions, proteins must be restricted to different plasma membrane domains in these two types of epithelia. An important example of this requirement is Na^+,K^+-ATPase.

In the choroid plexus epithelium (Wright, 1972; Masuzawa et al., 1984, 1985; Seigel et al., 1984; Ernst et al., 1986; Spector and Johanson, 1989) and in the retinal pigmented epithelium (Ghosh et al., 1990), Na^+,K^+-ATPase is restricted to the apical membrane domain. In contrast, Na^+,K^+-ATPase is localized at the basal-lateral plasma membrane in absorptive epithelia, such as renal and intestinal epithelia (Almers and Stirling, 1984). However, during early development of renal tubules, Na^+,K^+-ATPase is transiently localized to the lateral and apical membrane domains



B

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E-Cadherin  RRRTVVKEPLLPPDDDTRDNVYYYDEEGGGEEDQDFDLSQLHRGLDARPEVTRNDVAPTLMSVPQYRPRPANPDEIGNFIDE  658
B-Cadherin  ---K-------L-E------IF--G----------Y---------------I------P--AA-------------------  660

E-Cadherin  NLKAADSDPTAPPYDSLLVFDYEGSGSEAASLSSLNSSESDQDQDYDYLNEWGNRFKKLADMYGGGEDD   729
B-Cadherin  ------T---------------G----T--------A---------------------EL------EE  728
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Figure 11. E- and B-cadherin have different abilities to nucleate Na^+,K^+-ATPase localization to cell–cell contacts in transfected fibroblasts. (*A*) E-cadherin transfected L cells (*a*, *b*, and *c*), B-cadherin transfected L cells (*d*, *e*, and *f*), or untransfected control L cells (*g*, *h*, and *i*) were compared for their ability to nucleate Na^+,K^+-ATPase and fodrin relocalization to cell–cell contacts. Each cell type was stained for cadherin (*a*, *d*, and *g*), Na^+,K^+-ATPase (*b*, *e*, and *i*), or fodrin (*c*, *f*, and *i*) (for details, see Materials and Methods). Arrows

(Minuth et al., 1987; Avner et al., 1992). Also, Na^+,K^+-ATPase is found in apical membranes of cells in cystic kidney tubules of the *cpk* mouse mutant and in human patients with congenital polycystic kidney disease (Wilson et al., 1991; Avner et al., 1992). It has been proposed that localization of Na^+,K^+-ATPase to the apical membrane of renal cells in these circumstances contributes to an increase in lumen volume either directly by causing ion and solute secretion into the lumen, or indirectly by decreasing reabsorption of ions and solutes from the lumen. Together, these observations indicate that there is dynamic regulation of Na^+,K^+-ATPase distribution in simple epithelial cells, and emphasize the importance of Na^+,K^+-ATPase location in determining the direction of vectorial transport of ions and solutes across the epithelium. Our current studies have begun to analyze mechanisms used to generate the opposite polarities of Na^+,K^+-ATPase in secretory and absorptive simple epithelia.

The Membrane-Cytoskeletal Complex of the Choroid Plexus Includes Na^+,K^+-ATPase

Immunofluorescence microscopy revealed that Na^+,K^+-ATPase colocalized on the apical plasma membrane of chick and rat choroid plexus with fodrin (and ankyrin in chick; data not shown). That fodrin, ankyrin, and Na^+,K^+-ATPase in the choroid plexus may comprise a multi-protein complex is supported by three results. First, Na^+,K^+-ATPase is relatively resistant to extraction from the choroid plexus in buffers containing Triton X-100 (72%). The proportion of Na^+,K^+-ATPase the remained insoluble under these extraction conditions was similar to those of ankyrin and fodrin (84 and 87%, respectively). These data are consistent with Na^+,K^+-ATPase being associated with the Triton X-100 insoluble cytoskeleton. Another integral membrane protein, AE2, was localized to the basal-lateral membrane of the chick choroid plexus. However, only a small fraction of AE2 was found to be insoluble from choroid plexus under the same extraction conditions (37%). Subsequent fractionation of extracted AE2 in sucrose gradients did not reveal compelling evidence for membrane-cytoskeletal association of AE2 (see below).

Second, to provide more direct evidence for the presence of a membrane-cytoskeletal complex comprising ankyrin, fodrin, and Na^+,K^+-ATPase, proteins extracted from the cells in buffers containing Triton X-100 were analyzed by sucrose density gradients. Results showed that Na^+,K^+-ATPase cosedimented with fodrin and ankyrin in a peak at 10.5 S; these proteins were well separated from the majority of proteins solubilized from choroid plexus which sedimented at ~5 S, and had sedimentation profiles distinctly different from those of individual, purified proteins (Nelson and Veshnock, 1987*b*; Nelson and Hammerton, 1989; Nelson et al., 1990).

Third, cosedimenting proteins from sucrose gradients were subjected to further separation by electrophoresis in polyacrylamide gels under nondenaturing conditions. After transfer of proteins from the gel to nitrocellulose, we detected two distinct protein bands by staining with India ink. The electrophoretic mobility of these protein bands is closely similar to that of protein bands identified from MDCK cells after the same series of separations in sucrose gradient and non-denaturing polyacrylamide gels (Nelson and Hammerton, 1989; Nelson et al., 1990).

Analysis of protein distributions in the non-denaturing gel revealed that the slower migrating of the two protein bands reacted with antibodies specific for the α- and β-subunits of Na^+,K^+-ATPase, fodrin, and ankyrin. These results indicate strongly that the cosedimentation of these proteins in the sucrose gradient reflected the presence of a high molecular weight complex of fodrin, ankyrin, and Na^+,K^+-ATPase. The faster migrating of the two protein bands reacted with ankyrin and fodrin antibodies, but not with antibodies specific for either the α- or β-subunit of Na^+,K^+-ATPase. In some experiments, we detected fodrin staining predominantly in the faster migrating protein complex, suggesting that the membrane-cytoskeletal complex containing Na^+, K^+-ATPase is labile under these extraction and separation conditions. Again, the electrophoretic mobilities of these protein complexes were distinctly different from those of purified Na^+,K^+-ATPase, ankyrin, and fodrin (Nelson and Veshnock, 1987*a*; Nelson and Hammerton, 1989; Nelson et al., 1990).

Previous studies of membrane-cytoskeletal protein complexes isolated from MDCK cell extracts (Nelson and Hammerton, 1989; Nelson et al., 1990), or reconstituted in vitro from purified proteins (Nelson and Veshnock, 1987*a*) have characterized these two protein bands in non-denaturing gels as containing fodrin tetramers bound to ankyrin (faster migrating complex), and fodrin tetramers and ankyrin bound to integral membrane proteins (slower migrating complex). By analogy, we suggest that the slower migrating protein complex isolated from choroid plexus contains fodrin tetramers and ankyrin complexed with Na^+,K^+-ATPase.

Our results on the distribution and protein interactions of AE2 represents an interesting contrast to the findings of Na^+,K^+-ATPase. AE2 is a member of the anion exchanger family of proteins (Kopito, 1990); AE1 is expressed in erythrocytes and is the principle binding site for ankyrin to the plasma membrane (Hargreaves et al., 1980). Sequence analysis of AE2 revealed a high degree of identity with AE1 except for an additional COOH-terminal domain not found in AE1 (Lindsey et al., 1990). AE2 is localized to the basal-lateral membrane domain of choroid plexus epithelium, and is absent from the apical membrane (Lindsey et al., 1990). Hence, AE2 colocalizes with a subpopulation of ankyrin and fodrin in choroid plexus. However, our studies revealed that, in contrast to Na^+,K^+-ATPase, AE2 was susceptible to extraction from choroid plexus in buffers containing Triton X-100. Furthermore, when we analyzed the sedimentation profile of the solubilized AE2 in sucrose gradients, we found that it sedimented in a peak distinct from those of ankyrin

denote regions of staining of Na^+,K^+-ATPase and fodrin at cell–cell contacts in E-cadherin expressing fibroblasts. Bar, 50 μm. (*B*) Comparison of the cytoplasmic domain amino acid sequences of E- and B-cadherin (Ringwald et al., 1991; Napolitano et al., 1991). B-cadherin sequences which are identical to those of E-cadherin are represented by dashes and differences are shown. The two cadherin cytoplasmic domains are 88% identical and the differences are distributed over the length of the cytoplasmic domain.

and fodrin. However, there was a shoulder on the major AE2 peak which overlaps the sedimentation positions of ankyrin and fodrin which was consistently observed. Therefore, we cannot rule out at present the possibility that AE2 interacts with other isoforms of the membrane-cytoskeleton that have sedimentation characteristics different from those of fodrin and ankyrin identified here, or that the binding of AE2 to the membrane-cytoskeleton is labile under these extraction conditions. However, interactions of this type appear to be different from those involving Na^+,K^+-ATPase.

Generation of a Polarized Distribution of Na^+,K^+-ATPase: a Membrane-Cytoskeletal Complex May Retain the Sodium Pump in the Correct Membrane Domain

Polarized distributions of Na^+,K^+-ATPase may be generated by different mechanisms that are not mutually exclusive: targeting of the protein from the Golgi complex to the appropriate plasma membrane; retention of protein at the correct membrane domain by interaction with the membrane-cytoskeleton, and removal of improperly localized protein by endocytosis and degradation (Nelson, 1992). Recent studies have demonstrated that Na^+,K^+-ATPase may be delivered directly to the basal-lateral membrane in thyroid follicle cells (Zurzolo and Rodriguez-Boulan, 1983) and in strain II MDCK cells (Gottardi and Caplan, 1993), but is delivered to both the apical and basal-lateral plasma membrane domains in another clone of MDCK cells (Hammerton et al., 1991; Siemers [Krzeminski] et al., 1993). However, in the latter case, Na^+,K^+-ATPase still developed a polarized distribution in the basal-lateral membrane domain. Analysis of the fate of newly-synthesized protein that arrived at both membrane domains revealed that the pool of protein in the apical membrane was rapidly degraded. In contrast, the pool of Na^+,K^+-ATPase in the basal-lateral membrane domain was retained, presumably by stable assembly with the membrane-cytoskeleton (Hammerton et al., 1991). Our finding that Na^+,K^+-ATPase is present in a high molecular weight complex with ankyrin and fodrin in choroid plexus, similar to that described in MDCK cells, indicates that Na^+,K^+-ATPase is retained in the apical membrane by interactions with the membrane-cytoskeleton.

In MDCK cells, assembly of the membrane-cytoskeleton and accumulation of Na^+,K^+-ATPase to the (basal-) lateral membrane domain is induced by E-cadherin-mediated cell–cell adhesion (Nelson et al., 1990). Direct evidence for this inductive role of E-cadherin was obtained by analyzing protein distributions in confluent monolayers of fibroblasts transfected with E-cadherin, or with E-cadherin that contained a deletion of the cytoplasmic domain that included the binding site for cytoskeletal proteins (McNeill et al., 1990). Immunofluorescence showed that Na^+,K^+-ATPase was specifically localized to regions of cell–cell contact in the cells containing E-cadherin, but not in cells expressing the mutant E-cadherin that had a truncated cytoplasmic domain. Significantly, the distribution of fodrin paralleled that of Na^+,K^+-ATPase in both cell types. Since Na^+,K^+-ATPase and E-cadherin have been shown to be associated with the membrane-cytoskeleton (ankyrin and fodrin) in MDCK cells (Nelson and Hammerton, 1989; Nelson et al., 1990), it was concluded that E-cadherin initiates assembly of the membrane-cytoskeleton at regions of cell–cell contact, and Na^+,K^+-ATPase becomes stabilized within this complex.

We did not detect E-cadherin expression in the choroid plexus. However, our analysis of the complement of cadherins, and a previous study (Napolitano et al., 1991), showed that a different member of the cadherin superfamily, termed B-cadherin, is the predominant cadherin expressed in the choroid plexus epithelium. We localized cadherin to the lateral membrane domain, and showed that it colocalized with a subset of the membrane-cytoskeleton. Cadherins were relatively resistant to extraction by Triton X-100 (76% insoluble), similar to that of Na^+,K^+-ATPase (72% insoluble). Direct demonstration of a membrane-cytoskeletal complex containing B-cadherin was obtained by sucrose gradient and nondenaturing PAGE. In sucrose gradients, a $\sim$10.5 S complex containing cadherins was detected, also similar to that of Na^+,K^+-ATPase. Cadherin reactivity in nondenaturing gels was complex. The high molecular weight complex that contained ankyrin and fodrin also reacted with antiserum against this cadherin. The additional reactivity suggests that a considerable amount of cadherin was either not complexed with these membrane-cytoskeletal proteins, or the membrane-cytoskeletal complex was labile under these extraction conditions.

That cadherins and Na^+,K^+-ATPase are each complexed with the membrane-cytoskeleton, but have different membrane domain distributions is strikingly different from the situation in absorptive epithelia where all these proteins colocalize. To interpret these results with regard to our current views on how epithelial cells generate polarized distributions of Na^+,K^+-ATPase (see above), we propose two possible mechanisms for generating apically localized Na^+,K^+-ATPase in the choroid plexus epithelium.

First, Na^+,K^+-ATPase may be exclusively delivered from the TGN to the apical plasma membrane domain. In this way the cell would bypass the cadherin membrane-cytoskeletal complex which might stabilize Na^+,K^+-ATPase at the lateral plasma membrane. Na^+,K^+-ATPase delivered to the apical plasma membrane may accumulate at this location due to assembly with the membrane-cytoskeleton. At present, we have no insight into the intracellular trafficking of proteins in the choroid plexus; in the absence of an established cell line or methods for primary culture of these cells, this is difficult to resolve. However, the fact that B-cadherin is localized to the (basal-) lateral membrane in all cell types where it is expressed, including absorptive epithelia (kidney and intestine; Murphy-Erdosh, C., E. W. Napolitano, and L. F. Reichardt, manuscript in preparation) and secretory epithelia (choroid plexus, this study), indicates that polarized distributions of proteins are not simply reversed in the choroid plexus relative to absorptive epithelia. Rather, the change in polarity seems to be restricted to a subset of proteins that includes Na^+,K^+-ATPase and perhaps other ion transporters (Wright, 1972), and components of the membrane-cytoskeleton.

It is significant that the same subunits of Na^+,K^+-ATPase (α1, β1) are expressed in both choroid plexus and renal epithelium (this study), and in the apical membrane of renal epithelia in polycystic kidney disease and ischemia (Molitoris, 1991; Wilson et al., 1991; Avner et al., 1992). This indicates that the generation of different Na^+,K^+-ATPase distributions is not simply due to unique apical or basal-

lateral sorting signals within the protein, and implies that the sorting machinery must accommodate plasticity in the delivery of Na^+,K^+-ATPase to either membrane domain.

Second, Na^+,K^+-ATPase may be delivered to both cell surface domains, but due to differences between the cadherin-associated membrane-cytoskeletal proteins of absorptive and secretory epithelial cells, Na^+,K^+-ATPase is not stabilized in the lateral membrane and is internalized. This is supported by the fact that in fibroblasts expressing B-cadherin, neither Na^+,K^+-ATPase nor fodrin are induced to accumulate at sites of cell–cell contact; in the presence of E-cadherin both of these proteins accumulate at contact sites in these cells, a process which is dependent on the presence of the complete E-cadherin cytoplasmic domain (McNeill et al., 1990). These results indicate that, despite having 88% amino acid identity in their cytoplasmic domain (Fig. 11 *B* and Napolitano et al., 1991), B- and E-cadherin differ in their respective abilities to nucleate membrane-cytoskeleton assembly and to induce reorganization of Na^+,K^+-ATPase. Considering that the sequence differences between B- and E-cadherin are distributed throughout the cytoplasmic domain (Fig. 11 *B*), it is remarkable that these two cadherins have different abilities to produce Na^+,K^+-ATPase polarity. Further studies are underway to identify specific domains of these cadherins and the protein–protein interactions required to generate these different functions.

In the context of cell–cell adhesion, the recent analysis of Na^+,K^+-ATPase in the retinal pigmented epithelium is noteworthy. In these cells, Na^+,K^+-ATPase colocalized with the membrane-cytoskeletal proteins ankyrin and fodrin; a complex comprising these proteins was demonstrated by chemical cross-linking (Gundersen et al., 1991). Although the distribution of Na^+,K^+-ATPase, ankyrin, and fodrin are similar in retinal pigmented epithelium and choroid plexus epithelium, the apical surface of the retinal pigmented epithelium is in direct cell–cell contact with the neural retina. Recent studies demonstrate that N-CAM, a non-cadherin cell adhesion system, is expressed in retinal pigmented epithelial cells in the apical plasma membrane and may interact with N-CAM expressed in the rod outer segments of photoreceptor cells (Gundersen et al., 1993). It is also possible that cadherins are expressed at the apical membrane of retinal pigmented epithelial cells and accumulation of Na^+, K^+-ATPase in apical membrane of the retinal pigmented epithelium may result from cadherin-induced Na^+,K^+-ATPase localization to a region of cell–cell contact with the neural retina.

In summary, our current studies define the organization of the choroid plexus epithelium with respect to the membrane-cytoskeleton and cell–cell contact for generating apical polarity of Na^+,K^+-ATPase. We find that the apically localized Na^+,K^+-ATPase and the lateral B-cadherin are assembled into multi-protein complexes with the membrane-cytoskeletal proteins fodrin and ankyrin. The membrane-cytoskeleton may function as a retention system for maintaining the polarized distributions of these integral plasma membrane proteins. We present the novel finding that despite the assembly of cadherins with the membrane-cytoskeleton, Na^+,K^+-ATPase does not become colocalized with the cadherins as was previously observed in kidney epithelial cells. We assert that our current data points to cell type specific mechanisms for developing cell surface polarity which would account for the generation of the opposite polarized distributions of Na^+,K^+-ATPase in secretory vs absorptive epithelia.

The authors wish to thank Dr. Timothy Ryan for assistance with confocal microscopy, and we thank Dr. Stephen Smith for the use of his confocal microscope. We are grateful to JoAnn Buchanan and Nifisa Ghori (Stanford University Cell Sciences Imaging Facility) for performing electron microscopy. We thank the members of the Nelson laboratory for critical reading of the manuscript. Drs. Elizabeth Repasky, Ron Kopito, Robert Levenson, Masatoshi Takeichi, Rolf Kemler, and Kathleen Siemers provided antibodies used in these experiments, for which we are grateful. Dr. Rolf Kemler generously provided the cDNA encoding mouse E-cadherin and the E-cadherin transfected fibroblasts.

This work was supported by grants from the National Institutes of Health (NIH) (GM35527) and American Cancer Society to W. J. Nelson, and grants from the Howard Hughes Medical Institute and NIH to L. F. Reichardt. J. A. Marrs is the recipient of a postdoctoral fellowship from the NIH. E. W. Napolitano is a research associate of the Howard Hughes Medical Institute. Cynthia Murphy-Erdosh is supported by a training grant from the NIH (1T32EY07120-02). L. F. Reichardt is an Investigator of the Howard Hughes Medical Institute. W. J. Nelson is an Established Investigator of the American Heart Association.

Received for publication 5 February 1993 and in revised form 7 July 1993.

Note Added in Proof. Recent studies on retinal pigmented epithelial cell lines demonstrate that localization of Na^+,K^+-ATPase to cell-cell contacts is dependent on the specific expression of E-cadherin (Andersson-Fison, C., J. A. Marrs, W. J. Nelson, E. Rodriguez-Boulan, manuscript in preparation), confirming our current results with cadherin-transfected fibroblasts.

References

Almers, W., and C. Stirling. 1984. Distribution of transport proteins over animal cell membranes. *J. Membr. Biol.* 77:169–186.

Avner, E., W. E. Sweeney, and W. J. Nelson. 1992. Abnormal sodium pump distribution during renal tubulogenesis in congenital murine polycystic kidney disease. *Proc. Natl. Acad. Sci. USA.* 89:7447–7451.

Bennett, V. 1990. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* 70:1029–1065.

Bennett, V., and P. J. Stenbuck. 1980. Human erythrocyte ankyrin. Purification and properties. *J. Biol. Chem.* 255:2540–2548.

Bennett, V., K. Gardner, and J. P. Steiner. 1988. Brain adducin: a protein kinase C substrate that may mediate site-directed assembly at the spectrin-actin junction. *J. Biol. Chem.* 263:5860–5869.

Boller, K., D. Vestweber, and R. Kemler. 1985. Cell adhesion molecule uvomorulin is localized in the intermediate junction of adult intestinal epithelial cells. *J. Cell Biol.* 100:327–332.

Coleman, T. R., D. J. Fishkind, M. S. Mooseker, and J. S. Morrow. 1989. Contributions of the β-subunit to spectrin structure and function. *Cell Motil. Cytoskeleton.* 12:248–263.

Davis, J., and V. Bennett. 1990. The anion exchanger and Na^+,K^+-ATPase interact with distinct sites on ankyrin in *in vitro* assays. *J. Biol. Chem.* 265:17252–17256.

Ernst, S. A., J. R. Palacios, and G. J. Siegel. 1986. Immunocytochemical localization of Na^+,K^+-ATPase catalytic polypeptide in mouse choroid plexus. *J. Histochem. Cytochem.* 34:189–195.

Gardner, K., and V. Bennett. 1987. Modulation of spectrin-actin assembly by erythrocyte adducin. *Nature (Lond.).* 328:359–362.

Geiger, B., and O. Ayalon. 1992. Cadherins. *Annu. Rev. Cell Biol.* 8:307–332.

Ghosh, S., A. C. Freitag, V. P. Martin, and P. M. Coca. 1990. Cellular distribution and differential gene expression of the three α subunit isoforms of the Na,K-ATPase in the ocular ciliary epithelium. *J. Biol. Chem.* 265:2935–2940.

Gottardi, C. J., and M. J. Caplan. 1993. Vectorial targeting of newly synthesized Na,K-ATPase in polarized epithelial cells. *Science (Wash. DC).* 260: 552–554.

Gundersen, D., J. Orlowski, and E. Rodriguez-Boulan. 1991. Apical polarity of Na,K-ATPase in retinal pigment epithelium is linked to a reversal of the ankyrin-fodrin submembrane cytoskeleton. *J. Cell Biol.* 112:863–872.

Gunderson, D., S. K. Powell, and E. Rodriguez-Boulan. 1993. Apical polarization of N-CAM in retinal pigment epithelium is dependent on contact with the neural retina. *J. Cell Biol.* 121:335–343.

Hammerton, R. W., K. A. Krzeminski, R. W. Mays, T. A. Ryan, D. A. Wollner, and W. J. Nelson. 1991. Mechanism for regulating cell surface distribution of Na^+,K^+-ATPase in polarized epithelial cells. *Science (Wash. DC).* 254:847–850.

Hargreaves, W. R., K. N. Giedd, A. Verleij, and D. Branton. 1980. Reassociation of ankyrin with band 3 in erythrocyte membranes and in lipid vesicles. *J. Biol. Chem.* 255:11965–11972.

Kaiser, H. W., E. O'Keefe, and V. Bennett. 1989. Adducin: Ca^{++}-dependent association with sites of cell-cell contact. *J. Cell Biol.* 109:557–569.

Kemler, R. 1992. Classical cadherins. *Sem. Cell Biol.* 3:149–155.

Koob, R., M. Zimmerman, W. Schoner, and D. Dreukhahn. 1987. Colocalization and co-precipitation of ankyrin and Na^+,K^+-ATPase in kidney epithelial cells. *Eur. J. Cell Biol.* 45:230–237.

Kopito, R. R. 1990. Molecular biology of the anion exchanger gene family. *Int. Rev. Cytol.* 123:177–199.

Kudrycki, K. E., P. R. Newman, and G. E. Shull. 1990. cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl^-/HCL_3^- exchanger. *J. Biol. Chem.* 265:462–471.

Lindsey, A. E., K. Schneider, D. M. Simmons, R. Baron, B. S. Lee, and R. R. Kopito. 1990. Functional expression and subcellular localization of an anion exchanger cloned from choroid plexus. *Proc. Natl. Acad. Sci. USA.* 87:5278–5282.

Masuzawa, T., T. Ohta, M. Kawamura, N. Nakahara, and F. Sato. 1984. Immunohistochemical localization of Na^+,K^+-ATPase in the choroid plexus. *Brain Res.* 302:357–362.

Masuzawa, T., T. Ohta, K. Kawakami, and F. Sato. 1985. Immunocytochemical localization of Na^+,K^+-ATPase in the canine choroid plexus. *Brain.* 108:625–646.

McNeill, H., M. Ozawa, R. Kemler, and W. J. Nelson. 1990. Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell.* 62:309–316.

Minuth, W. W., P. Gross, P. Gilbert, and M. Kashgarian. 1987. Expression of the α-subunit of Na/K-ATPase in renal collecting duct epithelium during development. *Kidney Int.* 31:1104–1112.

Mische, S. M., M. S. Mooseker, and J. S. Morrow. 1987. Erythrocyte adducin: a calmodulin-regulated actin-bundling protein that stimulates spectrin-actin binding. *J. Cell Biol.* 105:2837–2845.

Molitoris, B. A. 1991. New insights into the cell biology of ischemic acute renal failure. *J. Am. Soc. Nephrol.* 1:1263–1270.

Morrow, J. S., C. D. Cianci, T. Ardito, A. S. Mann, and M. Kashgarian. 1989. Ankyrin links fodrin to the alpha subunit of Na^+,K^+-ATPase in Madin-Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108:455–465.

Mostov, K., G. Apodaca, B. Aroeti, and C. Okamoto. 1992. Plasma membrane protein sorting in polarized epithelial cells. *J. Cell Biol.* 116:577–583.

Nagafuchi, A., and M. Takeichi. 1989. Transmembrane control of cadherin-mediated cell adhesion: a 94 kd protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul.* 1:37–44.

Napolitano, E. W., K. Venstrom, E. F. Wheeler, and L. F. Reichardt. 1991. Molecular cloning and characterization of B-cadherin, a novel chicken cadherin. *J. Cell Biol.* 113:893–905.

Nelson, W. J. 1992. Regulation of cell surface polarity from bacteria to mammals. *Science (Wash. DC).* 258:948–955.

Nelson, W. J., and R. W. Hammerton. 1989. A membrane-cytoskeletal complex containing Na^+,K^+-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* 108:893–902.

Nelson, W. J., R. W. Hammerton, A. Z. Wang, and E. M. Shore. 1990*a*. Involvement of the membrane-cytoskeleton in the development of epithelial cell polarity. *Sem. Cell Biol.* 1:359–371.

Nelson, W. J., E. M. Shore, A. Z. Wang, and R. W. Hammerton. 1990*b*. Identification of a membrane-cytoskeletal complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 110:349–357.

Nelson, W. J., and P. J. Veshnock. 1986. Dynamics of membrane-skeleton (fodrin) organization during development of polarity in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 103:1751–1765.

Nelson, W. J., and P. J. Veshnock. 1987*a*. Ankyrin binding to Na^+,K^+-ATPase and implications for the organization of membrane domains in polarized cells. *Nature (Lond.).* 328:533–536.

Nelson, W. J., and P. J. Veshnock. 1987*b*. Modulation of fodrin (membrane skeleton) stability by cell-cell contact in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 104:1527–1537.

Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1711–1717.

Ringwald, M., H. Baribault, C. Schmidt, and R. Kemler. 1991. The structure of the gene coding of the mouse cell adhesion molecule uvomorulin. *Nucleic Acids Res.* 19:6533–6539.

Rodriguez-Boulan, E., and S. K. Powell. 1992. Polarity of epithelial and neuronal cells. *Annu. Rev. Cell Biol.* 8:395–427.

Seigel, G. J., C. Holm, J. H. Schreiber, T. Desmond, and S. A. Ernst. 1984. Purification of mouse brain ($Na^+ + K^+$)-ATPase catalytic unit, characterization of antiserum, and immunocytochemical localization in cerebellum, choroid plexus, and kidney. *J. Histochem. Cytochem.* 32:1309–1318.

Shore, E. M., and W. J. Nelson. 1991. Biosynthesis of the cell adhesion molecule uvomorulin (E-cadherin) in Madin-Darby canine kidney epithelial cells. *J. Biol. Chem.* 266:19672–19680.

Siemers, K., R. Wilson, R. W. Mays, T. A. Ryan, D. A. Wolner, and W. J. Nelson. 1993. Delivery of Na^+,K^+-ATPase in polarized epithelial cells. *Science (Wash. DC).* 260:554–556.

Sorkin, B. C., W. J. Gallin, G. M. Edelman, and B. A. Cunningham. 1991. Genes for two calcium-dependent cell adhesion molecules have similar structures and are arranged in tandem in the chicken genome. *Proc. Natl. Acad. Sci. USA.* 88:11545–11549.

Spector, R., and C. E. Johanson. 1989. The mammalian choroid plexus. *Sci. Am.* 131:68–74.

Sweadner, K. J. 1989. Isoenzymes of the Na^+/K^+-ATPase. *Biochem. Biophys. Acta.* 988:185–220.

Tyler, J., B. N. Reinhardt, and D. Branton. 1980. Associations of erythrocyte membrane proteins: binding of purified bands 2.1 and 4.1 to spectrin. *J. Biol. Chem.* 255:7034–7039.

Watts, A. G., G. Sanchez-Watts, J. R. Emanuel, and R. Levenson. 1991. Cell-specific expression of mRNAs encoding Na^+,K^+-ATPase alpha- and beta-subunit isoforms within the rat central nervous system. *Proc. Natl. Acad. Sci. USA.* 88:7425–7429.

Wilson, P. D., A. C. Sherwood, K. Palla, J. Du, R. Watson, and J. T. Norman. 1991. Reversed polarity of Na/K-ATPase: mislocalization to the apical plasma membranes in polycystic kidney disease epithelia. *Am. J. Physiol.* 260:F420–F430.

Wright, E. M. 1972. Mechanisms of ion transport across the choroid plexus. *J. Physiol.* 226:545–571.

Zurzolo, C., and E. Rodriguez-Boulan. 1993. Delivery of Na^+,K^+-ATPase in polarized epithelial cells. *Science (Wash. DC).* 260:550–552.