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

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Abstract. In polarized Madin-Darby canine kidney (MDCK) epithelial cells, ankyrin, and the α- and β-subunits of fodrin are components of the basolateral membrane-cytoskeleton and are colocalized with the Na^+,K^+-ATPase, a marker protein of the basolateral plasma membrane. Recently, we showed with purified proteins that the Na^+,K^+-ATPase is competent to bind ankyrin with high affinity and specificity (Nelson, W. J., and P. J. Veshnock. 1987. *Nature (Lond.).* 328:533–536). In the present study we have sought biochemical evidence for interactions between these proteins in MDCK cells. Proteins were solubilized from MDCK cells with an isotonic buffer containing Triton X-100 and fractionated rapidly in sucrose density gradients. Complexes of cosedimenting proteins were detected by analysis of sucrose gradient fractions in nondenaturing polyacrylamide gels. The results showed that ankyrin and fodrin cosedimented in sucrose gradient. Analysis of the proteins from the sucrose gradient in nondenaturing polyacrylamide gels revealed two distinct ankyrin:fodrin complexes that differed in their relative electrophoretic mobilities; both complexes had electrophoretic mobilities slower than that of purified spectrin heterotetramers. Parallel analysis of the distribution of solubilized Na^+,K^+-ATPase in sucrose gradients showed that there was a significant overlap with the distribution of ankyrin and fodrin. Analysis by nondenaturing polyacrylamide gel electrophoresis showed that the α- and β-subunits of the Na^+,K^+-ATPase colocalized with the slower migrating of the two ankyrin:fodrin complexes. The faster migrating ankyrin:fodrin complex did not contain Na^+,K^+-ATPase. These results indicate strongly that the Na^+,K^+-ATPase, ankyrin, and fodrin are coextracted from whole MDCK cells as a protein complex. We suggest that the solubilized complex containing these proteins reflects the interaction of the Na^+,K^+-ATPase, ankyrin, and fodrin in the cell. This interaction may play an important role in the spatial organization of the Na^+,K^+-ATPase to the basolateral plasma membrane in polarized epithelial cells.

THE plasma membrane of many specialized cell-types is organized into domains of distinct morphology and function. Cells of transporting epithelia exhibit a characteristic functional polarity that is a reflection of the asymmetrical distribution of enzymes and transport proteins between the apical and basolateral membrane domains (for reviews, see Rodriguez-Boulan, 1983; Simons and Fuller, 1985; Matlin, 1986). The mechanisms involved in the establishment and maintenance of the protein specificity of these domains are poorly understood. Studies of the topogenesis of viral (Rodriguez-Boulan and Sabatini, 1978; Misek et al., 1984; Rindler et al., 1984, 1985; Matlin and Simons, 1984) and endogenous glycoproteins (Caplan et al., 1986; Gottlieb et al., 1986; Urban et al., 1987) in Madin-Darby canine kidney (MDCK) cells indicate that newly synthesized proteins are targeted directly to the appropriate plasma membrane domain, whereupon their distribution becomes restricted. This restriction may be accomplished by the tight junction that is situated at the boundary of the apical and basolateral membrane domains (Dragsten et al., 1981; Hertzlinger and Ojakian, 1984; for review, see Gumbiner, 1987). Alternatively, lateral diffusion may be restricted by interactions between integral membrane proteins and cytoplasmic structural proteins (Nelson and Veshnock, 1986; for review, see Nelson, 1989). Such interactions have been extensively characterized in erythrocytes and involve the linkage of integral membrane proteins, e.g., the anion transporter, to a cytoplasmic protein meshwork composed of ankyrin, spectrin, and actin, collectively termed the membrane-cytoskeleton (for reviews, see Branton et al., 1981; Bennett, 1985; Marchesi, 1985).

Recent studies have identified ankyrin (M_r 215,000) and fodrin (α-subunit, M_r 240,000; β-subunit, M_r 230,000) as major components of the membrane-cytoskeleton in a variety of nonerythroid cells (for reviews, see Nelson and Lazarides, 1984; Bennett, 1985; Marchesi, 1985). In vitro studies with purified proteins have shown that ankyrin and fodrin hetero-

tetramers form a complex (Davis and Bennett, 1984), and that high-affinity binding sites for ankyrin exist on the plasma membrane of nonerythroid cells (Davis and Bennett, 1986). However, little is known about the molecular organization of the membrane-cytoskeleton in nonerythroid cells and the role of these protein complexes in the formation of membrane domains.

Recently, we initiated a study of the membrane-cytoskeleton in polarized Madin-Darby canine kidney (MDCK) epithelial cells. We found that during the development of cell polarity there is a gradual reorganization of ankyrin and fodrin, which results in the formation of a dense, insoluble, and metabolically stable protein meshwork that is localized predominantly if not exclusively to the basolateral plasma membrane in monolayers of polarized cells (Nelson and Veshnock, 1986). The formation of the membrane-cytoskeleton requires extensive cell–cell contact (Nelson and Veshnock, 1987*a*) and coincides temporally and spatially with the development of polarity of the Na^+,K^+-ATPase, a marker protein of the basolateral membrane domain and a high-affinity binding site for ankyrin (Nelson and Veshnock, 1987*b*).

To gain insight into the organization and function of the membrane-cytoskeleton in polarized epithelial cells, we have searched for interactions between structural cytoplasmic proteins (ankyrin and fodrin) and the Na^+,K^+-ATPase in extracts of whole cells. After fractionation of solubilized proteins in sucrose gradients and nondenaturing polyacrylamide gels, a discrete protein complex was identified that contained the Na^+,K^+-ATPase, ankyrin, and fodrin. The identification of this complex constitutes direct evidence of a molecular linkage between a specific integral membrane protein and the membrane-cytoskeleton in nonerythroid cells. The results strongly support a role of the membrane-cytoskeleton in the biogenesis and maintenance of membrane domains in polarized epithelial cells.

Materials and Methods

Cell Fractionation

A low passage stock of MDCK cells (clone 8; Nelson and Veshnock, 1986) was established on collagen-coated 35-mm petri dishes at a confluent cell density of 3×10^5 cells/cm^2 in DME containing 5 μM Ca^{2+} and 10% dialyzed FBS (LC medium); under these conditions there is little or no cell-cell contact (for details, see Nelson and Veshnock, 1987*a*). After ~12 h, the cells were placed on ice and processed at 4°C. The cells were rinsed twice with ice-cold buffer containing 15 mM Tris-HCl, pH 7.5, 120 mM NaCl, and 1 mM PMSF. The petri dish was completely drained of buffer. 200 μl of isotonic buffer containing Triton X-100 (0.5% [vol/vol] Triton X-100, 10 mM Tris-HCl, pH 7.5, 25 mM KCl, 120 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, and 0.5 mM PMSF) was added and the petri dish was rocked gently for 5–10 min. The monolayer of cells was scraped from the petri dish in extraction buffer with a rubber policeman, and centrifuged at 48,000 *g* for 5 min. The supernatant was layered onto 3.8 ml linear 5–20% (wt/wt) sucrose gradients prepared in extraction buffer without Triton X-100, and centrifuged at 486,000 *g* for 5 h in the SW60 rotor of the L8-70M ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). Gradients were fractionated from the bottom to the top into 20 fractions (200 μl). The following protein standards of known S values were centrifuged on replicate 5–20% (wt/wt) sucrose gradients: apoferritin, 17.2S; catalase 11.35S; aldolase, 7.35S; bovine serum albumin, 4.6S; cytochrome *c*, 1.7S; purified human erythrocyte spectrin heterodimers, 9.6S; and ankyrin, 6.9S. Their distributions in the sucrose gradients were determined by SDS-PAGE.

For surface labeling of MDCK cells with ^{125}I, confluent monolayers of the cells were established in LC-medium in 35-mm petri dishes, washed in PBS, and then processed as described by Antonicek et al. (1987). Under these culture conditions, the apical and basolateral plasma membranes were accessible to ^{125}I. Cells were extracted with isotonic buffer containing Triton X-100 and solubilized proteins were fractionated in 5–20% (wt/wt) sucrose gradients (see above). To determine the distribution of ^{125}I-labeled proteins, individual fractions were precipitated with TCA and collected on GF/C glass fiber filters (Whatman Inc., Clifton, NJ), dried, and counted in a gamma counter (model 5500; Beckman Instruments, Inc.).

PAGE

5–12.5% SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with silver (Oakley et al., 1980) using the Gelcode Kit (Pierce Chemical Co., Rockford, IL). 2–4% nondenaturing PAGE was performed in 1.5-mm gel slabs at 4°C (Morrow and Haigh, 1983); the duration of electrophoresis was varied between 24–60 h. Erythrocyte spectrin heterodimer ($[\alpha\beta]$) and heterotetramer ($[\alpha\beta]_2$) standards were purified as described previously (Bennett, 1983). Ankyrin and Na^+,K^+-ATPase were purified, incubated together and extracted with the nonionic detergent, octaethyleneglycoldodecyl ether ($C_{12}E_8$) as described (Nelson and Veshnock, 1987*b*).

Proteins separated in SDS or nondenaturing polyacrylamide gels were electrophoretically transferred to nitrocellulose sheets (0.45 μm) (Towbin et al., 1981). Silver staining of the gel showed that all proteins had been transferred to the nitrocellulose. The nitrocellulose was stained with India ink and processed for Western blotting with antibodies to ankyrin, α-fodrin, or the α- and β-subunits of the Na^+,K^+-ATPase (Nelson and Veshnock, 1986). Immune complexes were detected with ^{125}I-labeled protein A (~10 μCi/μg) as described previously (Nelson and Veshnock, 1986). The nitrocellulose was preincubated before addition of antibodies in a buffer containing 3% BSA and 1% FBS. The nitrocellulose was exposed to XAR-5 x-ray film at −80°C with two intensifying screens (DuPont Co., Wilmington, DE).

Results

Our previous studies of ankyrin and fodrin in MDCK cells showed that these proteins exist as soluble and insoluble pools of protein that are defined by their extractability in isotonic buffer containing Triton X-100 (see Materials and Methods; Nelson and Veshnock, 1986). In the absence of cell–cell contact, ~70% of the proteins are present in the soluble pool. However, this pool of protein can be rapidly recruited into an insoluble, metabolically stable pool upon induction of cell–cell contact; this insoluble pool comprised 60–70% of the total ankyrin and fodrin. These results suggested to us that the soluble pool might be a precursor to the insoluble pool (Nelson and Veshnock, 1987*a*). Hence, an analysis of the molecular organization of ankyrin and fodrin in the soluble pool may provide new insight into the nature of the insoluble pool.

To analyze the molecular organization of solubilized ankyrin and fodrin we have developed an analytical method for detecting protein complexes extracted from whole cells. Proteins are first fractionated in sucrose gradients and the distribution of individual proteins is determined by immunoblotting or immunoprecipitation with specific antibodies. Second, to determine whether cosedimenting proteins are in a complex, proteins from sucrose gradient fractions are separated by nondenaturing PAGE and their location in the gel determined by immunoblotting.

Ankyrin and Fodrin Solubilized from MDCK Cells Cosediment in Sucrose Density Gradients

Monolayers of MDCK cells were extracted for 5–10 min at 4°C in a buffer containing 0.5% (vol/vol) Triton X-100 and an isotonic salt concentration (see Materials and Methods). After centrifugation at 48,000 *g*, the supernatant was layered onto a linear 5–20% (wt/wt) sucrose gradient and centrifuged at 468,000 *g* for 5 h at 4°C. The distribution of anky-

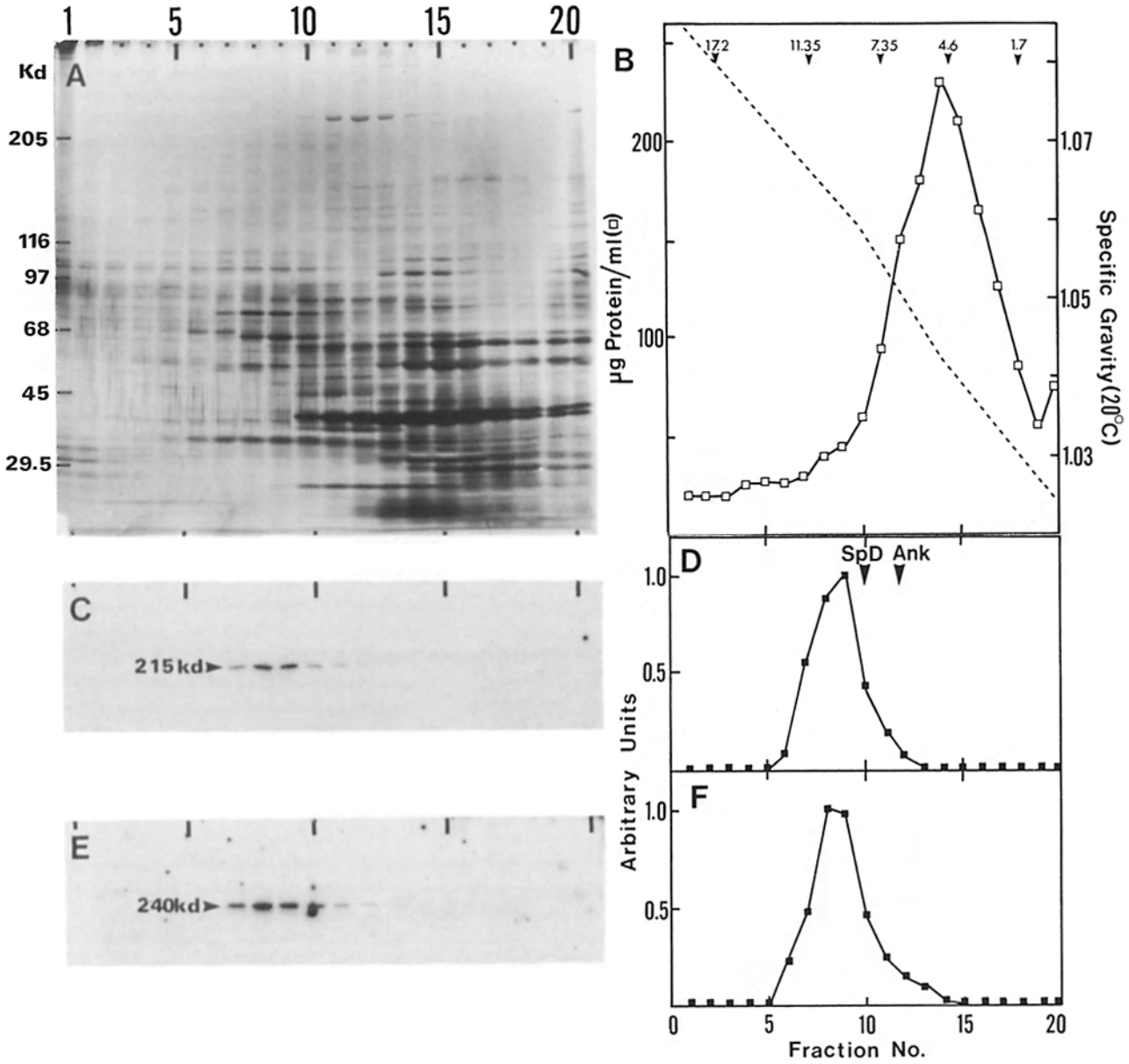

Figure 1. Sucrose gradient fractionation of ankyrin and fodrin solubilized from MDCK cells in isotonic buffer containing Triton X-100. (*A*) 5–12.5% SDS-polyacrylamide gel of total proteins stained with silver. Fraction 1 is at the bottom of the gradient. Molecular weight standards; *a*, myosin (M_r 205,000); *b*, β-galactosidase (M_r 116,000); *c*, phosphorylase *b* (M_r 97,000); *d*, BSA (M_r 68,000); *e*, ovalbumin (M_r 45,000); and *f*, carbonic anhydrase (M_r 29,500). (*B*) Profile of total protein concentration in the sucrose gradient determined using the Bio-Rad (Richmond, CA) protein assay. The specific gravity of the sucrose gradient was determined using a refractometer. The peak distribution of protein standards of known S value are shown (for details see Materials and Methods). (*C* and *E*) Western blot analyses of the distributions of ankyrin and α-fodrin in the sucrose gradient using affinity-purified antisera. (*D* and *F*) Corresponding densitometric analysis of the distributions of ankyrin and α-fodrin. In each case, the values determined by scanning densitometry were normalized to that of the peak fraction (8 or 9) given an arbitrary value of 1. The peak fractions of purified erythrocyte spectrin heterodimers (*SpD*) and ankyrin (*Ank*) are indicated.

rin and fodrin in the sucrose density gradient was determined by Western blotting (Fig. 1, *C–F*). Ankyrin and fodrin cosedimented in fractions 7–11 with an apparent S value of 9.5–10.5, well separated from the majority of solubilized proteins (Fig. 1, *C–F*); these fractions contained <4% of the total solubilized proteins (Fig. 1, *A* and *B*). The sedimentation rate of solubilized ankyrin and fodrin was faster than that of purified erythrocyte ankyrin and purified spectrin heterodimers (Fig. 1, *D* and *F*). When the centrifugation time was varied (3–6 h), ankyrin and fodrin invariably cosedimented (data not shown); the distribution of proteins varied with the time of centrifugation, demonstrating that cosedimentation was thus not the result of an equilibrium centrifugation.

Identification of an Ankyrin:Fodrin Complex in Nondenaturing Polyacrylamide Gels

To determine whether ankyrin and fodrin cosedimented as

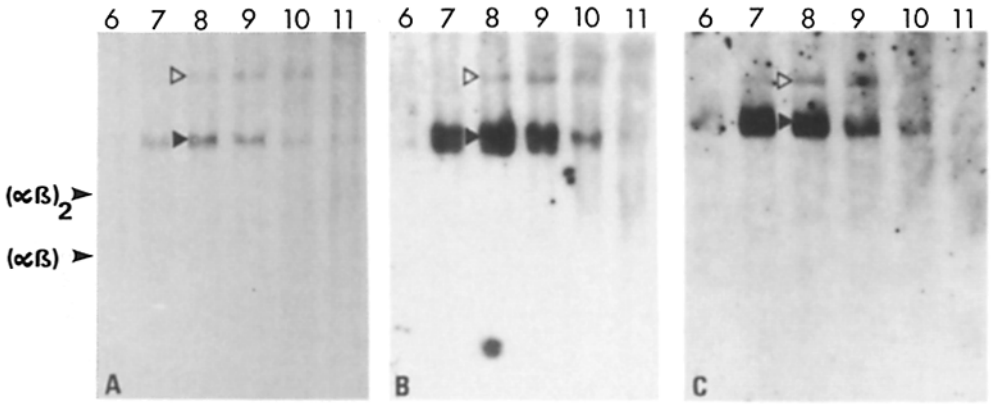

Figure 2. Analysis by nondenaturing PAGE and Western blotting of ankyrin:fodrin complexes solubilized from MDCK cells. Proteins solubilized with isotonic buffer containing Triton X-100 were fractionated as described in Materials and Methods. After sucrose density gradient centrifugation, 100–120 μl of fractions 6–11 were separated on 2–4% nondenaturing polyacrylamide gels. Proteins were transferred to nitrocellulose, stained with India ink (*A*) before incubation with ankyrin (*B*) or α-fodrin (*C*) antibodies. The gels are representative of three separate experiments. The electrophoretic mobilities of purified erythrocyte spectrin heterodimers ([αβ]) and heterotetramers ($[\alpha\beta]_2$) are indicated by arrows.

a complex, individual fractions of the sucrose gradients were separated in linear 2–4% nondenaturing polyacrylamide gels and transferred electrophoretically to nitrocellulose (Fig. 2). Staining of the nitrocellulose filter with India ink revealed two protein bands in fractions 6–11 from the sucrose gradient. These protein bands exhibited different relative electrophoretic mobilities in the gel. In addition, we noted that the protein bands showed slight, but reproducible, differences in their sedimentation rates in the sucrose gradient (Fig. 2 *A*). The more rapidly migrating band (*closed arrowhead*) on the nondenaturing polyacrylamide gel had a peak distribution in fractions 7–9 in the sucrose gradient (average 10.5S), whereas the more slowly migrating band (*open arrowhead*) had a peak distribution in fractions 8–11 (average 9.5S).

The distributions of ankyrin and fodrin on the nondenaturing polyacrylamide gel were determined directly by Western blotting. The faster migrating of the two protein bands stained more prominently with antibodies specific for ankyrin and fodrin, and had an electrophoretic mobility slower than that of purified human erythrocyte $(\alpha\beta)_2$spectrin heterotetramers (Fig. 2, *B* and *C*) and canine lens fodrin (data not shown). The slower migrating protein band also reacted with both antibodies, but appeared to contain <15% of the total ankyrin and fodrin solubilized from the cell compared with the faster migrating ankyrin:fodrin complex (>85%). This result demonstrates that there are two distinct populations of ankyrin:fodrin complexes, which are distinguishable by differences in their electrophoretic mobilities in nondenaturing polyacrylamide gels.

Separation of Na^+,K^+-ATPase from Other Solubilized Surface Glycoproteins on Sucrose Density Gradients

Our previous in vitro studies identified Na^+,K^+-ATPase as a high-affinity binding site for ankyrin, and showed that a detergent-solubilized complex of these proteins was not dissociated in sucrose gradients or nondenaturing polyacrylamide gels (Nelson and Veshnock, 1987*b*). Accordingly, we sought to determine whether Na^+,K^+-ATPase coisolated with the ankyrin and fodrin after sucrose gradient and nondenaturing polyacrylamide gel fractionation of proteins from whole MDCK cells.

Greater than 80% of the Na^+,K^+-ATPase in MDCK cells grown in LC-medium was solubilized in the isotonic buffer containing Triton X-100 (Fig. 3 *A*). In MDCK cell cultures that had extensive cell–cell contact the Na^+,K^+-ATPase exhibited different solubility properties (Fig. 3 *B*). Approximately 50% of the Na^+,K^+-ATPase was insoluble in the isotonic buffer containing Triton X-100 (compared with <20% in cells without cell–cell contact).

Na^+,K^+-ATPase was solubilized from MDCK cells grown in LC-medium, and analyzed on sucrose gradients. The peak of α-subunit (Fig. 4 *B*) sedimented in fractions 6–10 (peak of ~10.5S), well-separated from the bulk of plasma membrane glycoproteins, which were identified by surface-labeling with ^{125}I (fractions 15–20; Fig. 4 *A*). The β-subunit of the Na^+,K^+-ATPase had an identical sedimentation profile (data not shown). There was significant overlap in the distribution of the Na^+,K^+-ATPase with those of ankyrin and fodrin (Fig. 4 *C*); these three proteins had almost identical peak fractions (7–9). The distributions of the Na^+,K^+-ATPase, ankyrin, and fodrin overlapped even when the duration of the centrifugation varied (data not shown), demonstrating this was not the result of an equilibrium centrifugation of individual proteins and raising the possibility that all three proteins were cosedimenting as a complex.

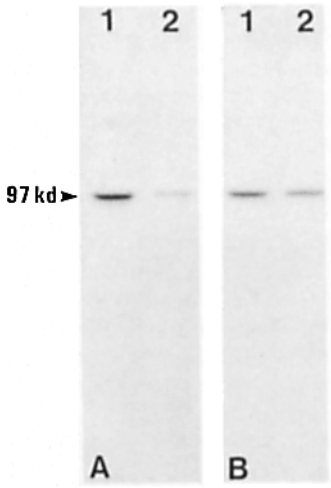

Figure 3. Solubility of Na^+,K^+-ATPase in MDCK cells. MDCK cells were grown in LC-medium (*A*) or DMEM (*B*) and extracted with isotonic buffer containing Triton X-100 (see Materials and Methods). Equivalent portions of the soluble and insoluble fractions were separated by 5–12.5% SDS-PAGE and the partitioning of the Na^+,K^+-ATPase in the two fractions determined by Western blotting and scanning densitometry.

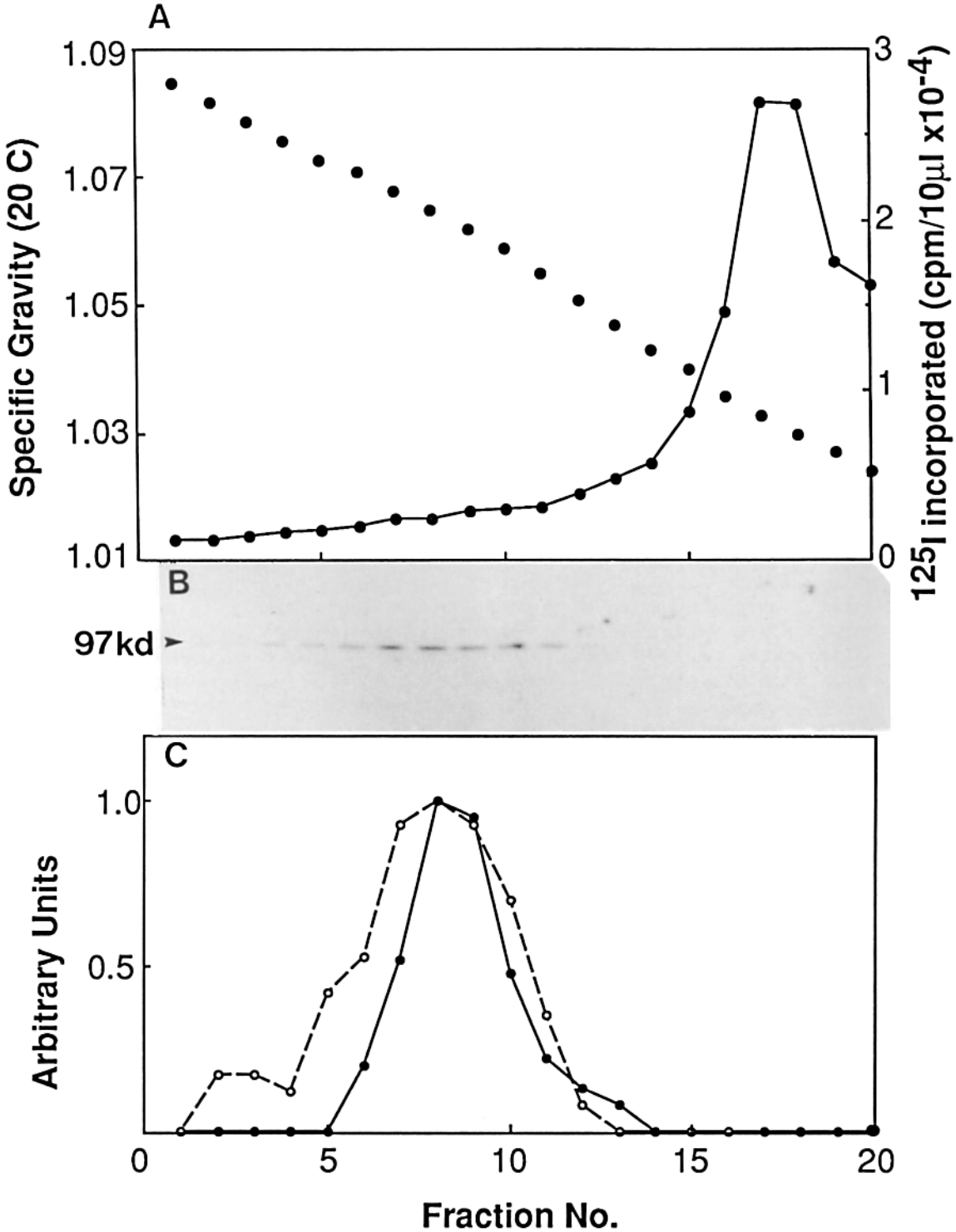

Figure 4. Distribution of solubilized α- and β-subunits of Na^+,K^+-ATPase on sucrose density gradients. Proteins solubilized in Triton X-100, isotonic buffer were fractionated on sucrose density gradients. (*A*) Distribution of ^{125}I-surface labeled proteins and proteins of known S value. Western blot (*B*) and corresponding densitometric analysis (*C*) of the α-subunit Na^+,K^+-ATPase (– – –), and comparison with the distribution of fodrin (——).

Identification of a Complex Containing Na^+,K^+-ATPase, Ankyrin, and Fodrin Solubilized from MDCK Cells

Protein fractions from the sucrose gradient were separated on linear 2–4% nondenaturing polyacrylamide gels and individual proteins were identified by Western blotting. The proteins in a single protein band (fractions 7–10) reacted with antibodies affinity purified against either the α- or β-subunit of the Na^+,K^+-ATPase (Fig. 5, *B* and *C*). The electrophoretic mobility of the Na^+,K^+-ATPase subunits was identical to that of the slower migrating of the two ankyrin:fodrin com-

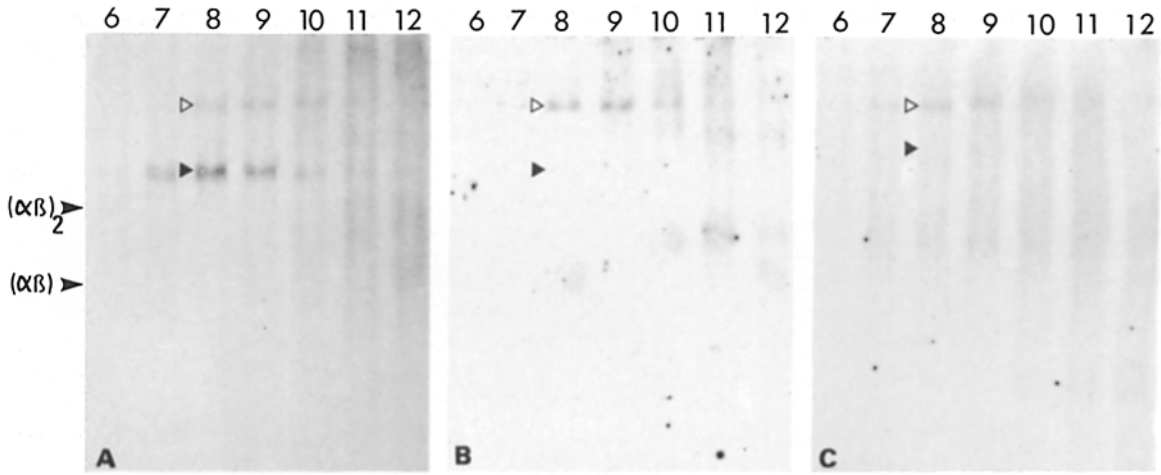

Figure 5. Nondenaturing PAGE and Western blotting of Na^+,K^+-ATPase solubilized from MDCK cells. After extraction with isotonic buffer containing Triton X-100 and sucrose gradient centrifugation, fractions 6–12 were resolved in 2–4% nondenaturing polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose, stained with India ink (*A*), and processed for Western blotting (*B* and *C*) with affinity-purified antibodies specific for the α- (*B*) or β-subunits (*C*) of the Na^+,K^+-ATPase. The electrophoretic mobilities of purified erythrocyte spectrin heterodimers ([αβ]) and heterotetramers ($[\alpha\beta]_2$) are indicated by arrows.

plexes (Fig. 6, *A–F*; see also Fig. 2). Little or no staining of the faster migrating ankyrin:fodrin complexes was detected with Na^+,K^+-ATPase antibodies. The same distributions were found for Na^+,K^+-ATPase, ankyrin, and fodrin when the nondenaturing gels were run for shorter or longer intervals (data not shown).

We compared the electrophoretic mobility of the complex containing the Na^+,K^+-ATPase, ankyrin, and fodrin (Fig. 6, lanes *A–F*) with that of the purified proteins and complexes of these proteins formed in vitro (Fig. 6, lanes *G–N*). The purified proteins were incubated at 4°C in the presence of a nonionic detergent (Triton X-100 or octaethyleneglycol-dodecyl ether) and DTT before electrophoresis (for details, see Nelson and Veshnock, 1987*b*). The electrophoretic mobility of purified ankyrin (Fig. 6, lanes *H* and *K*), spectrin heterodimers (Fig. 6, lanes *G* and *L*) and heterotetramers (Fig. 6, lane *L*) and solubilized Na^+,K^+-ATPase (Fig. 6, lane *I*) were distinctly faster than that of either of the two

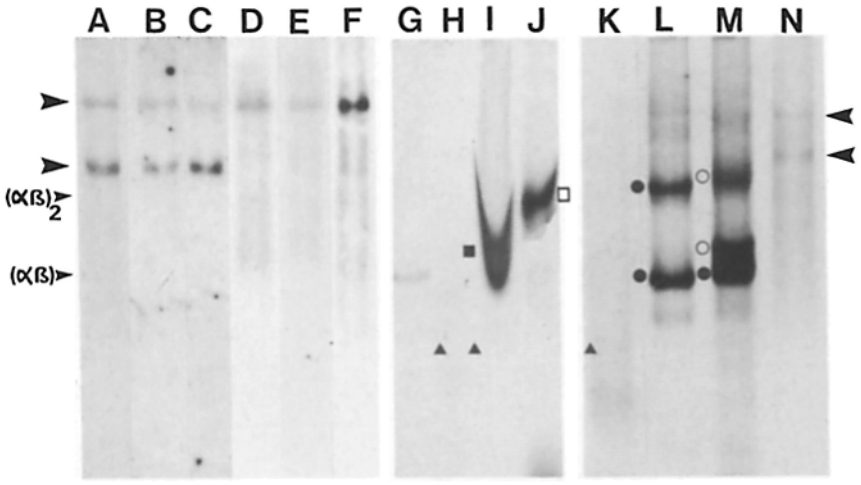

Figure 6. Comparative analysis of the electrophoretic mobilities in nondenaturing polyacrylamide gels of purified proteins and protein complexes solubilized from MDCK cells. (Lanes *A–F*) Analysis of protein complexes solubilized from MDCK cells. Proteins solubilized from MDCK cells in isotonic buffer containing Triton X-100 were separated in sucrose gradients. Peak fractions of ankyrin, fodrin, and Na^+,K^+-ATPase (fractions 7–9) were combined and analyzed in a nondenaturing polyacrylamide gel. After transfer to nitrocellulose, protein complexes (*large arrowhead*) were identified in replicate lanes by India ink staining (lane *A*) or Western blotting with antibodies specific for ankyrin (lane *B*), α-fodrin (lane *C*), or the α- (lane *D*) or β- (lane *E*) subunits of the Na^+,K^+-ATPase. In addition (lane *F*), the nitrocellulose was incubated with a mixture of lectins (wheat germ agglutinin, concanavalin A, *Helix pomentia*) that had been labeled with ^{125}I using iodogen (Pierce Chemical Co., Rockford, IL) as described in Nelson and Veshnock (1986). (Lanes *G–J*) Analysis of purified Na^+,K^+-ATPase and ankyrin. Human erythrocyte spectrin dimers (lane *G*) and ankyrin (lane *H*; ▲) were purified (Bennett, 1983), and the Na^+,K^+-ATPase (lane *I*; ■) was purified in a membrane-bound form (for details see Nelson and Veshnock, 1987*b*). Ankyrin and Na^+,K^+-ATPase (lane *J*; □) were incubated together at 4°C for 90 min as described in detail previously (Nelson and Veshnock, 1987*b*). Proteins were incubated in $C_{12}E_8$ before analysis in a 2–4% nondenaturing polyacrylamide gel. The gel was stained with Coomassie blue. (Lanes *K–N*) Analysis of purified spectrin and ankyrin. Ankyrin (lane *K*; ▲) and spectrin dimers and tetramers (lane *L*; ●) were purified from human erythrocytes (Bennett, 1983), and incubated at 4°C for 90 min (lane *M*). (Lane *N*) Peak fractions of ankyrin, fodrin, and Na^+,K^+-ATPase from sucrose gradients of proteins extracted from MDCK cells (see lanes *A–F*). The arrowheads indicate the protein complexes containing ankyrin, fodrin, and the Na^+,K^+-ATPase (see lanes *A–E*). The nondenaturing polyacrylamide gel was stained with silver (see Materials and Methods).

ankyrin:fodrin complexes extracted from MDCK cells (Fig. 6, lane *N*). Complexes of ankyrin and spectrin heterotetramers (Fig. 6, lane *M*) or ankyrin and Na^+,K^+-ATPase formed in vitro (Fig. 6, lane *J*) exhibited slower electrophoretic mobilities than that of the individual proteins, but faster electrophoretic mobilities than that of the complex containing Na^+,K^+-ATPase, ankyrin, and fodrin extracted from MDCK cells (Fig. 6, lane *N*); for instance, the complex formed between purified ankyrin and Na^+,K^+-ATPase had a relative electrophoretic mobility slightly slower than that of spectrin heterotetramers (Fig. 6, lanes *G* and *I*).

Taken together, these results indicate that the slower migrating band of proteins extracted from MDCK cells comprises a complex containing the Na^+,K^+-ATPase, ankyrin, and fodrin. Comparison of the distribution of the Na^+,K^+-ATPase on the nondenaturing polyacrylamide gel and sucrose gradient indicates that >80% of the total amount of solubilized Na^+,K^+-ATPase comigrated with ankyrin and fodrin on the nondenaturing polyacrylamide gel. Analysis by second-dimension SDS-PAGE revealed the presence of several other proteins in this complex (data not shown). Some of these may be glycoproteins, because they react with ^{125}I-labeled lectins (Fig. 6, lane *F*). Hence, a family of proteins that includes the Na^+,K^+-ATPase appears to be bound to the ankyrin:fodrin complex. Recently we have identified one of the other proteins in this complex as the cell adhesion protein, uvomorulin. Immunoprecipitates containing uvomorulin, ankyrin, and fodrin using uvomorulin antibodies do not appear to contain Na^+,K^+-ATPase, indicating that there are individual complexes of integral membrane proteins, ankyrin, and fodrin (Nelson, W. J., R. W. Hammerton, E. Shore, and R. Kemler, manuscript in preparation). Further details of the stoichiometry of proteins in these complexes await future characterization of the other comigrating components.

Discussion

Previous studies have demonstrated that ankyrin and fodrin are colocalized to the plasma membrane of many nonerythroid cell types (Nelson and Lazarides, 1984; Bennett, 1985; Marchesi, 1985), and that ankyrin interacts with fodrin heterotetramers and with high-affinity binding sites on the plasma membrane (Davis and Bennett, 1984, 1986). These results are consistent with the idea that ankyrin, fodrin, and integral membrane proteins form a membrane-cytoskeletal complex that may play an important role in the spatial organization of proteins on the plasma membrane (Nelson and Lazarides, 1984; Bennett, 1985; Marchesi, 1985). However, the nature of the plasma membrane binding sites for ankyrin in cells is poorly understood, although it has been reported recently that purified ankyrin binds with high affinity and specificity to purified, membrane-bound Na^+,K^+-ATPase (Nelson and Veshnock, 1987*b*) and to the voltage-dependent Na^+ channel (Srinivasan et al., 1987). In the present study, we have identified and characterized a protein complex comprising ankyrin, fodrin, and the Na^+,K^+-ATPase in extracts of whole MDCK epithelial cells after fractionation of solubilized proteins in sucrose gradients and nondenaturing polyacrylamide gels.

Central to our experimental approach to search for these protein interactions in extracts of whole cells is our previous demonstration that ankyrin and fodrin exist as soluble and insoluble pools of protein in MDCK cells (Nelson and Veshnock, 1986). These pools are operationally defined by the relative extractability of the proteins in a buffer containing 0.5% (vol/vol) Triton X-100 and an isotonic salt concentration. However, the characteristics of the soluble pool are consistent with it being a precursor of the insoluble membrane-cytoskeleton (Nelson and Veshnock, 1986, 1987*a*).

Identification of a Complex Containing the Na^+,K^+-ATPase, Ankyrin, and Fodrin

After fractionation of solubilized proteins in sucrose gradients and nondenaturing polyacrylamide gels, we detected two distinct protein complexes using antibodies specific for ankyrin, fodrin, and the Na^+,K^+-ATPase. The protein complexes differed in their relative electrophoretic mobilities in nondenaturing polyacrylamide gels.

One protein complex contained >85% of the total solubilized ankyrin and fodrin, but did not react with Na^+,K^+-ATPase antibodies. This ankyrin:fodrin complex had a sedimentation rate of 10.5S, and an electrophoretic mobility slower than that of purified human erythrocyte spectrin and canine fodrin heterotetramers. This ankyrin:fodrin complex did not contain the Na^+,K^+-ATPase or other glycoproteins as indicated by negative reactivity with ^{125}I-labeled lectins (Fig. 6). The electrophoretic mobility of this ankyrin:fodrin complex in the nondenaturing polyacrylamide gels indicates that it may comprise ankyrin bound to a fodrin heterotetramer $[(\alpha\beta)_2]$. Additional support for this conclusion comes from preliminary electron microscopic analysis of these fractions (Heuser, J. E., and W. J. Nelson, unpublished observations) which reveal structures similar in morphology to that of purified ankyrin-$(\alpha\beta)_2$fodrin complexes reported previously (Davis and Bennett, 1984). We are currently analyzing these structures further to confirm the presence of ankyrin-$(\alpha\beta)_2$fodrin complexes. Taken together, these results indicate that in the absence of cell–cell contact there is a large cytoplasmic pool of ankyrin:fodrin complexes which are not associated directly with integral membrane proteins.

The second protein complex detected in the nondenaturing polyacrylamide gel contained the remainder of the solubilized ankyrin and fodrin and >80% of the solubilized α- and β-subunits of the Na^+,K^+-ATPase. This protein complex was distinct from the ankyrin:fodrin complex described above by the criteria of its slower electrophoretic mobility in nondenaturing polyacrylamide gels and slightly different, but reproducible, sedimentation rate in sucrose gradients (9.5S). It is noteworthy that the electrophoretic mobility of the complex containing the Na^+,K^+-ATPase, ankyrin, and fodrin was very different from that of each of the proteins or a complex of ankyrin and the Na^+,K^+-ATPase formed in vitro (see Fig. 6). The interaction of ankyrin, fodrin, and the Na^+,K^+-ATPase in this complex appears to be noncovalent because the association of ankyrin and fodrin with the Na^+,K^+-ATPase is dissociated with high concentrations of salt (data not shown); note also that DTT is present at all times to inhibit sulfhydryl exchange.

These results indicate that the Na^+,K^+-ATPase and a portion of the solubilized ankyrin:fodrin complex are coisolated after sucrose gradient centrifugation and nondenaturing polyacrylamide gel electrophoresis. Whereas it is possible that other, as yet unidentified, proteins in the complex may

mediate the binding of these proteins, we suggest that the solubilized complex reflects the direct interaction of ankyrin, fodrin, and the Na^+,K^+-ATPase in the cell. This is supported principally by the fact that ankyrin has been shown to bind directly and with high affinity to fodrin heterotetramers (Davis and Bennett, 1984) and to purified, membrane-bound Na^+,K^+-ATPase (Nelson and Veshnock, 1987*b*).

Additional evidence also supports this conclusion. First, the method used to identify this complex involved the rapid separation of proteins by high-speed sucrose gradient centrifugation (Merlie and Lindstrom, 1983); this procedure reduces the chance of promiscuous interactions between solubilized proteins that may occur during slow separation of proteins, e.g., gel filtration chromatography. Second, ankyrin, fodrin, and the Na^+,K^+-ATPase cosedimented on the sucrose gradient as a single, symmetrical peak that was separated from >96% of the total solubilized proteins. Third, immunocytochemical studies have shown that ankyrin, fodrin, and Na^+,K^+-ATPase are colocalized at the basolateral plasma membrane of MDCK cells (Lamb et al., 1981; Caplan et al., 1986; Nelson and Veshnock, 1986) and kidney tubule cells (Koob et al., 1987). Fourth, complexes of purified ankyrin and fodrin heterotetramers, and ankyrin and Na^+, K^+-ATPase formed in vitro are biochemically stable in the presence of nondenaturing detergents, and are not dissociated in sucrose gradients or in nondenaturing polyacrylamide gels (Davis and Bennett, 1984; Nelson and Veshnock, 1987*b*). Fifth, the sedimentation rate and electrophoretic mobilities of ankyrin, fodrin, and the Na^+,K^+-ATPase in the complex were different from those of the individual purified proteins (Figs. 1, 5, and 6). Taken together, the evidence indicates strongly that the isolated complex containing ankyrin, fodrin, and Na^+,K^+-ATPase represents the molecular organization of the protein complex in the cell.

What is the relationship between this soluble complex of ankyrin, fodrin, and Na^+,K^+-ATPase and the insoluble membrane-cytoskeleton? We have shown previously that upon induction of extensive cell–cell contact there is a dramatic increase in the size of the insoluble pool of ankyrin, fodrin, and Na^+,K^+-ATPase and a commensurate decrease in the size of the soluble pool of these proteins. At present we do not know the molecular basis for the difference in the extractability of these proteins. However, we suspect that the difference is due to the degree of assembly of complexes of ankyrin, fodrin, and the Na^+,K^+-ATPase into higher-ordered structures on the plasma membrane (the membrane-cytoskeleton) which are insoluble in isotonic buffer containing Triton X-100. That a portion of ankyrin, fodrin, and the Na^+,K^+-ATPase remains relatively soluble after cell–cell contact (see Nelson and Veshnock, 1986, and Fig. 3) may be a reflection of incomplete assembly of some of the complexes into these higher-ordered structures, or the maintenance of small pools of proteins in the cytosol or on intracellular vesicles.

If, as we propose, the soluble complex of ankyrin, fodrin, and Na^+,K^+-ATPase is a precursor of the insoluble pool of proteins, then it should be possible under appropriate extraction conditions to dissociate the insoluble complexes to the soluble protomeric complex. We have found that buffers containing high concentrations of salt or denaturing agents are required to dissociate the insoluble complex of these proteins. However, under these conditions we have been unable to detect a complex of ankyrin:fodrin with bound Na^+,K^+-ATPase (Hammerton, R. W., and W. J. Nelson, unpublished observation). This result is not inconsistent with our proposal, because buffers containing high salt concentrations have been reported to dissociate ankyrin from high-affinity binding sites on nonerythroid plasma membranes (Davis and Bennett, 1986).

A Role for the Membrane-Cytoskeleton in the Biogenesis of Epithelial Cell Polarity

The results of this present study, together with those of our previous studies on the dynamics of membrane-skeleton organization upon cell–cell contact (Nelson and Veshnock, 1986, 1987*a*), lead us to propose a simple model for the role of the membrane-cytoskeleton in the biogenesis of epithelial cell polarity. We suggest that the complex containing ankyrin, fodrin, and the Na^+,K^+-ATPase detected in this study is representative of the smallest repeating denominator of the membrane-cytoskeleton (termed a UNIT). In the absence of cell–cell contact, these UNITs are distributed at the entire plasma membrane or on cytoplasmic vesicles (Nelson and Veshnock, 1986). In addition, there exists a large pool of ankyrin-$(\alpha\beta)_2$ fodrin complexes that is dispersed in the cytosol. UNITs are relatively soluble in isotonic buffer containing Triton X-100 and are metabolically unstable (t 1/2 ~15 h; Nelson and Veshnock, 1987*a*). Upon cell–cell contact, there is a rapid recruitment of these UNITs together with cytosolic ankyrin:fodrin complexes into relatively insoluble higher-order polymers on the plasma membrane ($[UNIT]_N$), perhaps by the self-assembly of fodrin heterotetramers into oligomers as shown for erythrocyte spectrin (Morrow and Marchesi, 1981), posttranslational modifications of ankyrin (Staufenbiel and Lazarides, 1986) or interactions between spectrin binding proteins (Bennett et al., 1988; Ungewickell et al., 1979). Cytosolic ankyrin:fodrin complexes may play a role in interconnecting UNITs on the membrane. This process does not appear to require protein synthesis (Nelson and Veshnock, 1987*a*), indicating that recruitment proceeds directly from these precursor UNIT complexes and cytosolic ankyrin:fodrin. Because the induction of cell–cell contact coincides with the increase in insolubility of ankyrin, fodrin, and the Na^+,K^+-ATPase, we suggest that the formation of the higher-order polymers ($[UNIT]_N$) is initiated at areas of cell–cell and cell-substratum contacts through "micropatching" of membrane proteins and associated protomeric ankyrin-$(\alpha\beta)_2$fodrin complexes, in a manner analogous to that of ligand-induced receptor-cytoskeleton patching in lymphocytes (Nelson et al., 1983; Bourguignon and Bourguignon, 1984); these events may be initiated by uvomorulin (Gumbiner et al., 1988) which is also contained in a complex with ankyrin and fodrin (Nelson, W. J., R. W. Hammerton, E. Shore, and R. Kemler, manuscript in preparation). Micropatching may increase the number and size of nucleation sites on the plasma membrane for ankyrin:fodrin interactions (see above) resulting in a progressive recruitment of these complexes from the cytoplasm to the plasma membrane. The formation of relatively insoluble polymers of $[UNIT]_N$ on the plasma membrane results in the increased metabolic stability of the constituent proteins ($t_{1/2}$ >72 h; Nelson and Veshnock, 1986, 1987*a*). We suggest that UNITs trapped at the apical membrane, as a result of the formation

of the tight junction after cell–cell contact (reviewed by Gumbiner, 1987), do not aggregate and therefore remain metabolically unstable ($t_{1/2}$ ~15 h). As a consequence, these "misplaced" UNITs are gradually lost from the membrane by protein turnover processes. This process of differential stabilization and accumulation of protein complexes in separate plasma membrane domains may play an important role in the overall morphogenesis of the polarized epithelium. The formation of insoluble, metabolically stable complexes of ankyrin, fodrin, and Na^+,K^+-ATPase on the basolateral plasma membrane may function to generate and maintain the polarized distribution of the Na^+,K^+-ATPase and other proteins to this domain of the plasma membrane.

This study has focused on the molecular organization of the membrane-cytoskeleton at steady state, and has proposed a model of how protein complexes present at steady state may play a role in remodeling of the plasma membrane in the biogenesis of epithelial cell polarity. However, at present we know little about the role of the membrane-cytoskeleton in the targeting to and capture of newly synthesized proteins (e.g., Na^+,K^+-ATPase) in plasma membrane domains. Future studies will address this problem by analyzing the steps in the assembly of these complexes from newly synthesized proteins.

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