

# Membrane Domains of Intestinal Epithelial Cells: Distribution of $\text{Na}^+, \text{K}^+$ -ATPase and the Membrane Skeleton in Adult Rat Intestine during Fetal Development and after Epithelial Isolation

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**Abstract.** The organization of the basolateral membrane domain of highly polarized intestinal absorptive cells was studied in adult rat intestinal mucosa, during development of polarity in fetal intestine, and in isolated epithelial sheets. Semi-thin frozen sections of these tissues were stained with a monoclonal antibody (mAb 4C4) directed against  $\text{Na}^+, \text{K}^+$ -ATPase, and with other reagents to visualize distributions of the membrane skeleton (fodrin), an epithelial cell adhesion molecule (uvomorulin), an apical membrane enzyme (aminopeptidase), and filamentous actin. In intact adult epithelium,  $\text{Na}^+, \text{K}^+$ -ATPase, membrane-associated fodrin, and uvomorulin were concentrated in the lateral, but not basal, subdomain. In the stratified epithelium of fetal intestine, both fodrin and uvomorulin were localized in areas of cell-cell contact at 16 and 17 d gestation, a stage when  $\text{Na}^+, \text{K}^+$ -ATPase was not yet expressed. These molecules were excluded from apical domains and from cell surfaces in contact with basal lamina. When  $\text{Na}^+, \text{K}^+$ -ATPase appeared at 18-

19 d, it was codistributed with fodrin. Detachment of epithelial sheets from adult intestinal mucosa did not disrupt intercellular junctions or lateral cell contacts, but cytoplasmic blebs appeared at basal cell surfaces, and a diffuse pool of fodrin and actin accumulated in them. At the same time,  $\text{Na}^+, \text{K}^+$ -ATPase moved into the basal membrane subdomain, and extensive endocytosis of basolateral membrane, including  $\text{Na}^+, \text{K}^+$ -ATPase, occurred. Endocytosis of uvomorulin was not detected and no fodrin was associated with endocytic vesicles. Uvomorulin, along with some membrane-associated fodrin and some  $\text{Na}^+, \text{K}^+$ -ATPase, remained in the lateral membrane as long as intercellular contacts were maintained. Thus, in this polarized epithelium, interaction of lateral cell-cell adhesion molecules as well as basal cell-substrate interactions are required for maintaining the stability of the lateral membrane skeleton and the position of resident membrane proteins concentrated in the lateral membrane domain.

**T**HE plasma membranes of polarized epithelial cells are divided by junctional complexes into apical and basolateral domains that differ both biochemically and morphologically. In epithelia such as intestine and kidney, the polarized distribution of membrane enzymes, channels and transport proteins provides the basis for the vectorial transport functions of the tissue. The basolateral plasma membrane of epithelial cells is commonly considered a single domain for the purposes of biochemical and functional studies, but there is mounting evidence that regions of distinct protein composition exist within it. Recent studies have shown that certain membrane components are enriched in lateral membranes but absent from the basal membrane that contacts the substrate: these include a 100/90/68-kD polypeptide in hepatocytes (Maurice et al., 1988), an unidentified antigen in pancreatic acinar cells (DeLisle et al., 1988) and  $\text{Na}^+, \text{K}^+$ -ATPase in kidney proximal tubule cells (Koob et al., 1987; Morrow et al., 1989). Even within the lateral domain, certain proteins may concentrate in specialized subdomains; for example, the cell adhesion molecule uvomorulin,

is concentrated in the apical adherens junctions of intestinal cells (Boller et al., 1985).

The maintenance of membrane domains and subdomains of distinct composition depends on the ability of the cell to selectively restrict the movement of proteins in the plane of the membrane. Various mechanisms for this have been proposed, in addition to the intramembrane "fence" formed by the tight junction (Simons and Fuller, 1985). Proteins such as receptors that are endocytosed from the basolateral domain may cluster in coated pit subdomains because they associate with each other and/or with adjacent membrane or submembrane proteins (Goldstein et al., 1985). The position of some resident basolateral membrane proteins may be stabilized by specific interactions with molecules on adjacent cells or on the substrate (Ekblom et al., 1986), while others may be stably tethered to the membrane skeleton or other components of the cytoskeleton (Nelson and Hammerton, 1989). The degree to which each of these mechanisms controls the distribution of particular membrane proteins in polarized epithelial cells is not yet clear.

It is clear that association with the membrane skeleton may be sufficient to limit the mobility of specific membrane proteins in erythrocytes, (Fowler and Bennett, 1978; Bennett, 1985) and also in polarized epithelial cells (Nelson, 1989). In intercalated cells of rat kidney, a Band 3-like protein colocalizes with ankyrin and nonerythroid spectrin (fodrin) in the basolateral plasma membrane (Drenckhahn and Merte, 1987; Drenckhahn et al., 1985). In rat kidney epithelial cells  $\text{Na}^+, \text{K}^+$ -ATPase colocalizes and coprecipitates with ankyrin and fodrin (Koob et al., 1987) and ankyrin directly binds  $\text{Na}^+, \text{K}^+$ -ATPase in membrane vesicles derived from canine kidney (Nelson and Veshnock, 1987; Morrow et al., 1989). It has been proposed that the association of these and other "resident" basolateral membrane proteins with the membrane skeleton is the primary mechanism for maintaining the distinct composition of the basolateral domain in kidney epithelial cells (Nelson, 1989).

If so, fodrin and other elements of the membrane skeleton should consistently colocalize with  $\text{Na}^+, \text{K}^+$ -ATPase in other transporting epithelial cells, and the distribution of  $\text{Na}^+, \text{K}^+$ -ATPase in the plasma membrane should be dependent on the integrity of the membrane skeleton. To see if these predictions hold true, we examined the distribution of  $\text{Na}^+, \text{K}^+$ -ATPase, fodrin and actin, along with the cell adhesion molecule uvomorulin and other apical and basolateral membrane proteins, in the highly polarized cells of the simple columnar epithelium of adult rat intestine. We then followed the development of polarized membrane domains in the stratified epithelium of differentiating fetal intestine. Finally, we documented changes in the distribution of these molecules after detachment of the epithelium from the basal lamina and isolation of intact epithelial sheets.

## Materials and Methods

### Reagents and Antibodies

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Sprague Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Reagents used in SDS-PAGE and Western blotting were from Bio-Rad Laboratories (Richmond, CA). Dr. Daniel Louvard (Institut Pasteur, Paris, France) provided rabbit polyclonal antisera raised against laminin and against the alpha subunit of  $\text{Na}^+, \text{K}^+$ -ATPase. Rabbit polyclonal antiserum against human brain fodrin (Harris et al., 1985) was provided by Dr. Alan Harris (Yale University, New Haven, CT). Dr. Rolf Kemler (Max Planck Institute, Tübingen, FRG) provided rabbit antiserum raised against the 80-kD extracellular domain of uvomorulin (Vestweber and Kemler, 1984). Monoclonal antibodies against the following rat intestinal membrane proteins were provided by Dr. Andrea Quaroni (Cornell University, Ithaca, NY): aminopeptidase (Quaroni and Iselbacher, 1985), a 35/40-kD basolateral antigen (Quaroni, A., personal communication), and the beta subunit of  $\text{Na}^+, \text{K}^+$ -ATPase (Marxer et al., 1989). These generous gifts are gratefully acknowledged.

$\text{Na}^+, \text{K}^+$ -ATPase was localized by immunofluorescence using a monoclonal antibody (mAb 4C4) obtained after immunization of a Robertsonian RBF/DN mouse with a membrane preparation from epithelial cells of suckling rat ileum. Methods for production and cloning of these hybridomas were previously described (Wilson et al., 1987). Supernatants were screened by a dot-blot assay of 0.2  $\mu\text{g}$  aliquots of membrane proteins denatured in SDS sample buffer, using rabbit anti-mouse IgG conjugated to horseradish peroxidase or alkaline phosphatase (HyClone Laboratories, Logan, UT). Positive wells were screened by immunofluorescence of 1- $\mu\text{m}$  frozen sections of rat intestinal tissue as described below.

mAb 4C4 was selected as a candidate anti- $\text{Na}^+, \text{K}^+$ -ATPase because it produced specific staining of intestinal cell plasma membranes in a pattern similar to an antibody known to be directed against the beta subunit of  $\text{Na}^+, \text{K}^+$ -ATPase.

### Immunoprecipitation, SDS-PAGE, and Immunoblotting

To establish the antigen specificity of mAb 4C4, the antibody was used for immunoprecipitation from an intestinal cell membrane preparation. Intestinal epithelial cells were isolated from a young adult rat by incubation of inverted intestinal segments in ice-cold cell dissociation buffer containing 76 mM  $\text{Na}_2\text{HPO}_4$ , 19 mM  $\text{KH}_2\text{PO}_4$ , 200 mM sucrose, 60 mM NaOH, and 20 mM EDTA, pH 7.4, for 15 min. This and subsequent solutions contained 0.5 M PMSF, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, and 1  $\mu\text{g}/\text{ml}$  aprotinin. Epithelial cells and sheets were dissociated by shaking, pelleted, and resuspended in cold homogenization solution containing 5 mM histidine, 5 mM imidazole, 0.25 M sucrose, and 0.5 mM EDTA, pH 7.0 (Weiser et al., 1978). After homogenization with 70 strokes of Pestle A in a Dounce homogenizer, the homogenate was spun at 1,000 g for 10 min to pellet nuclei and brush borders, and the supernatant was spun at 20,000 g for 30 min. The resulting membrane pellet was resuspended in 5 mM Tris-HCl containing 0.25 M sucrose and 0.5 mM  $\text{MgCl}_2$ , pH 7.4, and stored at  $-80^\circ\text{C}$ .

Immunoprecipitations were done according to the method of Bartles et al. (1985). Membrane extracts ( $\sim 5$  mg protein) were pre-adsorbed with nonimmune mouse IgG/Sepharose 4B, incubated overnight with 1.5 ml mAb 4C4 culture supernatant, and then adsorbed with Sepharose 4B coupled to anti-mouse IgG (affinity purified; HyClone Laboratories). Beads were washed, boiled in sample buffer, and pelleted; the supernatants were reduced by the addition of 1.5  $\mu\text{l}$  of 130 mg/ml DTT, heated for an additional 2 min, and alkylated with 15  $\mu\text{l}$  of 36 mg/ml iodoacetamide in 2 M sucrose. Samples were run in 7.5% discontinuous polyacrylamide gels (Laemmli, 1970). For immunoblot analysis, proteins were transferred to nitrocellulose filters using a Genie Western blotting apparatus (Idea Scientific, Corvallis, OR).

To identify the antigen precipitated by mAb 4C4, its molecular weight was estimated on Ponceau S-stained Western blots and its reactivity with antibodies against known membrane antigens of similar cellular distribution was tested. Western blots of 4C4 immunoprecipitates and of the crude intestinal cell membrane preparation were immunostained with rabbit polyclonal antisera directed against the alpha subunit of  $\text{Na}^+, \text{K}^+$ -ATPase, or against uvomorulin, and were analyzed in parallel. Antibody binding was visualized with goat anti-rabbit IgG conjugated to alkaline phosphatase (HyClone Laboratories).

### Tissue Preparation

Intact ileal mucosa was collected from ether-anesthetized, young adult rats and fixed by immersion for 3 h at room temperature in either PLP fixative (McLean and Nakane, 1974) containing 10 mM  $\text{NaIO}_4$ , 75 mM lysine, 37 mM  $\text{Na}_2\text{HPO}_4$ , and 2% (wt/vol) freshly depolymerized formaldehyde, or in PF fixative (Matlin et al., 1981) containing 1.75% formaldehyde, 0.1 mM  $\text{CaCl}_2$ , and 0.1 mM  $\text{MgSO}_4$  in PBS. Tissue was equilibrated with 1 M and then 2.3 M sucrose in PBS containing 0.01% thimerosal. Sucrose-infiltrated tissue was mounted on ultramicrotome chucks with Tissue Tek II (Miles Laboratories, Naperville, IL), frozen in liquid freon, and stored in liquid nitrogen. To obtain fetal tissue, 16-19-d timed pregnant rats were anesthetized with 25% (wt/vol) urethane in PBS. Fetuses were removed one at a time without compromising uterine blood circulation, and ileum and colon rapidly excised, fixed, and processed as described above.

Intact sheets of ileal epithelium were isolated from young adult rats by brief intravascular perfusion with EDTA, using the method of Bjerknes and Cheng (1981) modified by Phillips et al. (1984). Epithelial sheets were released by brief agitation, collected, and maintained in RPMI 1640 containing 10% FBS at  $23^\circ\text{C}$  for 5-45 min before fixation. Samples were fixed either in PLP or PF for immunocytochemistry or in the LM/EM fixative described below. PLP- and PF-fixed epithelia were pelleted in a warm solution of 5% gelatin in PBS in a microfuge tube. After hardening the gelatin at  $4^\circ\text{C}$ , the pellet was excised, further fixed for 1 h at room temperature, and then infiltrated with sucrose, mounted and frozen for immunocytochemistry as described above.

### Immunofluorescence

Frozen sections 0.5-1.0  $\mu\text{m}$ -thick were cut with a Reichert-Jung Ultracut E cryomicrotome, mounted on microscope slides coated with a solution of 1% gelatin and 0.1% potassium chromosulfate, and stained with primary antibody followed by rabbit anti-mouse IgG conjugated to rhodamine, or goat anti-rabbit IgG conjugated to rhodamine or fluorescein (HyClone Laboratories). Some gelatin-embedded tissue sections were predigested for 2 min at  $37^\circ\text{C}$  with collagenase (0.5 mg/ml in RPMI with 10% FBS) before immuno-

staining. After rinsing and mounting, sections were photographed with a Zeiss universal PM3 light microscope using Kodak TMax film at ASA 800.

### Light and Electron Microscopy

For microscopy, mucosal tissue or isolated epithelium was immersed in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub> for 1 h at room temperature. Tissues were postfixated in OsO<sub>4</sub>, stained en bloc with 0.5% uranyl acetate, dehydrated through a series of ethanol solutions, and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100C transmission electron microscope. Semithin sections (0.5–1.0 μm) were stained with 1% toluidine blue in 1% sodium borate and photographed with Kodak Ektapan film, ASA 100.

## Results

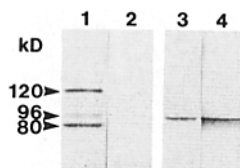
### Na<sup>+</sup>,K<sup>+</sup>-ATPase Is Located Primarily in Lateral Membranes of Intestinal Epithelial Cells

Immunoblot analysis of the material immunoprecipitated from intestinal epithelial membrane extracts by mAb 4C4 confirmed that this monoclonal antibody is directed against Na<sup>+</sup>,K<sup>+</sup>-ATPase (Fig. 1). Anti-uvomorulin antiserum stained bands at 120, 96, and 80 kD in crude membrane protein samples but did not recognize any antigens in 4C4 immunoprecipitates (Fig. 1, lanes 1 and 2). Specific polyclonal antibodies against the alpha subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase recognized a 96-kD band in crude membrane protein samples, and this band was also recognized in 4C4 immunoprecipitates (Fig. 1, lanes 3 and 4).

Immunofluorescent staining of intact intestinal mucosa of adult rats with mAb 4C4 revealed that Na<sup>+</sup>,K<sup>+</sup>-ATPase is located primarily on lateral membranes of intestinal epithelial cells (Fig. 2 a). Immunoreactive Na<sup>+</sup>,K<sup>+</sup>-ATPase was excluded from the apical membrane domain and was sparse or absent in the basal domain. Application of a monoclonal antibody directed against the beta subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase resulted in uniform staining of lateral cell surfaces in a pattern indistinguishable from that of mAb 4C4 (data not shown). mAb 4C4 bound to lateral (but not apical) surfaces of intact intestinal cells in isolated, formalin-fixed epithelial sheets exposed to hybridoma supernatant in suspension (not shown). Thus, the epitope recognized by mAb 4C4 is on the exoplasmic membrane face.

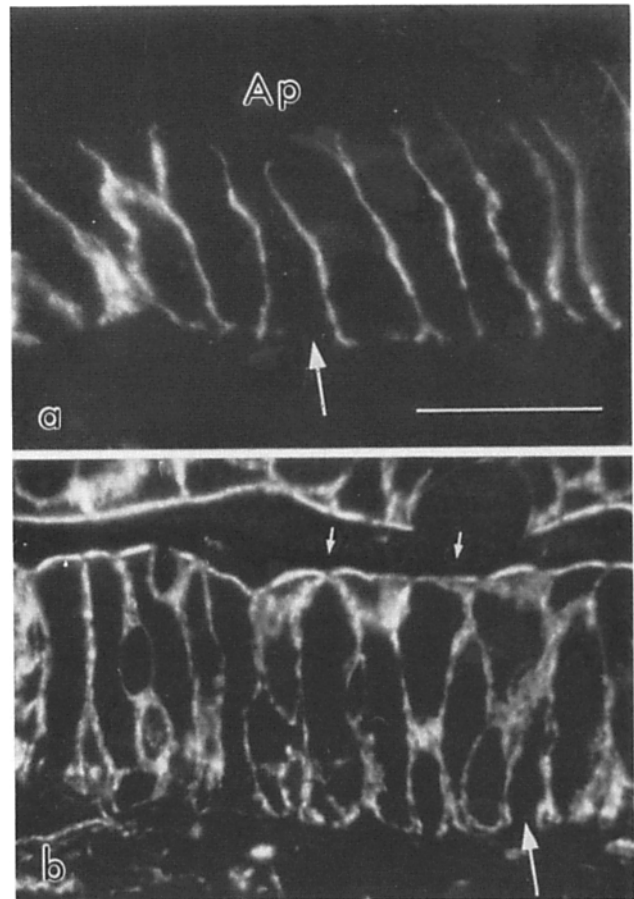
### Codistribution of Na<sup>+</sup>,K<sup>+</sup>-ATPase and Membrane Skeleton in Adult Intestinal Epithelial Cells In Vivo

Having demonstrated that Na<sup>+</sup>,K<sup>+</sup>-ATPase is concentrated



**Figure 1.** Monoclonal antibody 4C4 recognizes Na<sup>+</sup>,K<sup>+</sup>-ATPase. Lanes 1 and 3: SDS-solubilized cell membranes. (Lanes 2 and 4) mAb 4C4 immunoprecipitates. Specific bands at 96 kD were visualized in lanes 2 and 4 with Ponceau red (not shown). Lanes

1 and 2, stained with anti-uvomorulin antiserum, show that in the crude membrane preparation (lane 1), the antiserum recognizes major bands at 80 and 120 kD as previously described (Ekblom et al., 1986) as well as a minor 96-kD band, but the antiserum does not recognize any protein immunoprecipitated by mAb 4C4 (lane 2). Antiserum against the alpha subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase recognizes a 96-kD band in crude cell membranes (lane 3); this band is also recognized in mAb 4C4 immunoprecipitates (lane 4).

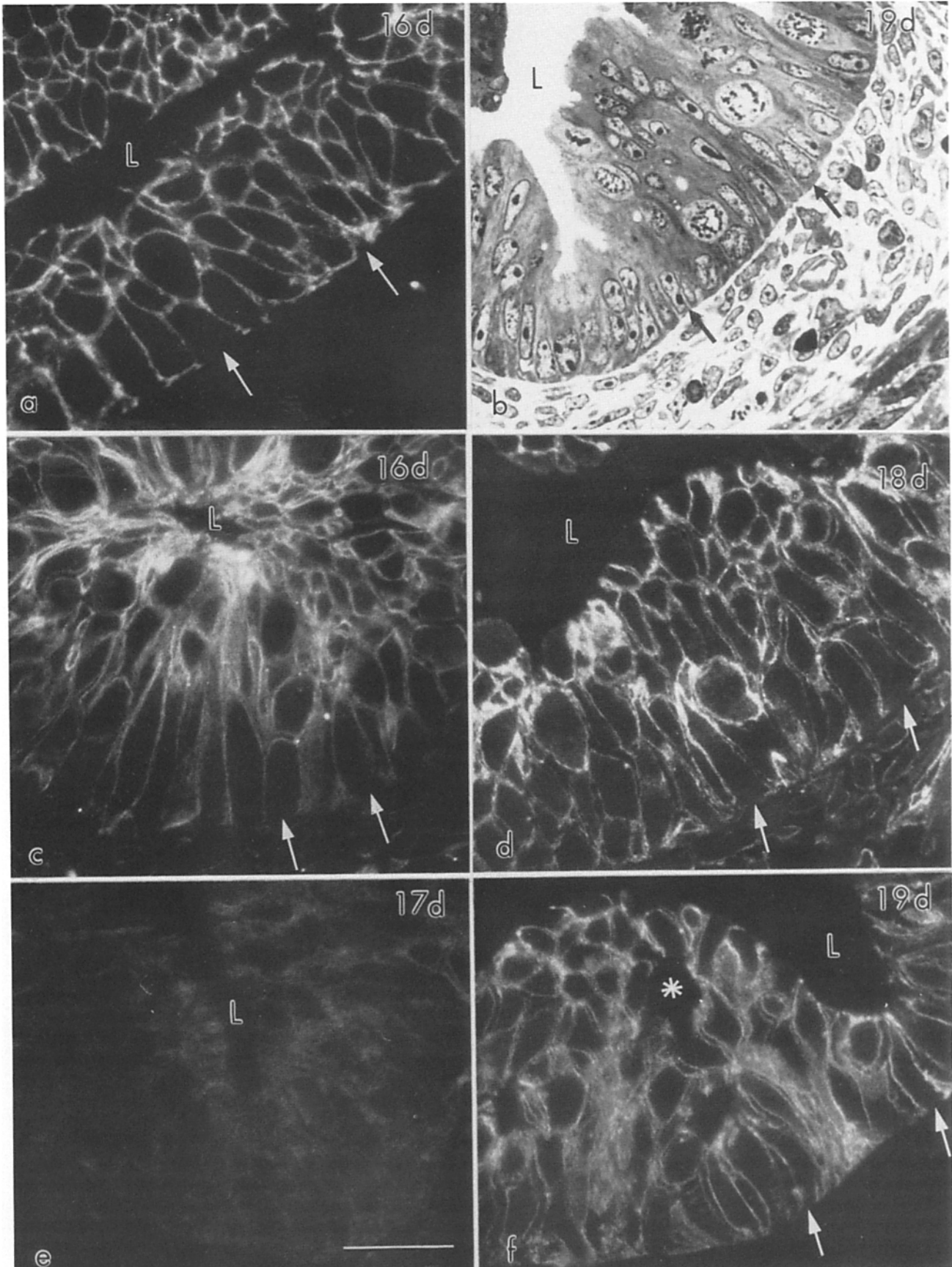


**Figure 2.** Immunofluorescent localization of Na<sup>+</sup>,K<sup>+</sup>-ATPase and fodrin in adult intestinal epithelium. (a) Staining with mAb 4C4 shows that Na<sup>+</sup>,K<sup>+</sup>-ATPase is concentrated in lateral membranes. Note the absence of staining on apical (Ap) and basal (arrow) surfaces of the epithelium. (b) Staining with anti-fodrin antiserum shows that fodrin, like Na<sup>+</sup>,K<sup>+</sup>-ATPase, is associated with lateral membranes but is largely absent from the basal surface of epithelial cells (large arrow). Apical staining is due to cross-reactivity with brush border spectrin in the terminal web. Small arrows mark the position of microvillar tips. Bar, 20 μm.

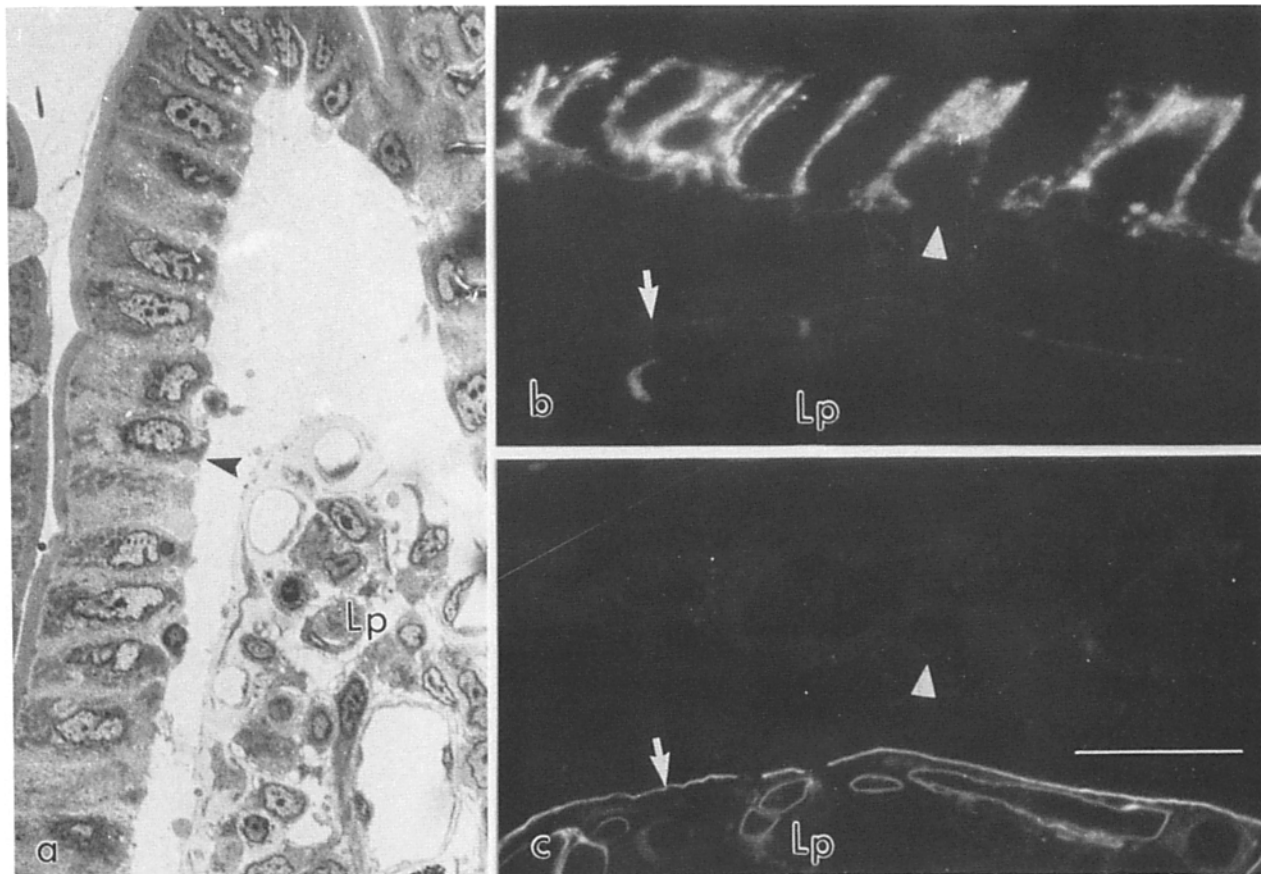
in the lateral plasma membrane, we sought to determine whether fodrin shows a similar distribution. The anti-fodrin antibodies used in this study recognized both of the spectrin-like proteins known to be present in intestinal epithelial cells: fodrin and a brush border-specific spectrin associated with the apical terminal web (Mooseker, 1985). The latter protein could be distinguished from fodrin by immunofluorescence because it is known to be associated only with the terminal web cytoskeleton immediately below the microvillus border (here verified with Nomarski optics) and not with the plasma membrane. Membrane-associated immunostaining presumably representing fodrin was associated only with the basolateral cell surface and was largely restricted to the lateral membrane (Fig. 2 b).

### Distribution of Domain-specific Membrane Proteins and Membrane Skeleton in the Stratified Intestinal Epithelium during Fetal Development

We examined the developing intestine to determine to what extent Na<sup>+</sup>,K<sup>+</sup>-ATPase, membrane-associated fodrin, and



**Figure 3.** Immunofluorescent localization of uvomorulin (*a*), fodrin (*c* and *d*), and  $\text{Na}^+, \text{K}^+$ -ATPase (*e* and *f*) in fetal intestine. At the gestational ages examined, the epithelium of both ileum and colon is stratified, as shown in a toluidine blue-stained section (*b*). Uvomorulin (*a*) and fodrin (*c* and *d*) are present from early in development and are both localized on plasma membranes involved in cell-cell contact.



**Figure 4.** Detachment of intestinal epithelium immediately after EDTA perfusion. (a) Toluidine blue stained section showing separation of an epithelial sheet from the lamina propria (*Lp*). (b) Immunofluorescent staining with mAb 4C4 shows that immediately after separation epithelial  $\text{Na}^+, \text{K}^+$ -ATPase is still concentrated in the lateral membrane. (c) The same tissue section as in *b* stained with anti-laminin shows that the basal lamina (*arrow*) remains on the lamina propria (*Lp*) and is not associated with the basal surface of the epithelium (*arrow-head*). Bar, 20  $\mu\text{m}$ .

the cell adhesion molecule uvomorulin are colocalized before establishment of the simple columnar epithelium. During the last week of gestation of fetal rats, the entire intestinal epithelium is converted from stratified to simple columnar. At 16 d gestation, the epithelium consists of a multilayer of undifferentiated cells. Cells in the uppermost layer that contact the lumen are joined by tight junctions and thus have apical domains, cells in contact with the basal lamina have basal domains, and cells in between are not polarized. Between 17 and 20 d, the epithelium undergoes a complex process involving the establishment of secondary lumens with tight junctions within the stratified epithelium, fusion of these lumens with the primary lumen, and sloughing of cells (Mathan et al., 1976; Madara et al., 1981; Colony and Neutra, 1983).

Application of uvomorulin antibodies to sections of stratified fetal ileal and colonic epithelium revealed that, as expected, this cell adhesion molecule was present at 16 d gestation, the earliest stage examined (Fig. 3 *a*). Uvomorulin was present in all areas of cell-cell contact; in some sections there were points of higher concentration corresponding to

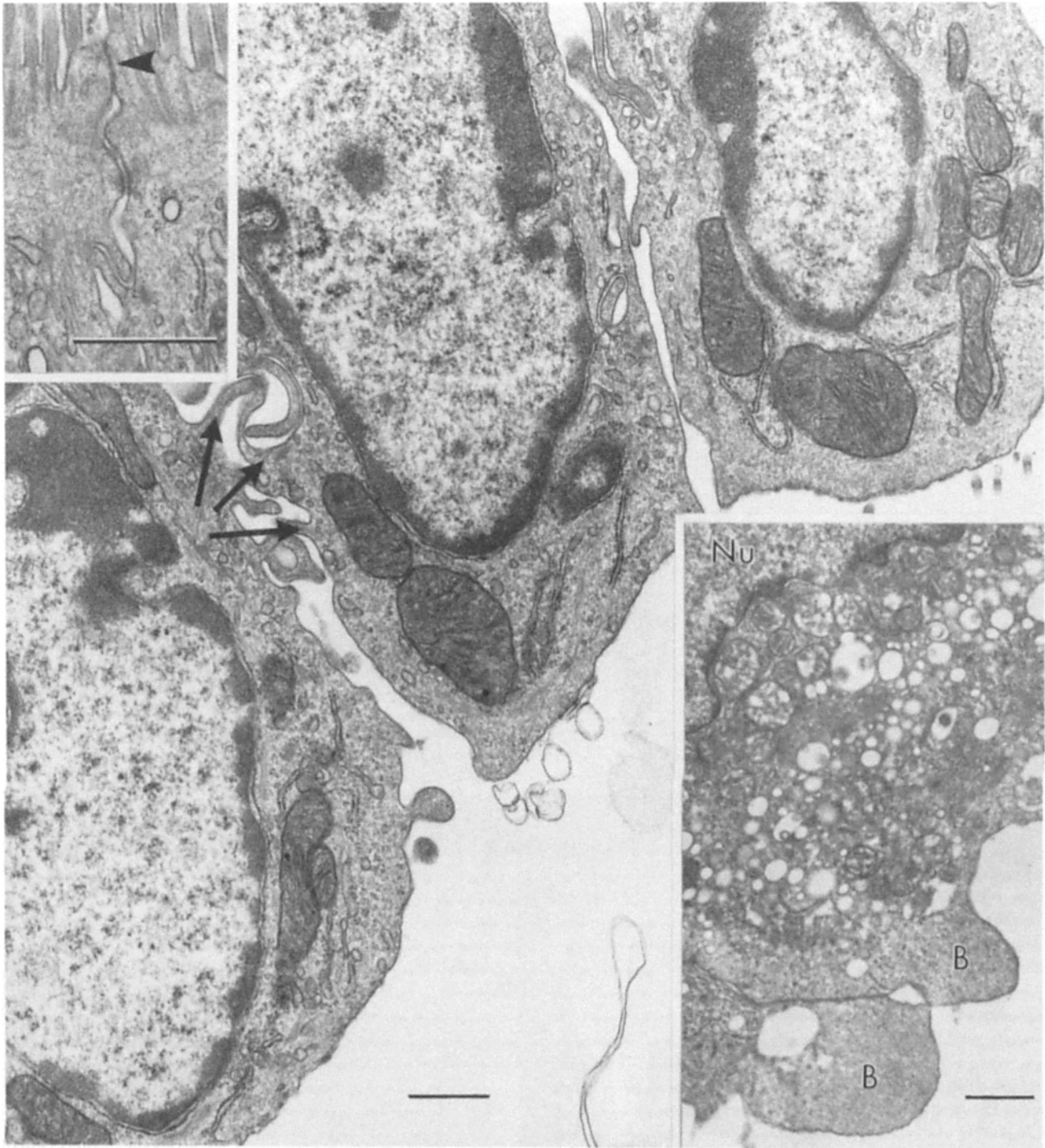
apical adherens junctions (Boller et al., 1985) and to other close contacts of lateral membranes. The distribution of uvomorulin was unchanged at 19 d gestation (not shown). At 19 d, the conversion process is underway in fetal ileum and colon, but the epithelium is still stratified (Fig. 3 *b*).

At all stages examined, fodrin was uniformly distributed on plasma membranes of unpolarized cells but was sparse or absent on membranes contacting the basal lamina (Fig. 3, *c* and *d*). At 16 and 18 d, there was no anti-fodrin immunoreactivity associated with apical cell poles facing either primary or secondary lumens (Fig. 3 *d*), presumably because brush border spectrin was not yet present. Immunoreactivity in the terminal web region appeared at 19–20 d, a time when the brush border cytoskeleton is undergoing rapid assembly.

In contrast to uvomorulin and fodrin,  $\text{Na}^+, \text{K}^+$ -ATPase was not present at 16 d gestation (Fig. 3 *e*). It first appeared in ileum at 17 d and in colon at 18 d, coinciding with initial appearance of basolateral phosphatase activity (Colony and Neutra, 1983). At 19 d, it was distributed uniformly over the entire plasma membrane of cells within the stratified epithelium but was excluded from the apical membrane of cells

In contrast,  $\text{Na}^+, \text{K}^+$ -ATPase is not present at 17 d gestation but is present on all cells at 19 d (*f*). All three proteins are excluded from membranes facing the lumen (*L*) or contacting the basal lamina (*arrows*) but are distributed over the entire surface of unpolarized cells within the stratified epithelium. The asterisk indicates an intra-epithelial secondary lumen. Bar, 20  $\mu\text{m}$ .





**Figure 5.** Electron micrograph of isolated epithelium immediately after separation from the basal lamina. Cell ultrastructure is normal, lateral interdigitations are present (*arrows*), and the basal plasma membrane is intact. (*Upper inset*) The apical junctional complex, including tight junctions (*arrowhead*), and lateral cell-cell contacts are unaffected by the isolation procedure. (*Lower inset*) Basal surface of an intestinal epithelial cell 15 min after separation from the basal lamina. A large population of endocytic vesicles appears in the basal cytoplasm between the nucleus (*Nu*) and actin-rich blebs (*B*) Bars: 0.1  $\mu\text{m}$ ; (*insets*) 0.05  $\mu\text{m}$ .

contacting the main lumen or secondary lumens, and was sparse or absent on the basal membrane of cells contacting the basal lamina (Fig. 3 *f*).

***Loss of Substrate Contact Results in Lateral Diffusion and Endocytosis of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, and Alterations in the Membrane Skeleton***

To explore the role of cell-substrate contact in maintaining

the positions of resident plasma membrane proteins and elements of the membrane skeleton, we examined the distribution of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and other membrane proteins, as well as fodrin and actin, in sheets of intestinal epithelium immediately after loss of contact with basal lamina, and during subsequent maintenance for up to 45 min *in vitro*. Brief intravascular perfusion with  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free buffer containing 30 mM EDTA resulted in detachment of basal sur-

faces of the intestinal epithelium from the basal lamina (Fig. 4 *a*). Immediately after detachment (time 0), Na<sup>+</sup>,K<sup>+</sup>-ATPase maintained a lateral distribution (Fig. 4 *b*). All detectable laminin immunoreactivity remained associated with the surface of the lamina propria, indicating that basal lamina did not adhere to the basal surface of the isolated epithelium (Fig. 4 *c*). Electron microscopy of the isolated epithelial sheets at time 0 revealed no ultrastructural changes at lateral cell surfaces: lateral interdigitations and cell-cell contacts were intact (Fig. 5) and tight junctions were of normal structure (Fig. 5, *top inset*).

Epithelial sheets were rapidly harvested by shaking from the mucosa into serum-supplemented culture medium containing normal concentrations of calcium and magnesium ions. At 5 and 15 min after isolation, tight junctions and lateral cell-cell contacts were still intact, but basal cell surfaces had developed cytoplasmic processes and blebs, as previously reported in intestine (Phillips et al., 1984) and in other epithelia (Sugrue and Hay, 1981). Electron microscopy of epithelial sheets 15 min after isolation revealed that the basal blebs contained a filamentous matrix but no organelles, and numerous clear vesicles were located between the blebs and the nucleus (Fig. 5, *bottom inset*). By 30 min, lateral intercellular interdigitations had disappeared, but close lateral cell-cell contacts and tight junctions were intact. After 45 min, however, many cells had detached from the epithelial sheets. Within some sheets, cells showed open tight junctions and loss of lateral contacts, while other sheets were intact.

Immunostaining of isolated epithelia 5 and 15 min after isolation revealed that Na<sup>+</sup>,K<sup>+</sup>-ATPase was no longer confined to the lateral domain; it was present in the basal domain and also in internal vesicles along the lateral membrane and below the nucleus (Fig. 6 *a*). The rapid appearance of these vesicles suggests that they represent an endocytic compartment derived from plasma membrane. Na<sup>+</sup>,K<sup>+</sup>-ATPase was excluded from the apical membrane domain and apical cytoplasmic vesicles. A 35/40-kD basolateral antigen was also internalized into basal vesicles over the same time course as Na<sup>+</sup>,K<sup>+</sup>-ATPase (Fig. 6 *b*). Uvomorulin, in contrast, maintained its lateral position and focal concentrations; it did not move to the basal domain and did not appear in internal vesicles (Fig. 6 *c*). By 15 min after isolation, staining of lateral membranes with anti-fodrin antiserum decreased in intensity and diffuse fodrin immunoreactivity appeared in the basal cytoplasmic blebs (Fig. 6 *d*). There was no detectable fodrin associated with the internal vesicular compartment containing Na<sup>+</sup>,K<sup>+</sup>-ATPase. Apical staining of the terminal web, presumably representing brush border spectrin, showed no changes in isolated epithelial sheets as long as tight junctions were intact. Actin associated with the apical brush border also appeared to be unaffected by epithelial isolation, but the distribution of basolateral actin changed such that with time, an increasing amount of actin was present in the blebs at the basal surface, and a decreasing amount was associated with the lateral membrane (Fig. 6 *e*). Aminopeptidase, a microvillar hydrolase normally restricted to the apical membrane, remained in the apical domain as long as tight junctions were intact and did not enter internal compartments (Fig. 6 *f*).

By 45 min after isolation, cells that had lost junctional complexes and lateral intercellular contacts were rounded, but microvilli and cytoplasmic blebs nevertheless remained at opposite poles (Fig. 7). In such cells, immunostaining re-

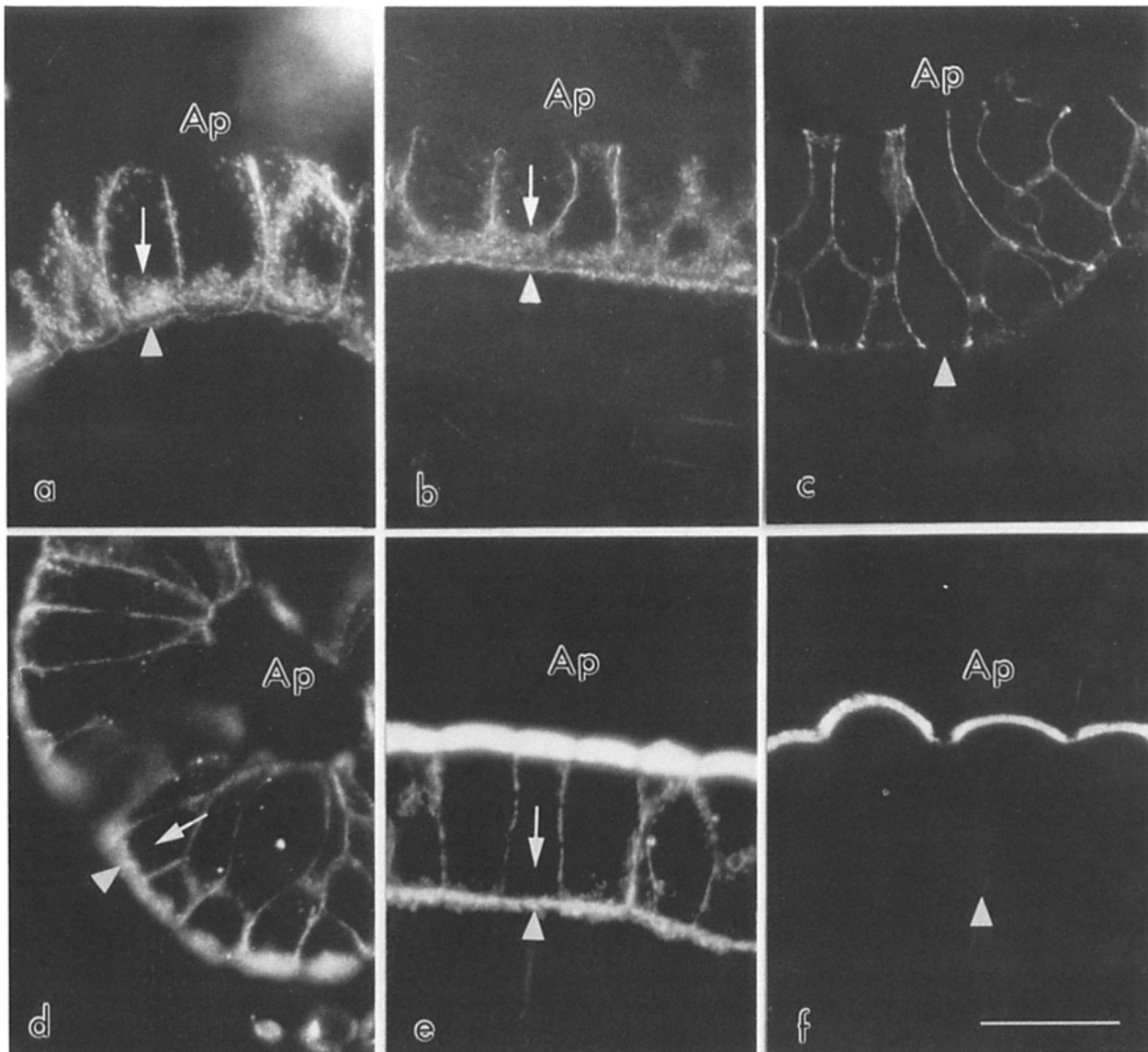
vealed that all Na<sup>+</sup>,K<sup>+</sup>-ATPase had been internalized (Fig. 7 *a*). Diffuse anti-fodrin staining remained in the cytoplasmic blebs and some immunoreactivity (presumably brush border spectrin) remained associated with the expanded brush border, but no detectable fodrin was associated with endocytic vesicles (Fig. 7 *b*). With the loss of tight junctions, aminopeptidase was no longer confined to the microvillus border, but had diffused in the plane of the membrane over the entire cell surface (Fig. 7 *c*) as previously described (Ziomek et al., 1980). Unlike Na<sup>+</sup>,K<sup>+</sup>-ATPase, however, aminopeptidase did not appear in intracellular vesicles.

## Discussion

In this study we have shown that Na<sup>+</sup>,K<sup>+</sup>-ATPase is concentrated in the lateral plasma membranes of intestinal epithelial cells, and is largely excluded from basal cell surfaces. Lateral distribution of this membrane ion pump in villus absorptive cells is consistent with current understanding of ion and water absorption in the intestinal villus epithelium. According to the standing gradient model for production of an isotonic absorbate proposed by Diamond and Bossert (1967), an osmotic gradient is established within the epithelium by active pumping of sodium ions along the length of the lateral membrane. Because villus cell tight junctions are relatively impermeable, the highest osmotic concentration develops at the sealed (apical) end of the intercellular space, and water is drawn into this space transcellularly through the interdigitated lateral membranes of adjacent cells, to produce an isotonic absorbate that enters the lamina propria. In the crypts, lateral Na<sup>+</sup>,K<sup>+</sup> pump sites would also increase intercellular Na<sup>+</sup> concentration, but because crypt tight junctions are relatively permeable to monovalent cations, the osmotic gradient would result in net movement of Na<sup>+</sup> and water through the junctions into the lumen (Armstrong, 1987). The efficiency of both villus absorptive and crypt secretory systems would be increased by exclusion of pump sites from basal cell surfaces.

Restriction of Na<sup>+</sup>,K<sup>+</sup>-ATPase to the lateral subdomain of the basolateral cell surface has not been reported in cultured MDCK cell monolayers but has been observed in transporting epithelial cells of kidney distal tubules *in vivo* (Koob et al., 1987). In the epithelial cells of bile ducts, that also function in ion/water transport, we have observed that Na<sup>+</sup>,K<sup>+</sup>-ATPase (visualized by immunofluorescent staining) was also concentrated on lateral surfaces (data not shown). Other membrane antigens of unknown function have been found to be concentrated in lateral membranes of hepatocytes (Maurice et al., 1988) and pancreatic acinar cells (DeLisle et al., 1988), and a membrane-associated proteoglycan was restricted to the lateral surfaces of a variety of mouse epithelial cells (Hayashi et al., 1987). Taken together, these data establish that the lateral membrane can be considered a chemically and functionally distinct subdomain of the basolateral cell surface.

How is the distinct protein composition of the lateral membrane subdomain maintained? A current hypothesis derived from MDCK cells is that interaction between the membrane-associated ankyrin/fodrin network and specific integral membrane proteins, including Na<sup>+</sup>,K<sup>+</sup>-ATPase, is responsible for immobilization of "resident" proteins in the basolateral membrane and thus for the distinct composition of this domain (Nelson and Veshnock, 1986). Our data show that in intestinal cells, the fodrin-based membrane skeleton is asso-

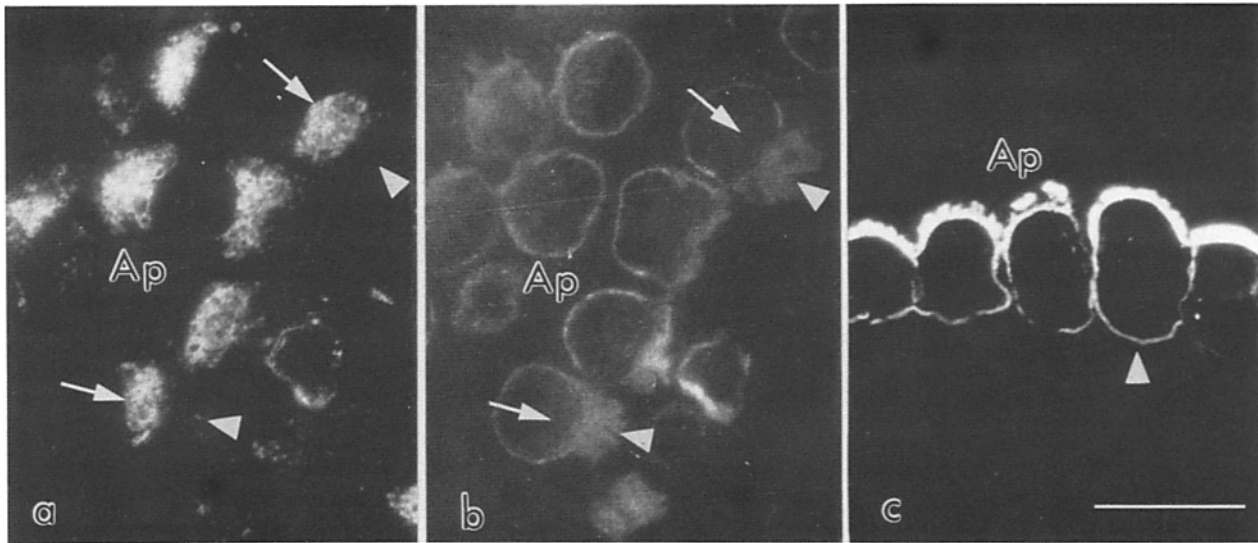


**Figure 6.** Distribution of basolateral membrane proteins and cytoskeletal proteins in intestinal epithelium 15 min after isolation of the epithelium from the basal lamina. (a)  $\text{Na}^+, \text{K}^+$ -ATPase, (b) 35/40-kD membrane glycoprotein, (c) uvomorulin, (d) fodrin, (e) actin, and (f) aminopeptidase. Actin is stained with Phalloidin-rhodamine; all other proteins are stained immunocytochemically. Dramatic reorganization of the basolateral membrane domain occurs after epithelial isolation.  $\text{Na}^+, \text{K}^+$ -ATPase (a) and the 35/40-kD glycoprotein (b) are present in both lateral and basal domains. In addition, they are endocytosed and appear on the membranes of endocytic vesicles (arrows) which accumulate in the basal cytoplasm. Fodrin (d) and actin (e) are not associated with these internalized membrane proteins; they appear as a diffuse pool in the blebs (arrowheads) at the basal surface of the epithelium. Uvomorulin (c) and aminopeptidase (f) distribution are not affected by the isolation procedure as long as intercellular contacts remain intact. Bar, 20  $\mu\text{m}$ .

ciated only with the lateral membrane subdomain; the basal membrane must be stabilized by other mechanisms such as interaction of actin-linked membrane proteins with the extracellular matrix (Ingber et al., 1986). We further show that loss of contact with the basal lamina in intestinal cells allows movement of both  $\text{Na}^+, \text{K}^+$ -ATPase and fodrin into the basal domain, even when lateral cell-cell contacts are intact. It is possible that both proteins are excluded from the basal domain in intact epithelium by steric hindrance of basal actin-ECM receptor complexes. This is supported by our (unpublished) observation that in hepatocytes,  $\text{Na}^+, \text{K}^+$ -ATPase is not excluded from the sinusoidal domain, a basal cell surface lacking a well-organized basal lamina.

It is not clear how the membrane skeleton itself is stabilized on lateral membranes of intestinal epithelial cells. Initial assembly would likely depend on association with a membrane protein that is polarized by other factors. It is unlikely that this membrane protein anchor is  $\text{Na}^+, \text{K}^+$ -ATPase, since our observations of fetal epithelium demonstrate that the membrane skeleton is assembled and maintained on lateral cell surfaces before synthesis of  $\text{Na}^+, \text{K}^+$ -ATPase. Uvomorulin, on the other hand, is expressed early in the fetal epithelium and is polarized by binding to ligands on adjacent cells. In MDCK cells, lateral cell-cell contact is a prerequisite for stable assembly of the basolateral membrane skeleton and the polarized distribution of  $\text{Na}^+, \text{K}^+$ -ATPase (Nelson and Vesh-





**Figure 7.** Distribution of basolateral proteins in intestinal cells dissociating from epithelial sheets 45 min after isolation of the epithelium. *a* and *b* show the same section double stained with  $\text{Na}^+, \text{K}^+$ -ATPase (*a*) and anti-fodrin (*b*). Arrowheads indicate the blebbing basal surface where fodrin accumulates; arrows indicate the intracellular compartment containing  $\text{Na}^+, \text{K}^+$ -ATPase. (*c*) The apical membrane protein, aminopeptidase, moves into the basolateral domain as cells begin to dissociate but, unlike  $\text{Na}^+, \text{K}^+$ -ATPase, it is not endocytosed. Bar, 20  $\mu\text{m}$ .

nock, 1986) and  $\text{Na}^+, \text{K}^+$ -ATPase, ankyrin, fodrin, and uvomorulin can be co-extracted from MDCK cells as a complex (Nelson and Hammerton, 1989). In isolated intestinal epithelium some uvomorulin remained on the lateral cell surface as long as cell contacts were maintained, and some fodrin was consistently colocalized with uvomorulin. In contrast, uvomorulin was not present in the  $\text{Na}^+, \text{K}^+$ -ATPase-containing endocytic compartment formed after loss of substrate contact and no fodrin was associated with this compartment. These results provide further evidence that interaction of cell-cell adhesion molecules is required for maintaining the polarized distribution of the membrane skeleton (Nelson, 1989). In addition, this phenomenon may provide a useful model for examining the association and dissociation of these protein assemblies.

Our observation that isolation of the intestinal epithelium from the substrate results in rapid endocytosis of basolateral membrane, including  $\text{Na}^+, \text{K}^+$ -ATPase, is also relevant to studies of epithelial cell physiology. Internalization of basolateral membrane and  $\text{Na}^+, \text{K}^+$ -ATPase after loss of normal epithelial cell-substrate contact may be a general phenomenon, as it has also been reported in MDCK cells (Sabatini et al., 1983; Smith et al., 1988; Zuk et al., 1989). This phenomenon should be taken into account when interpreting experiments in which isolated epithelial cells or epithelial sheets are used to study basolateral receptor-ligand interactions or ion transport activities.

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