

Studies on the Development and Maintenance of Epithelial Cell Surface Polarity with Monoclonal Antibodies

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ABSTRACT We examined epithelial cell surface polarity in subconfluent and confluent Madin-Darby canine kidney (MDCK) cells with monoclonal antibodies directed against plasma membrane glycoproteins of 35,000, 50,000, and 60,000 mol wt. The cell surface distribution of these glycoproteins was studied by immunofluorescence and immunoelectron microscopy. At the ultrastructural level, the electron-dense reaction product localizing all three glycoproteins was determined to be uniformly distributed over the apical and basal cell surfaces of subconfluent MDCK cells as well as on the lateral surfaces between contacted cells; however, after formation of a confluent monolayer, these glycoproteins could only be localized on the basal-lateral plasma membrane. The development of cell surface polarity was followed by assessing glycoprotein distribution with immunofluorescence microscopy at selected time intervals during growth of MDCK cells to form a confluent monolayer. These results were correlated with transepithelial electrical resistance measurements of tight junction permeability and it was determined by immunofluorescence that polarized distributions of cell surface glycoproteins were established just after electrical resistance could be detected, but before the development of maximal resistance. Our observations provide evidence that intact tight junctions are required for the establishment of the apical and basal-lateral plasma membrane domains and that development of epithelial cell surface polarity is a continuous process.

The specialized functions of epithelia such as absorption, secretion, and vectorial ion transport are dependent on the existence of two distinct cell surfaces; the apical (or mucosal) domain which is separated from the basal-lateral (or serosal) surface by a circumferential occluding belt of tight junctions (1). These two membrane domains are in contact with different environments and each exhibits unique structural and biochemical components that are responsible for the specialized physiological properties of epithelia.

Ultrastructural examination of a variety of epithelial tissues has demonstrated structural polarity with plasma membrane specializations such as microvilli and cilia being confined to the apical cell surface while intercellular junctions are localized to the basal-lateral membrane (1, 2). Structural polarity has also been observed by freeze-fracture electron microscopy with the intramembrane particle density being higher on the basal-lateral than the apical plasma membrane (3-5). Biochemical studies of purified apical and basal-lateral mem-

brane preparations from renal proximal tubule and small intestine have shown that these plasma membrane regions differ in composition with each membrane domain having their own characteristic set of enzymes, polypeptides, and lipids (6-11). The asymmetric distribution of cell surface components has been confirmed by autoradiography and immunocytochemistry (12-16).

Although epithelial cell surface polarity has been demonstrated structurally and biochemically, we still have limited insight into what cellular processes are involved in the biogenesis of the biochemically unique apical and basal-lateral membrane domains. Tight junctions have been proposed to play a role in the maintenance of epithelial polarity, acting as a molecular barrier to the intermixing of mobile cell surface macromolecules from the apical and basal-lateral domains (5, 17-19). The subject of epithelial polarity, its maintenance and biogenesis has been extensively documented in two recent reviews (20, 21).

The Madin-Darby canine kidney (MDCK)¹ epithelial cell line is ideally suited for studying cell polarity. It is morphologically polarized having apical microvilli and basal-lateral membranes with lateral interdigitations (22–25) and asymmetric distributions of plasma membrane intramembrane particles (18, 26) similar to those described for native epithelia (3–5). Biochemical and immunological studies have demonstrated that the MDCK cell surface has asymmetric distributions of membrane proteins (27–29) and lipids (30). When grown on permeable substrates MDCK monolayers can generate transepithelial electrical resistances of 100–300 ohms · cm² (23–25, 31–33) demonstrating the presence of functional tight junctions (34). These results are supported by morphological studies showing that tight junctions seal the lateral space between adjacent MDCK cells (24, 33).

We used the hybridoma technique of Kohler and Milstein (35) to produce monoclonal antibodies directed against the MDCK cell surface (36) to study the development of epithelial cell surface polarity and the role of tight junctions in this process. In this report we present evidence that establishment of epithelial cell surface polarity is a continuous process that is closely linked to tight junction assembly. Portions of this work have been published elsewhere (37, 38).

MATERIALS AND METHODS

Cell Culture

MDCK and P3U-1 mouse myeloma cell lines were grown and maintained as described previously (36). For experimental procedures, MDCK cells were plated on coverglasses and 0.45- μ m pore size micropore filters (Millipore/Continental Water Systems, Bedford, MA) in 24 cluster multiwells at a density of 1.5×10^5 cells/well. Cultures were fed daily with complete Eagle's minimal essential medium with Earle's salts, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% fetal calf serum. Primary dog kidney fibroblasts were obtained from adult dog kidneys according to the procedure of Taub et al. (39) and maintained as described previously (36). All cell culture materials were obtained from Gibco Laboratories (Grand Island, NY) and culture plasticware from Falcon Labware (Oxnard, CA).

Hybridoma Cell Lines

For these studies we used monoclonal antibody 11B8 that had been extensively characterized previously (36) as well as produced additional hybridomas that secrete antibodies directed against the MDCK cell surface. BALB/c mice were immunized with two separate intraperitoneal injections of 10^7 MDCK cells and hybridoma cell lines produced according to the protocol of Gefer et al. (40) as described previously (36).

Radioimmunoassay

A solid phase indirect radioimmunoassay (RIA) was used to screen for hybridomas secreting mouse antibodies directed against antigens on the MDCK cell surface. ¹²⁵I-labeled goat anti-mouse IgG (GAM) was prepared according to published procedures (41). Briefly, GAM (all antibody reagents were purchased from Cappel Laboratories, Inc., Cochranville, PA) was bound to an affinity column containing mouse IgG bound to cyanogen bromide-activated Sepharose 4B and iodinated on the column in the presence of chloramine-T (42). The ¹²⁵I-labeled GAM was eluted from the column with 0.2 M glycine (pH 2.3) –1% bovine serum albumin (BSA) into an equal volume of 0.2 M Tris-HCl (pH 8.6) –1% BSA. All radioisotopes were purchased from New England Nuclear (Boston, MA). Aggregates of ¹²⁵I-GAM were removed by gel filtration on a column of Sephacryl 300 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and the pooled samples stored at –20°C. Specific activity for ¹²⁵I-GAM was 10 μ Ci/ μ g protein.

The RIA was performed on subconfluent monolayers of MDCK in 96 cluster microtiter plates (2×10^4 cells/well) to ensure that antibodies had access

¹ Abbreviations used in this paper: BSA, bovine serum albumin; BSA-PBS, phosphate-buffered saline containing 2% BSA; GAM, goat anti-mouse IgG; HRP-GAM, GAM coupled to horseradish peroxidase; MDCK, Madin-Darby canine kidney; and RIA, radioimmunoassay.

to both the apical and basal cell surfaces. Parallel measurements were done on the dog kidney fibroblasts to determine whether positive MDCK antibodies were also epithelial specific. Live intact cells and fibroblasts were screened by RIA as follows: after incubation with hybridoma supernatant for 30 min at 4°C the cells were washed extensively with cold phosphate-buffered saline containing 2% BSA (BSA-PBS), then incubated in ¹²⁵I-GAM (10⁵ cpm/well) for 30 min at 4°C. After a thorough washing, cells were solubilized in 30 μ l of 2% Triton X-100 at 24°C and the radioactivity measured in a Beckman gamma counter.

To determine if any of the antibodies used in this study were against dog kidney Na⁺-K⁺ ATPase we obtained purified ATPase and antibody against the enzyme (provided by B. Forbush and D. Biemesderfer, Yale University School of Medicine) and performed the RIA as described previously (36).

Characterization of Membrane Proteins

METABOLIC LABELING: 10⁶ MDCK cells were allowed to grow in 100-mm plates, for 24 h. The medium was removed and replaced by methionine-deficient MEM (3 μ g methionine/ml) containing [³⁵S]methionine (100 μ Ci/ml) and 10% dialyzed fetal calf serum. To label membrane glycoproteins, we grew MDCK in glucose-deficient MEM (50% of normal glucose), [³H]glucosamine (50 μ Ci/ml) and 10% fetal calf serum. After 24 h growth, the medium was removed and the labeled cells solubilized in 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 10 U/ml aprotinin for 1 h at 4°C. The detergent-extracted material was centrifuged for 2 min at 4°C in an Eppendorf microcentrifuge (Brinkman Instruments, Palo Alto, CA), the supernatant was removed, aliquoted, and stored at –20°C.

IMMUNOPRECIPITATION: Membrane proteins from detergent extracts of isotopically labeled MDCK were immunoprecipitated according to the procedures of Mellman and Unkeless (43). 100 μ l of extract was incubated with 200 μ l of hybridoma supernatant in a microcentrifuge tube with constant agitation for 2 h at 4°C. Then 50 μ l of a 50% slurry of Sepharose 4B coupled to goat anti-mouse F(ab')₂ (Cappel Laboratories, Cochranville, PA) was added to the extract and agitated for an additional 2 h at 4°C. The Sepharose beads were then washed five times at 24°C in the microcentrifuge tube, with a solution containing: 0.25% Triton X-100, 0.1 M, NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 U/ml aprotinin, 1 mg/ml ovalbumin, and 50 mM Tris-HCl (pH 7.2). After the last wash, the Sepharose was pelleted in the microcentrifuge and the immunoprecipitated antigens eluted from the beads with 2% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml ovalbumin 1% β -mercaptoethanol, and 50 mM Tris-HCl (pH 6.7) by placing the tube in boiling H₂O for 3 min.

SDS PAGE: Immunoprecipitated MDCK cell surface proteins were identified by 12.5% SDS PAGE with a discontinuous buffer system (44). The gels were fixed and stained in a solution containing 0.4% Coomassie Blue, 50% methanol, and 7% acetic acid, then prepared for fluorography (45). Kodak XR1 x-ray film was exposed by the dried gels at –70°C.

Immunofluorescence Microscopy

MDCK cells grown on coverglasses and micropore filters were fixed with 4% paraformaldehyde–0.1% glutaraldehyde in PBS (pH 7.4) for 30 min (all steps were done at 4°C). The fixed cells were washed for at least 1 h in PBS and incubated in PBS-BSA for 15 min to quench free aldehyde groups. While washing in PBS, the coverglasses and filters were gently scored with forceps to induce small tears in the monolayer. The PBS-BSA was removed and the MDCK cells stained for immunofluorescence by incubating sequentially for 30 min each in (a) hybridoma supernatant, (b) rabbit anti-mouse IgG, and (c) affinity-purified rhodamine-labeled goat anti-rabbit IgG. After each incubation, the cells were thoroughly washed with BSA-PBS and then PBS alone. The cells were postfixed with 4% paraformaldehyde–0.1% glutaraldehyde for 15 min, mounted in glycerol, viewed with Zeiss epifluorescence optics, and photographed with Kodak Tri-X film. For comparative purposes, all photographs are taken at the same exposure, developed for the same length of time at 68°F and printed with identical exposure times.

Electron Microscopy

All steps were carried out at 4°C unless noted. Subconfluent MDCK cells grown on both coverglasses and micropore filters and confluent cells grown in culture dishes were fixed with 4% paraformaldehyde–0.1% glutaraldehyde for 30 min, washed overnight in PBS and then for 30 min in BSA-PBS. During the BSA-PBS incubation, confluent monolayers were scraped from the culture dish and pelleted between all subsequent steps for 15 s in the microcentrifuge. To localize cell surface proteins, we incubated MDCK cells for 45 min in hybridoma supernatant, washed in BSA-PBS and then incubated 45 min in GAM coupled to horseradish peroxidase (HRP-GAM). After washing in PBS,

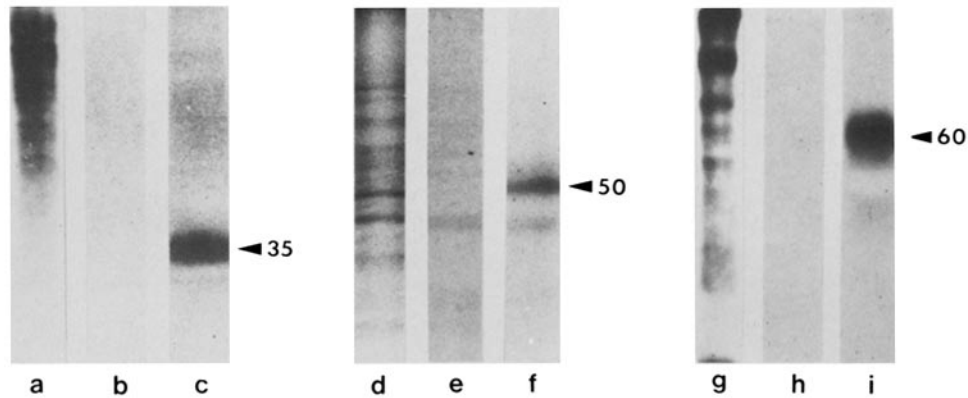


FIGURE 1 Identification of MDCK cell surface glycoproteins by immunoprecipitation and SDS PAGE. Subconfluent MDCK cells were grown in either [^3H]glucosamine (lanes a-c and g-i) or [^{35}S]methionine (lanes d-f) to label membrane glycoproteins isotopically. The labeled proteins were extracted with detergent, immunoprecipitated, analyzed by SDS PAGE and identified by fluorography as described in Materials and Methods. Monoclonal antibody 11B8 (lane c) immunoprecipitated a glycoprotein of 35,000 mol wt, antibody H6 (lane f) a glycoprotein of 50,000 mol wt (experiments using [^3H]glucosamine to label this protein are not presented here), and antibody G12 (lane i) a glycoprotein of 60,000 mol wt. These results are compared to whole cell lysates (lanes a, d, and g) or control samples (lanes b, e, and h) using a monoclonal antibody that recognizes the $\text{Na}^+\text{-K}^+$ ATPase of embryonic chick muscle membranes (a generous gift from Drs. Grace Migliorisi and Thomas Easton).

the cells were fixed in 2% glutaraldehyde-0.1 M NaH_2PO_4 (pH 7.4) for 30 min. An electron-dense reaction product was developed by incubating cells in 1 mg/ml diaminobenzadine-0.01% H_2O_2 in a citrate buffer (pH 6.0) for 45 min at room temperature (46). The cells were postfixed in 1% OsO_4 -0.1 M NaH_2PO_4 (pH 7.4) for 1 h, then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut with a diamond knife, then examined and photographed in a JEOL 100C operating at 80 kV.

Transepithelial Electrical Resistances

Micropore filters with MDCK cells were placed between two halves of a lucite Ussing-type chamber connected to Hg/HgCl_2 electrodes with 3 M KCl-agar bridges. Transepithelial electrical resistances, a measure of tight junction permeability (34) were determined by passing 10 microamperes of current across 0.32 cm^2 of the cell monolayer and recording the voltage change on a Keithley 610 electrometer (Keithley Instruments, Inc., Cleveland, OH).

RESULTS

Immunoprecipitation of MDCK Cell Surface Proteins

For the studies reported here, we used monoclonal antibodies secreted by hybridoma clones that we have designated 11B8, H6, and G12. SDS PAGE and Ouchterlony immunodiffusion analysis have determined that antibodies 11B8 and H6 are IgG1 and G12 is IgG_{2a} (data not shown).

Identification of MDCK cell surface proteins recognized by these monoclonal antibodies was carried out by growing MDCK cells in medium containing either [^{35}S]methionine or [^3H]glucosamine. Detergent extracts of isotopically labeled cells were incubated with Sepharose 4B immunoabsorbants and the immunoprecipitated polypeptides identified by SDS PAGE and fluorography. With these procedures we have identified three different MDCK cell surface glycoproteins (Fig. 1). Each antibody immunoprecipitates a single glycoprotein with 11B8 recognizing a species of ~35,000 mol wt, antibody H6 and G12 glycoproteins of 50,000 and 60,000 mol wt, respectively. The 50,000-mol-wt glycoprotein does not appear to be heavily glycosylated and can be identified much more readily after [^{35}S]methionine labeling (Fig. 1). RIA studies have determined that these proteins do not share antigenic determinants with dog kidney $\text{Na}^+\text{-K}^+$ ATPase (Herzlinger and Ojakian, data not shown).

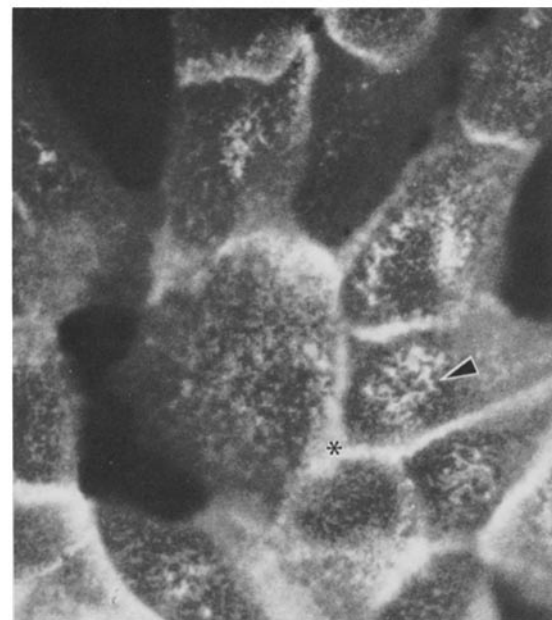


FIGURE 2 Immunofluorescence localization of the 50,000-mol-wt glycoprotein by monoclonal antibody H6 on subconfluent MDCK cells. This glycoprotein is expressed by every cell in the culture and can be observed on apical cell surface microvilli (arrowhead) as well as the lateral membranes (*) between contacted cells. $\times 500$.

Localization of Cell Surface Glycoproteins on Subconfluent Cells

LIGHT MICROSCOPY: Previous studies have determined that the 35,000-mol-wt glycoprotein was evenly distributed over the surface of all cells in subconfluent MDCK cultures (36). Identical observations have also been made for both the 50,000- and 60,000-mol-wt glycoproteins. When localized by immunofluorescence microscopy, the 60,000-mol-wt glycoprotein can be clearly visualized on apical surface microvilli as well as the lateral membranes in regions where the cells are in contact (Fig. 2). This has been a consistent observation for the 35,000-, 50,000-, 60,000-mol-wt glycoproteins in all sub-

confluent cultures studied and we have never observed a lack of apical staining in these cultures.

ELECTRON MICROSCOPY: Since it was not possible to determine by immunofluorescence microscopy whether these glycoproteins were also present on the basal cell surface that contacts the substratum, immunoelectron microscopic studies were initiated utilizing GAM-HRP as a localizing reagent. Thin sections of subconfluent MDCK demonstrated that the 35,000-, 50,000-, and 60,000-mol-wt glycoproteins were distributed over the entire apical and basal surfaces of subconfluent MDCK cells including apical surface microvilli (Fig. 3). In regions where growing cells were in contact, the 50,000-mol-wt glycoprotein was localized on the lateral membranes between contacted cells (Fig. 3*b*). The 35,000- and 60,000-mol-wt glycoproteins have also been localized on the lateral membranes of contacted, subconfluent MDCK cells (data not shown). The electron-dense reaction product localizing each of the three glycoproteins appeared to be of the same intensity on the apical, basal and lateral membranes (Fig. 3).

Localization of Cell Surface Glycoproteins on Confluent Cells

For physiological studies, MDCK cells must cover the entire permeable substrate upon which they were plated to allow the formation of tight junctions with occluding properties (23–25). At the plating densities utilized here, MDCK cells were confluent after 40–48 h growth. However, MDCK can continue to divide after completely covering the substratum and do not reach maximum density until 72 h after plating (see Fig. 7).

LIGHT MICROSCOPY: The 35,000-, 50,000-, and 60,000-mol-wt glycoproteins were localized by both light and electron microscopy (Figs. 4–6) on MDCK monolayers that had been confluent for 48 h. When confluent monolayers grown to maximum cell density were stained with fluorescent antibodies, only low levels of the three glycoproteins could be detected on the apical cell surface and none on the basal-lateral membrane (Figs. 4 and 5). The levels of membrane glycoproteins

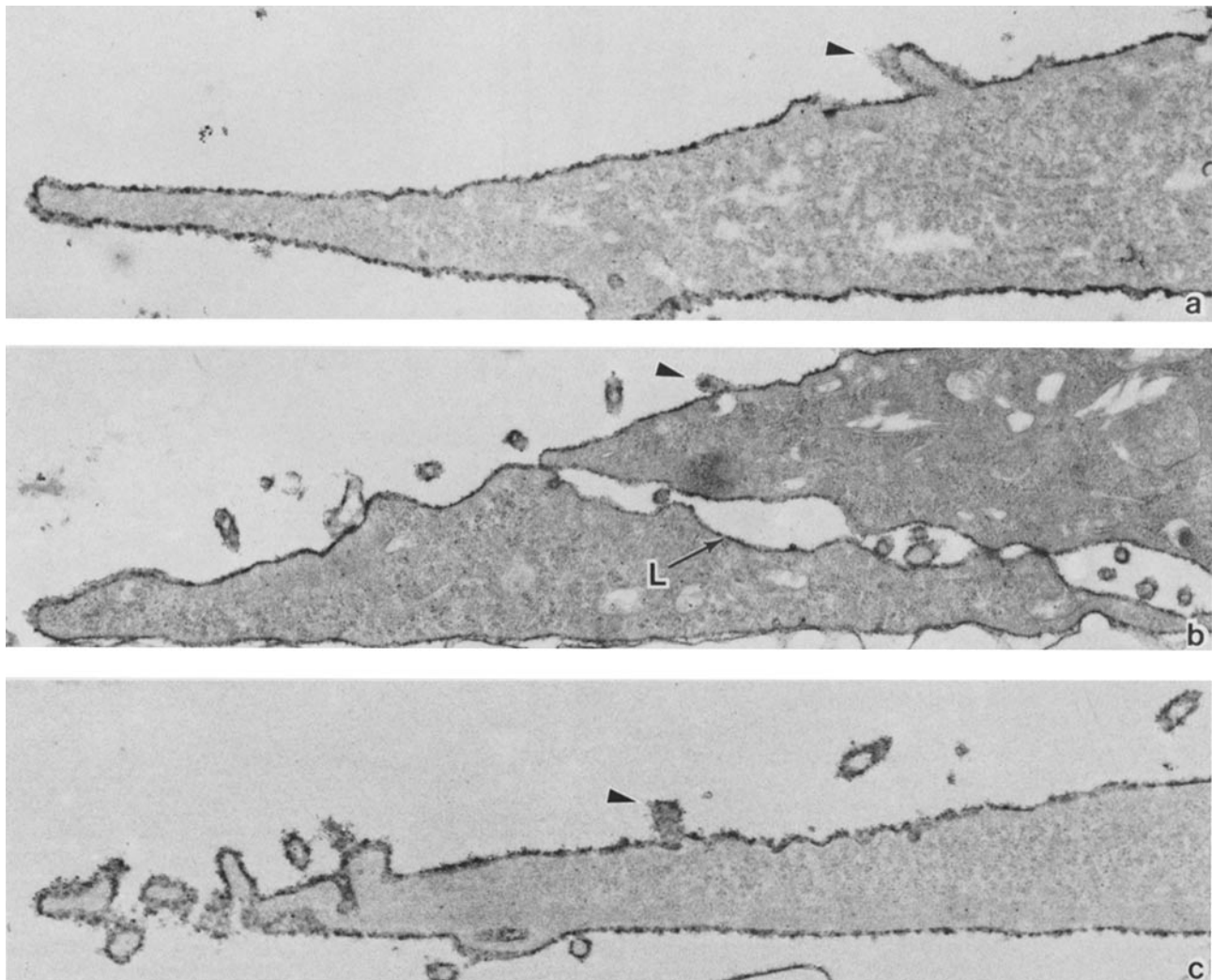


FIGURE 3 Ultrastructural distribution of cell surface glycoproteins on subconfluent MDCK cells. The 35,000- (a), 50,000- (b), and 60,000-mol-wt (c) glycoproteins have been localized by incubation in monoclonal antibody followed by HRP-GAM. All micrographs are oriented with the apical surface at the top (microvilli are designated by arrowheads) and the basal surface at the bottom. Electron-dense reaction product appears to be uniformly distributed over both apical and basal cell surfaces (a–c) and could also be localized on the lateral membranes (L) of cells in contact (b). In b and c, the MDCK cells have been grown on micropore filters. In control cultures incubated with chick muscle anti- $\text{Na}^+\text{-K}^+$ ATPase little, if any, reaction product could be visualized. (a) $\times 20,000$; (b) $\times 17,500$; (c) $\times 22,500$.

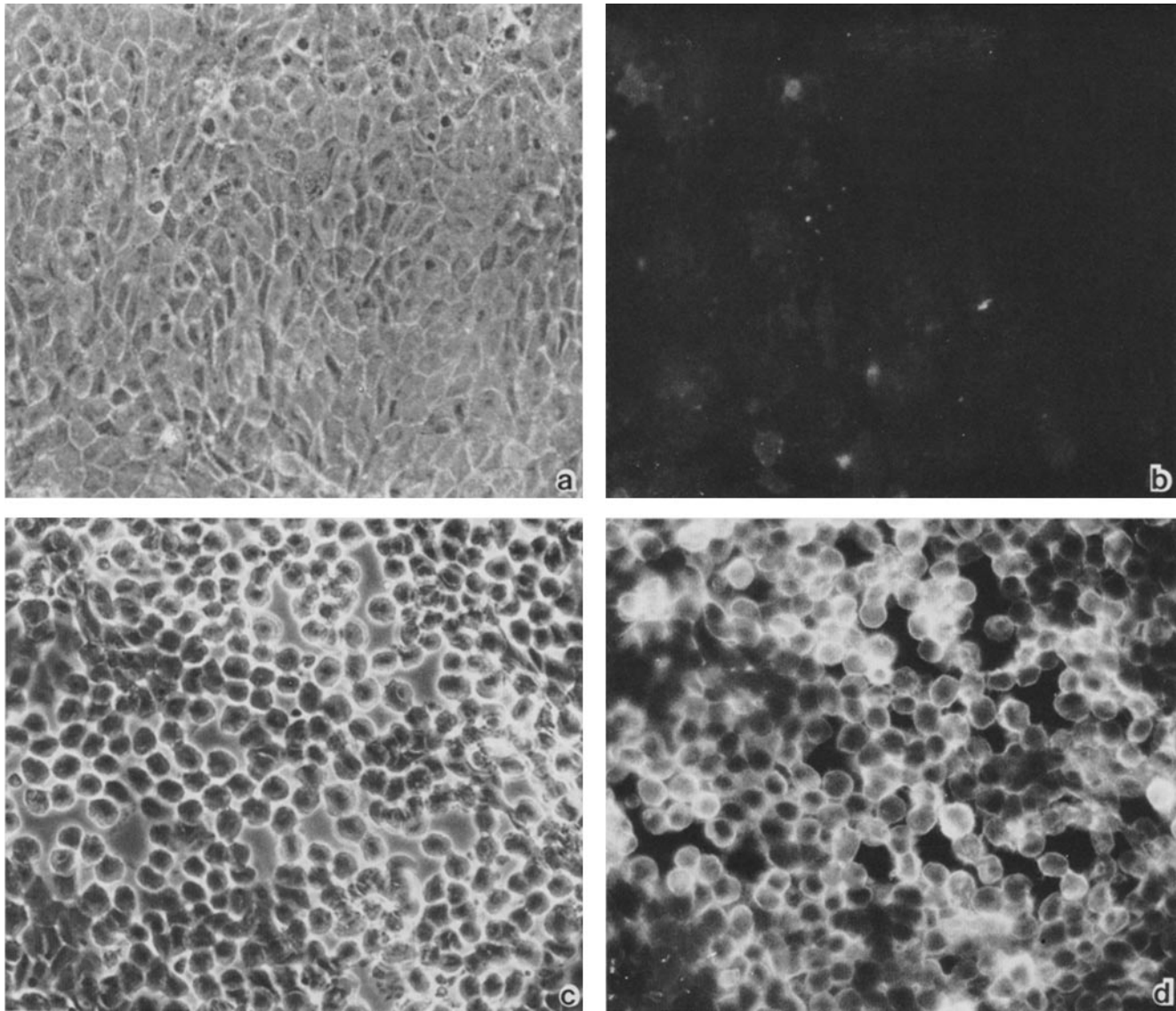


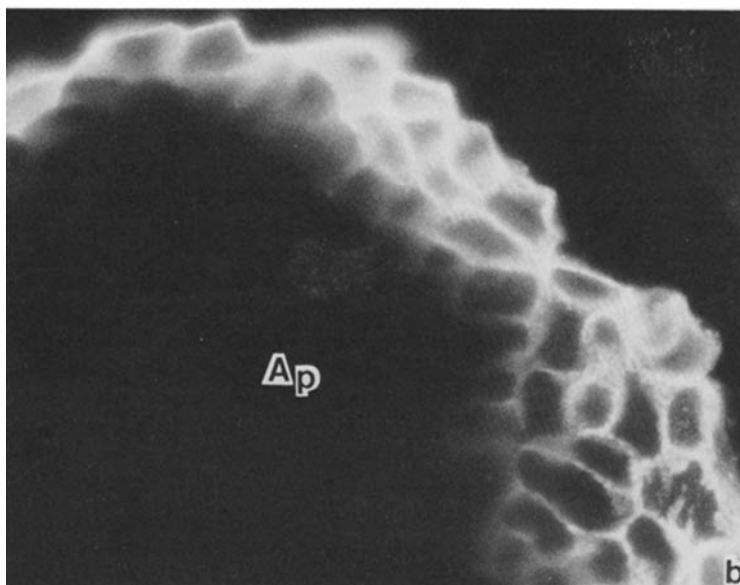
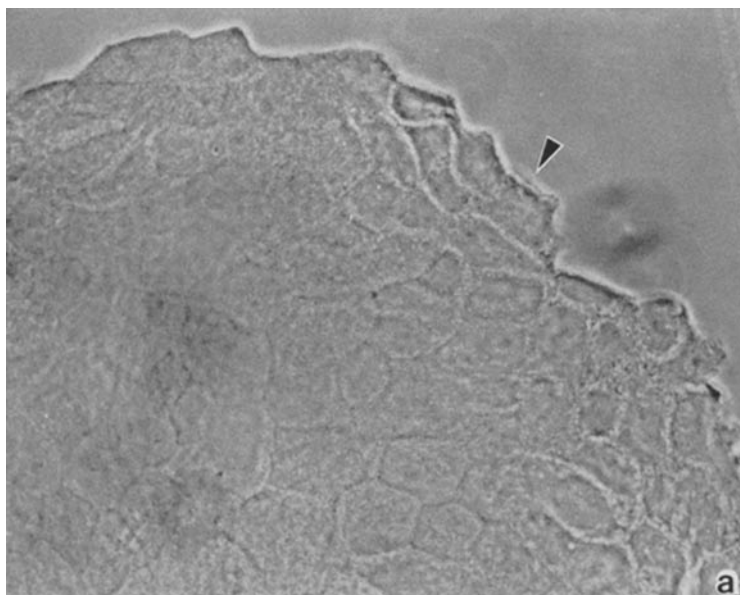
FIGURE 4 Immunofluorescence localization of the 35,000-mol-wt glycoprotein on confluent MDCK cells. Corresponding phase contrast (a) and immunofluorescence (b) micrographs of confluent MDCK monolayers. Low levels of fluorescence staining using monoclonal antibody 11B8 (b) demonstrate that only a small amount of the 35,000-mol-wt glycoprotein is present on the apical cell surface. MDCK cells were treated with 2.5 mM EGTA in PBS (Ca^{++} , Mg^{++} -free), at 37°C for a time (45 min) sufficient for rounding of the majority of cells (c) allowing the antibody access to the basal-lateral membrane. Increased levels of fluorescent staining (d) demonstrate that the 35,000-mol-wt glycoprotein is distributed primarily on the basal-lateral membrane in confluent monolayers. $\times 300$.

found on the apical surface were considerably lower than those detected on subconfluent cells by immunofluorescence microscopy (Fig. 2b). Since confluent MDCK monolayers are sealed by tight junctions, two strategies were developed to demonstrate the presence of proteins on the basal-lateral membrane. Confluent monolayers were incubated in EGTA to disrupt tight junctions (18, 24, 47) and allow the antibodies access to the basal-lateral space (Fig. 4). Under our incubation conditions (2.5 mM EGTA for 45 min at 37°C), the majority of MDCK cells round up (Fig. 4c) and intense immunofluorescence staining on every cell demonstrated the presence of large quantities of the 35,000-mol-wt glycoprotein on the basal-lateral membrane (Fig. 4d). By focusing on the upper surface of the rounded cells, apical staining was detected (data not shown), suggesting that some of the 35,000-mol-wt glycoprotein has moved laterally from the basal-lateral mem-

brane during the EGTA incubation. Since very long incubation times (45 min) were required to disrupt the majority of tight junctions in the confluent MDCK monolayers, we cannot exclude the alternate possibility that basal-lateral membrane glycoproteins are taken up by the cells and transported to the apical cell surface. The increased immunofluorescence staining on the basal-lateral membranes after EGTA was probably not due to the exposure of latent sites since, in experiments where portions of the MDCK monolayer remain unaffected by the EGTA, the unrounded cells do not exhibit an increase in staining.

As an alternative to the EGTA method, tears were experimentally induced in MDCK monolayers to allow the immunofluorescence visualization of basal-lateral antigens without the breakage of tight junctions (Fig. 5). After fixation in paraformaldehyde-glutaraldehyde, monolayers were scratched

FIGURE 5 Immunofluorescence localization of the 50,000-mol-wt glycoprotein on confluent MDCK cells. Tears were induced in the fixed monolayer to allow antibodies access to the basal-lateral as well as the apical cell surfaces. With phase-contrast microscopy (a) the edge of the tear (arrowhead) can be visualized in the upper portion of the field. The 50,000-mol-wt glycoprotein was localized by incubating the monolayer in monoclonal antibody H6 as described in Materials and Methods. In the corresponding immunofluorescence micrograph (b), the 50,000-mol-wt glycoprotein was not detected on the apical cell surface (Ap), however, antibody H6 diffused under the monolayer at the edge of the tear and the rings of fluorescence staining outlining the cells demonstrated that the 50,000-mol-wt glycoprotein was localized to the basal-lateral membrane. $\times 465$.



with forceps then stained with fluorescent antibodies. In the data presented here, the rings of fluorescent staining that surround each cell (Fig. 5b) indicate that the antibodies were able to diffuse under the cells adjacent to the tear (Fig. 5a) and localize the 50,000-mol-wt glycoprotein on the basal-lateral membrane (Fig. 5b). Similar patterns of immunofluorescence staining have been obtained when localizing $\text{Na}^+ - \text{K}^+$ ATPase (12, 28), a basal-lateral membrane protein of MDCK cells (12, 28, 29). Simultaneous visualization of the apical surface demonstrates that the 50,000-mol-wt glycoprotein cannot be detected by immunofluorescence in this membrane domain (Fig. 5b), and that this protein as well as the 35,000- and 60,000-mol-wt glycoproteins (data not shown) reside almost exclusively in the basal-lateral membrane of confluent MDCK cells. It should also be pointed out that portions of the monolayer removed by the forceps were frequently retained on the coverglass as flattened sheets in an inverted position (basal side up). When these sheets were stained for the 35,000-, 50,000, and 60,000-mol-wt glycoproteins every cell stained with the fluorescent antibodies on the

basal-lateral membrane (see Fig. 9).

ELECTRON MICROSCOPY: The basal-lateral distributions of the 35,000-, 50,000-, and 60,000-mol-wt glycoproteins on confluent MDCK cells, were confirmed at the ultrastructural level. Fixed MDCK monolayers were scraped from culture plates and allowed to incubate with monoclonal antibodies and then, GAM-HRP. Ultrastructural observations demonstrated that the 50,000-mol-wt glycoprotein was localized predominantly on the infoldings of the basal-lateral membrane (Fig. 6) and that little, if any, electron-dense reaction product could be detected on the apical cell surface (Fig. 6). Similar results were obtained for the 35,000- and 60,000-mol-wt glycoproteins (data not shown) confirming the immunofluorescence data (Figs. 4 and 5).

Development of Tight Junctions and Cell Surface Polarity

To study the development of cell surface polarity, we plated MDCK cells at subconfluent densities on coverglasses and

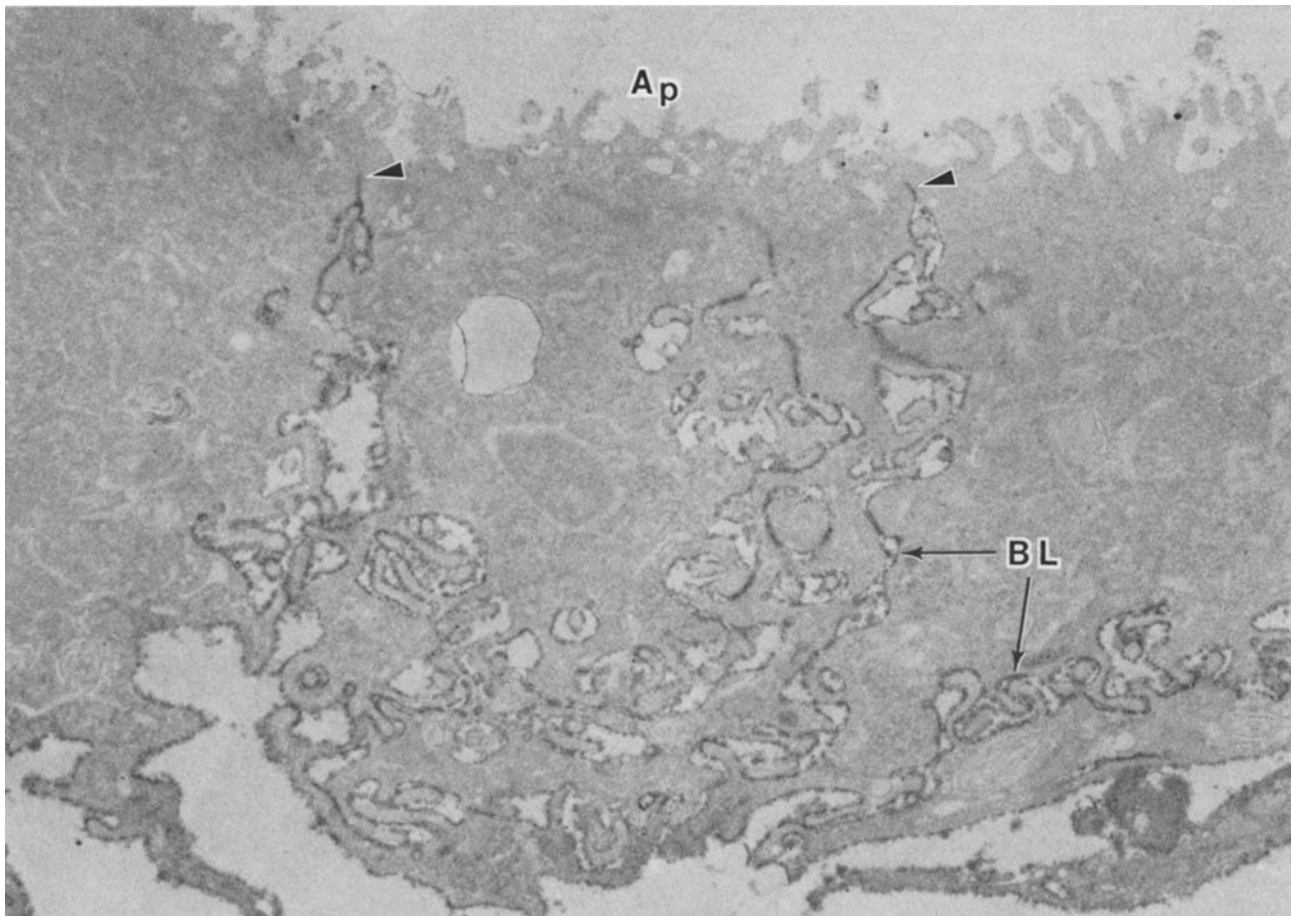


FIGURE 6 Ultrastructural localization of the 50,000-mol-wt glycoprotein on confluent MDCK cells. Monolayers were fixed, scraped from the culture dish and incubated in monoclonal antibody H6 followed by HRP-GAM. The distribution of electron-dense reaction product demonstrates that the 50,000-mol-wt glycoprotein is localized to the basal-lateral membrane (BL) up to the tight junctions (arrowheads) and could not be visualized on the apical cell surface (Ap). $\times 21,500$.

micropore filters (24) to allow the simultaneous monitoring of cell surface glycoprotein levels and distribution, cell growth, and the formation of tight junctions. At intervals of time after plating, transepithelial electrical resistances of MDCK monolayers on micropore filters were measured to determine tight junctional permeability (23–25). During the same time periods, MDCK cells grown on coverglasses and micropore filters were fixed and processed for immunofluorescence microscopy and cell density measurements.

For the initial 40 h after plating, transepithelial electrical resistance could not be detected. However, this was followed by slight increases in resistance to 6 ohms·cm² at 48 h and 28 ohms·cm² at 64 h (Fig. 7). Between 64–72 h a dramatic increase was recorded as the transepithelial electrical resistance rose sharply, peaking at 150 ohms·cm² and remained at this level (Fig. 7). During this same period, increases in cell density followed an entirely different time course rising steadily from 25 to 64 h before leveling off at 72 h (Fig. 7). These results suggest that even though tight junctions are present throughout the monolayer by 48–64 h, it is only after the cessation of growth that they became completely functional. This five-fold increase in electrical resistance over an 8-h period is comparable to that recorded by others using non-growing cultures (24, 49) and could be due to either the assembly of pre-existing junctional elements or the synthesis of new components (49). By measuring the density of MDCK cells plated on coverglasses and micropore filters, we deter-

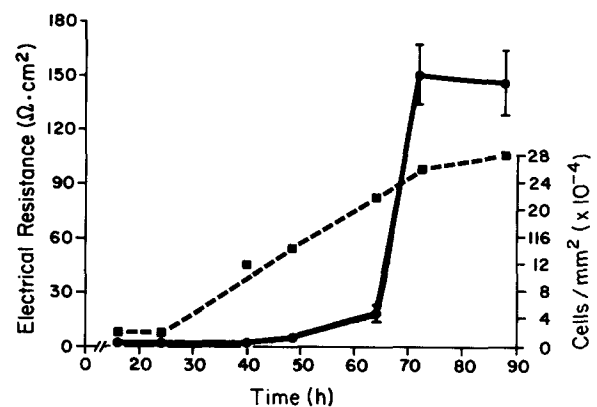


FIGURE 7 Development of MDCK tight junctions. MDCK cells were plated on micropore filters of 1.5×10^5 cells/well and transepithelial electrical resistance ($\Omega \cdot \text{cm}^2$) measurements recorded at the times indicated (solid line). Electrical resistances for four filters were recorded for each time point and the data is presented as the mean \pm standard error. Similar results were recorded for three experiments. Density measurements of cells grown on micropore filters were taken at the same intervals (dashed line). Standard errors were $<5\%$.

mined that cell growth occurs at the same rate on both substrates and identical maximum saturation densities were obtained (data not shown).

The temporal development of cell polarity was examined by studying the cell surface distribution of the 35,000-, 50,000-, and 60,000-mol-wt glycoproteins by immunofluorescence microscopy. Since identical results were obtained for

all glycoproteins only the data for the 60,000-mol-wt glycoprotein will be presented. After 24 h in culture, the MDCK monolayer is not complete (Fig. 8*a*) and the 60,000-mol-wt glycoprotein can be observed on both apical and lateral cell

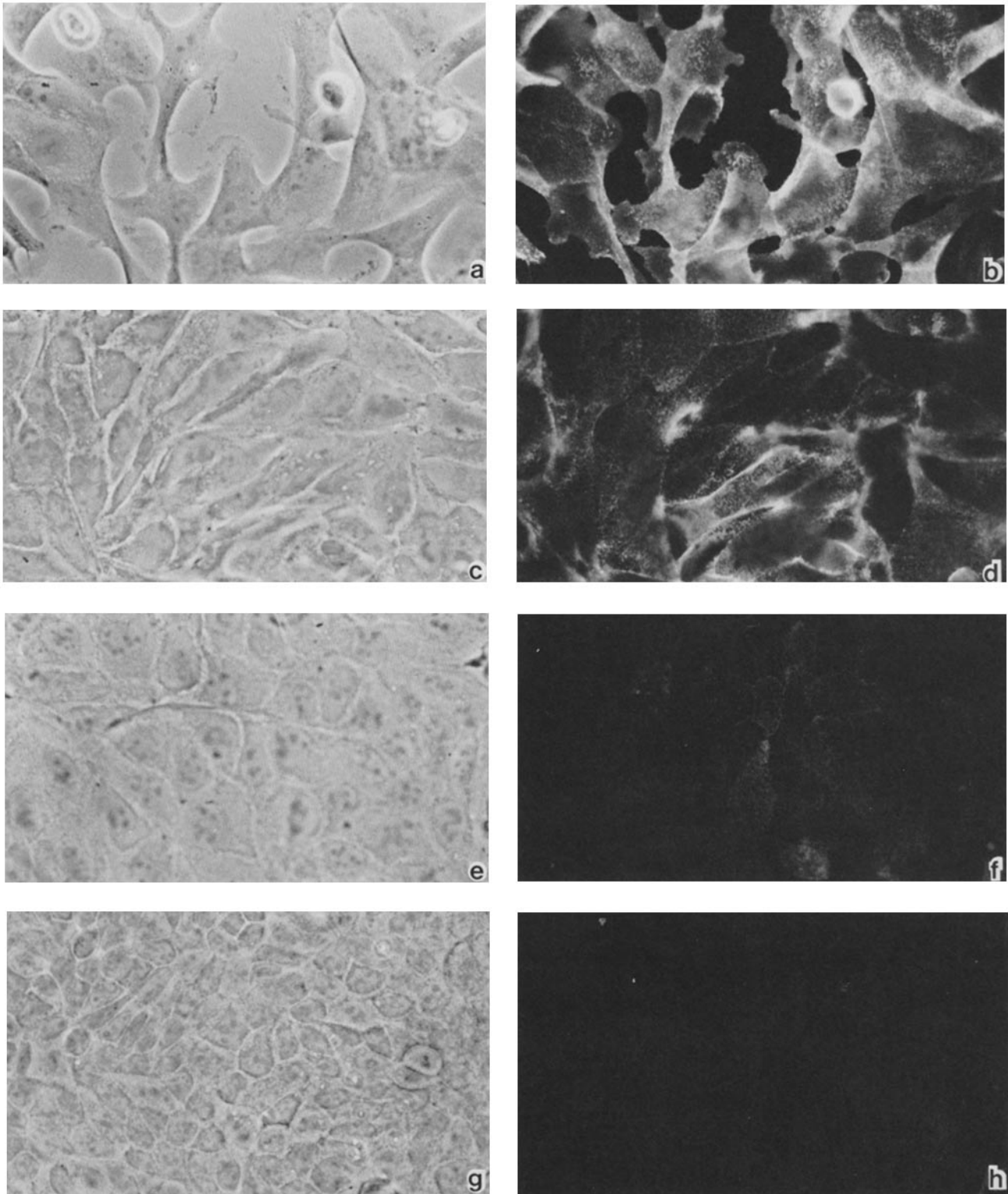


FIGURE 8 Development of MDCK cell surface polarity. Corresponding phase contrast (a, c, e, and g) and immunofluorescence (b, d, f, and h) micrographs of developing MDCK monolayers stained with monoclonal antibody G12 to localize the 60,000-mol-wt glycoprotein. When examined 24 h after plating (a and b) intense apical cell surface staining was observed (b) on the subconfluent cells (a). By 40 h (c and d) many of the cells still had the 60,000-mol-wt glycoprotein on their apical cell surface in reduced amounts (d). 48 h after plating (e and f) only a small amount of apical staining was observed (f) and these levels remained unchanged at 88 h (g and h). $\times 300$.

surfaces (Fig. 8*b*) indicating a nonpolarized distribution identical to that presented in Fig. 2. By 40 h, the monolayer appeared complete with all the cells in contact (Fig. 8*c*). However, many cells still exhibit detectable levels of glycoprotein on their apical surface (Fig. 8*d*). 48 h after plating, the monolayer appeared polarized as only small quantities of the 60,000-mol-wt glycoprotein could be detected on the apical surface by immunofluorescence microscopy (Fig. 8*f*) while intense basal-lateral staining was observed on cells adjacent to experimentally induced tears (Fig. 9, *a* and *b*). The immunofluorescence image of the apical cell surface, at 48 h (Fig. 8*f*) was identical to that of cells 88 h after plating (Fig. 8*h*), however, the basal-lateral staining at 88 h was more intense (Fig. 9*d*). Identical results were obtained when the cell surface distributions of the 35,000-, 50,000-, and 60,000-mol-wt glycoproteins were examined by immunofluorescence microscopy on MDCK cells grown on micropore filters (data not shown).

DISCUSSION

Monoclonal antibodies generated against the plasma membrane of MDCK cells have been used to study the development of cell surface polarity during the growth and establishment of a functional epithelial monolayer. For these studies, we used the hybridoma clones designated H6 and G12 that secrete antibodies that recognize cell surface glycoproteins of 50,000 and 60,000 mol wt, respectively, and clone 11B8, a hybridoma cell line isolated for another study (36), which secretes an antibody that recognizes a cell surface glycoprotein of 35,000 mol wt. We have recently demonstrated that antibody 11B8 does not bind to purified dog kidney Na⁺-K⁺ ATPase nor does it have the same distribution along the nephron as this enzyme (36). Here we provide conclusive evidence that antigen 11B8 is not a component of the Na⁺-K⁺ ATPase since its molecular weight of 35,000 when examined by SDS PAGE (44) is considerably different than either subunit of the dog kidney enzyme (48).

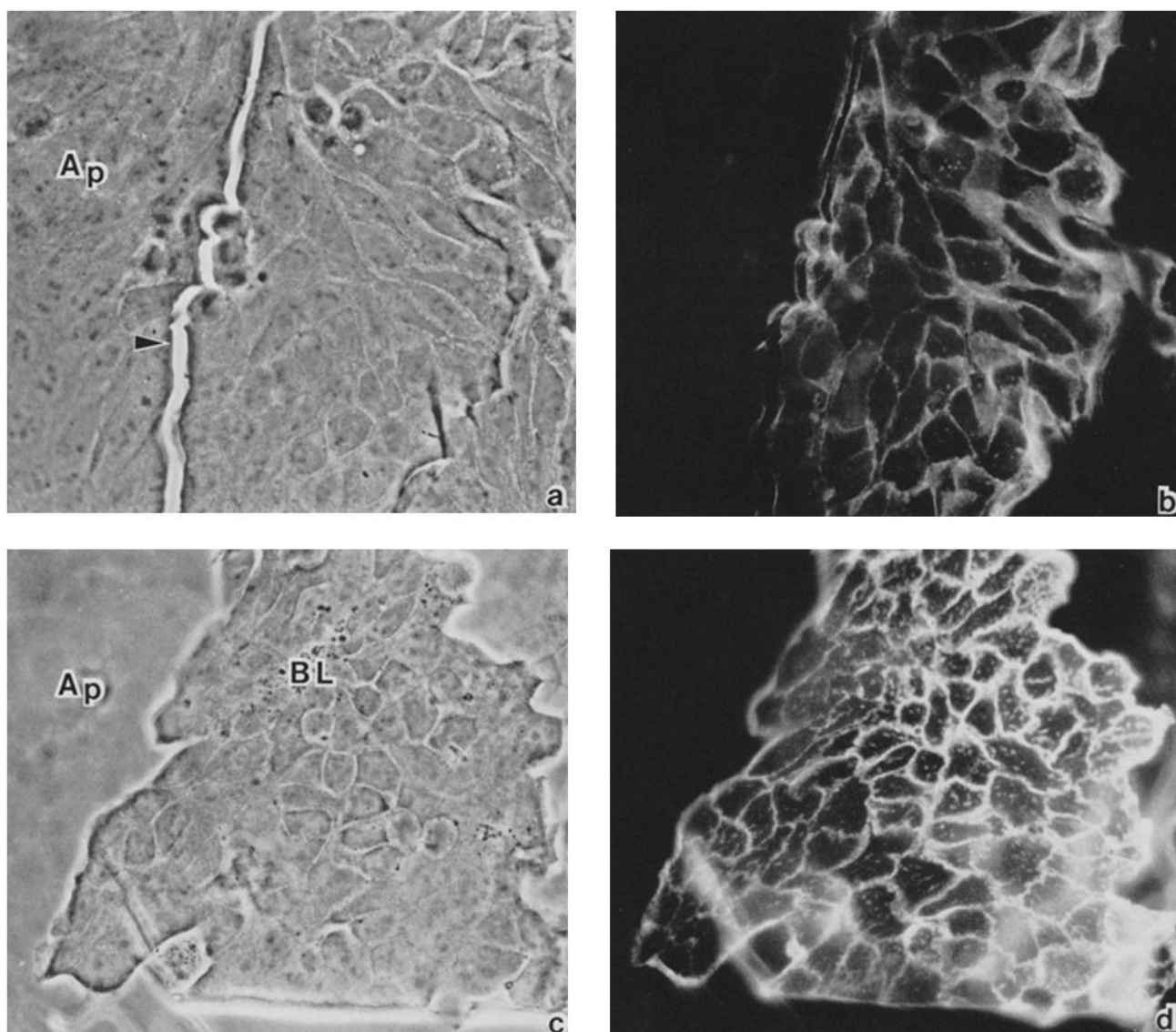


FIGURE 9 Localization of the 60,000-mol-wt glycoprotein on the basal-lateral membranes of confluent MDCK monolayers. Corresponding phase-contrast (*a* and *c*) and immunofluorescence (*b* and *d*) micrographs taken at 48 h (*a* and *b*) and 88 h (*c* and *d*) after plating. An experimentally tear (arrowhead) in the 48 h monolayer (*a*) allowed the visualization of the 60,000-mol-wt glycoprotein on the basal-lateral membranes (*b*). A polarization distribution was also observed on the basal-lateral membranes (BL) of a folded-over portion of an 88-h monolayer (*d*) however, the staining at 88 h is more intense than at 48 h (*b*). At both 48 and 88 h, apical cell (Ap) surface staining was extremely low and comparable to data presented in Fig. 8. $\times 300$.

In confluent monolayers, the 35,000-, 50,000-, and 60,000-mol-wt glycoproteins have a polarized distribution and can be localized primarily on the basal-lateral plasma membrane by both immunofluorescence and immunoelectron microscopy providing further evidence that MDCK are polarized cells (12, 26–30). Ultrastructural examination of subconfluent cells demonstrates that the electron-dense HRP reaction product that localizes the glycoproteins appears to be of the same intensity on the apical, basal, and lateral plasma membrane. Since HRP is not a quantitative assay at the electron microscopic level, we cannot state with absolute certainty that the distribution of each of the three glycoproteins studied is completely symmetrical. Further clarification of these observations will have to await studies with either colloidal gold or ferritin-labeled antibodies. Our studies contradict a recent report showing $\text{Na}^+\text{-K}^+$ ATPase is localized primarily to the apical surface of subconfluent MDCK cells (50). We do not have an explanation for this discrepancy at the present time; however, it should be noted that their incubation time in diaminobenzidine is considerably shorter (3–5 min) than we used (45 min) for our studies.

We do not have any evidence as to the site of insertion for newly synthesized MDCK cell surface glycoproteins during growth into a confluent monolayer but two possibilities seem likely. The first is that glycoprotein insertion takes place at random over the entire cell surface and the second is that newly synthesized proteins are sorted-out within the cell (20, 21), inserted in a polarized manner into either the apical or the basal domain and then disperse laterally within the membrane plane due to the absence of occluding tight junctions. Studies of viral membrane glycoprotein biogenesis provide considerable evidence to support the second possibility. Confluent MDCK cells infected with lipid envelope viruses will support the polarized budding of lipid envelope viruses (51) and the newly synthesized viral membrane glycoproteins are inserted into the proper membrane domain before budding of the virion (52). Polarized viral budding has also been observed in subconfluent MDCK cells suggesting that tight junctions are not required for the sorting-out of viral membrane glycoproteins (53). These results (53) are in marked contrast to those reported here for the 35,000-, 50,000-, and 60,000-mol-wt glycoproteins that are uniformly distributed over the entire cell surface of subconfluent MDCK; however, we do not have any information as to whether these cell surface glycoproteins are inserted in either a polarized or nonpolarized manner. Studies done on viral membrane glycoprotein biogenesis have determined that localized insertion followed by a uniform redistribution can occur in subconfluent cells (54, 55). One explanation for the polarized release of viruses from subconfluent MDCK cells is that the nonenvelope viral proteins or the nucleocapsid are associated with the inner surface of the plasma membrane in a polarized manner and provide nucleation sites for randomly inserted viral envelope proteins. Confirmation of this suggestion awaits a study of the distribution of newly inserted viral envelope proteins into the plasma membrane of subconfluent MDCK cells. For confluent epithelia, this possibility does not seem likely since a recent study has demonstrated the polarized insertion of an influenza membrane glycoprotein can occur in the absence of the nucleocapsid (55).

Examination of MDCK cells during the formation of a confluent monolayer has provided evidence that the devel-

opment of cell surface polarity is a continuous process. Progressive loss of apical immunofluorescence staining of cell surface glycoproteins was observed from 40 h as the monolayers became sealed and antibodies did not have access to the basal-lateral membrane due to the formation of tight junctions. By 64 h, the combination of low levels of apical staining and intense levels of basal-lateral immunofluorescence demonstrated that the monolayers had polarized distributions of the cell surface glycoproteins. These observations provide evidence that cell surface polarity had developed prior to the complete assembly of tight junctions. Although MDCK cells appear polarized, cell surface polarity can continue to develop as shown by an increase in the intensity of basal-lateral staining after tight junctions have been assembled. Regardless of whether this observed increase in basal-lateral glycoproteins is due to more molecules per unit area or to an increase in total basal-lateral membrane area, or both, the generation of asymmetric protein distribution is essential to the establishment of vectorial physiological functions. Our results confirm those of Rabito and Tchao (57) who have demonstrated that ouabain binding peaks several hours before development of maximal transepithelial electrical resistance in MDCK monolayers and proposed that complete development of tight junctions occurs after polarization of the $\text{Na}^+\text{-K}^+$ ATPase. Our studies also support Hoi Sang et al. (18) who have demonstrated that, after EGTA disruption of MDCK tight junctions, the re-establishment of a polarized intramembrane particle distribution appears to be coupled to tight junction formation.

The generation of cell surface polarity probably used the membrane protein turnover and sorting-out mechanisms that normally operate in the cell. Although the specific cellular mechanisms that generate epithelial polarity have not been completely elucidated (see references 20 and 21 for a complete discussion), the information obtained from our studies suggest that epithelial polarity could develop in the following manner. After growth of MDCK cells into a confluent monolayer, newly synthesized proteins are inserted in a polarized manner (20, 21, 52, 56) and retained in the proper membrane due to the presence of functional tight junctions. Since proteins from both epithelial cell surface domains are probably turned over by the normal cellular degradative processes, the preferential accumulation of protein in either the apical or basal-lateral membrane could then occur, allowing the progressive establishment of cell polarity. This proposal is supported by two recent papers demonstrating that a viral membrane glycoprotein experimentally inserted into the incorrect membrane domain (apical) of confluent MDCK cells is rapidly taken up and recycled to the basal-lateral plasma membrane (58, 59).

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REFERENCES

1. Farquhar, M. J., and G. E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375-412.
2. Berridge, M. J., and J. L. Oschman. 1972. *Transporting Epithelia*, Academic Press, Inc., New York.
3. Camilli, P., D. Peluchetti, and J. Meldolesi. 1974. Structural differences between luminal and lateral plasmalemma in pancreatic acinar cells. *Nature (Lond.)* 248:245-247.
4. Galli, P., A. Brenna, P., Decamilli, and J. Meldolesi. 1976. Extracellular calcium and the organization of tight junctions in pancreatic acinar cells. *Exp. Cell Res.* 99:178-183.
5. Meldolesi, J., G. Castiglioni, R. Parma, N. Nassivera, and P. De Camilli. 1978. Ca⁺⁺-dependent disassembly and reassembly of occluding junctions in guinea pig pancreatic acinar cells: effect of drugs. *J. Cell Biol.* 79:156-172.
6. Fujita, M., K. Kawai, S. Asane, and M. Nakae. 1973. Protein components of two different regions of an intestinal epithelial cell membrane. *Biochim. Biophys. Acta.* 307:141-151.
7. Kawai, K., M. Fujita, and M. Nakao. 1974. Lipid components of two different regions of an intestinal epithelial cell membrane of mouse. *Biochim. Biophys. Acta.* 369:222-233.
8. Ebel, H., E. Aulbert, and H. J. Merker. 1976. Isolation of the basal and lateral plasma membrane of rat kidney tubule cells. *Biochem. Biophys. Acta.* 433:531-546.
9. Semenza, G. 1976. Small intestinal disaccharidases: their properties and role as sugar translocators across natural and artificial membranes. In *The Enzymes of Biological Membranes*. A. Martonosi, editor. Plenum Press, New York. 3:349-382.
10. Kinsella, J. L., P. D. Holohan, N. I. Pessah, and C. R. Ross. 1979. Isolation of luminal and antiluminal membranes from dog kidney cortex. *Biochim. Biophys. Acta.* 552:468-477.
11. Murer, H., and R. Kinne. 1980. The use of isolated membrane vesicles to study epithelial transport processes. *J. Membr. Biol.* 55:81-95.
12. Reggio, H., E. Courdrier, and D. Louvard. 1982. Surface and cytoplasmic domains in polarized epithelial cells. In *Membranes in Growth and Development*. Alan R. Liss, New York. 89-105.
13. Stirling, C. E. 1972. Radioautographic localization of sodium pump sites in rabbit intestine. *J. Cell Biol.* 53:704-712.
14. Kyte, J. 1976. Immunoferritin determination of the distribution of (Na⁺-K⁺)ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment. *J. Cell Biol.* 68:287-303.
15. Kyte, J. 1976. Immunoferritin determination of (Na⁺-K⁺)ATPase over the plasma membrane of renal convoluted tubules. II. Proximal segment. *J. Cell Biol.* 68:304-318.
16. Ernst, S. A., and J. W. Mills. 1977. Basolateral plasma membrane localization of ouabain-sensitive sodium transport sites in the secretory epithelium of the avian salt gland. *J. Cell Biol.* 75:74-94.
17. Pisam, M., and P. Ripoche. 1976. Redistribution of surface macromolecules in dissociated epithelial cells. *J. Cell Biol.* 71:907-920.
18. Hoi Sang, U., M. H. Saier, and M. H. Ellisman. 1979. Tight junction formation is closely linked to the polar redistribution of intramembranous particles in aggregating MDCK epithelia. *Exp. Cell Res.* 122:384-391.
19. Ziomek, C. A., S. Scholman, and M. Edidin. 1980. Redistribution of membrane proteins in isolated mouse intestinal epithelial cells. *J. Cell Biol.* 86:849-857.
20. Rindler, M. J., I. E. Ivanov, E. Rodriguez-Boulan, and D. D. Sabatini. 1982. Biogenesis of epithelial cell plasma membranes. *CIBA Found. Symp.* 92:184-202.
21. Rodriguez-Boulan, E. 1983. Membrane biogenesis, enveloped RNA viruses, and epithelial polarity. In *Modern Cell Biology*. B. Satir, editor. Alan R. Liss, New York. 1:119-170.
22. Leighton, J., L. W. Estes, S. Mansukhani, and Z. Brada. 1970. A cell line derived from dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. *Cancer* 26:1022-1028.
23. Misfeldt, D. S., S. T. Hammamoto, and D. R. Pitelka. 1976. Transepithelial transport in cell culture. *Proc. Natl. Acad. Sci. USA.* 73:1212-1216.
24. Cerejido, M., E. S. Robbins, W. J. Dolan, C. A. Rotunno, and D. D. Sabatini. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* 77:853-880.
25. Rabito, C. A., T. Tchao, J. Valentich, and J. Leighton. 1978. Distribution and characteristics of the occluding junctions in a monolayer cell line (MDCK) derived from dog kidney. *J. Membr. Biol.* 43:351-365.
26. Cerejido, M., J. Ehrenfeld, I. Meza, and A. Martinez-Palomo. 1980. Structural and functional membrane polarity in cultured monolayers of MDCK cells. *J. Membr. Biol.* 52:147-159.
27. Richardson, J. C. W., and N. L. Simmons. 1979. Demonstration of protein asymmetries in the plasma membrane of cultured renal (MDCK) epithelial cells by lactoperoxidase-mediated iodination. *FEBS (Fed. Eur. Biochem. Soc.) Letts.* 105:201-204.
28. Louvard, D. 1980. Apical membrane aminopeptidase appears at sites of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:4132-4136.
29. Lamb, J. F., P. Ogdan, and N. L. Simmons. 1981. Autoradiographic localization of (³H)ouabain bound to cultured epithelial cell monolayers of MDCK cells. *Biochim. Biophys. Acta.* 644:333-340.
30. Van Meer, G., and K. Simons. 1982. Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cell have unique phospholipid compositions. *EMBO (Eur. Mol. Biol. Organ.)* 1:847-852.
31. Cramer, E. B., L. C. Milks, and G. K. Ojakian. 1980. Transepithelial migration of human neutrophils: an *in vitro* model system. *Proc. Natl. Acad. Sci. USA.* 77:4069-4073.
32. Ojakian, G. K. 1981. Tumor promoter-induced changes in the permeability of epithelial cell tight junctions. *Cell.* 23:95-103.
33. Milks, L. C., M. J. Brontoli, and E. B. Cramer. 1983. Epithelial permeability and the transepithelial migration of human neutrophils. *J. Cell Biol.* 96:1241-1247.
34. Fromter, E., and J. Diamond. 1972. Route of passive ion permeation in epithelia. *Nature New Biol.* 235:9-13.
35. Kohler, G., and C. Milstein. 1975. Continuous culture of fused cells secreting antibody of pre-defined specificity. *Nature (Lond.)* 256:495-497.
36. Herzlinger, D. A., T. G. Easton, and G. K. Ojakian. 1982. The MDCK epithelial cell line expresses a cell surface antigen of the kidney distal tubule. *J. Cell Biol.* 93:269-277.
37. Herzlinger, D. A., and G. K. Ojakian. 1982. Development and maintenance of epithelial polarity in MDCK cells. *J. Cell Biol.* 95(2, Pt. 2): 105a. (Abstr.)
38. Ojakian, G. K., and D. A. Herzlinger. 1983. Studies of kidney epithelial cell surfaces using monoclonal antibodies. *Anat. Rec.* 1(Suppl.):85-102.
39. Taub, M., L. Chuman, M. H. Saier, and G. Sato. 1979. Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. *Proc. Natl. Acad. Sci. USA.* 76:3338-3342.
40. Gefter, M. L., D. H. Margulies, and M. D. Scharff. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genet.* 3:231-236.
41. Herzenberg, L. A., and L. A. Herzenberg. 1978. Mouse immunoglobulin allotypes: description and special methodology. In *Handbook of Experimental Immunology*. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. 12.11-12.23.
42. Greenwood, E. C., W. M. Hunter, and S. Glover. 1963. The preparation of ¹³¹I-labeled human growth hormone of high specific radioactivity. *J. Biochem.* 89:114-122.
43. Mellman, I. S., and J. C. Unkeless. 1979. Purification of a functional mouse Fc receptor through the use of a monoclonal antibody. *J. Exp. Med.* 152:1048-1069.
44. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
45. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
46. Fahimi, H. D. 1979. As assessment of the DAB methods for cytochemical detection of catalase and peroxidase. *J. Histochem. Cytochem.* 27:1365-1366.
47. Martinez-Palomo, A., I. Meza, G. Beaty, and M. Cerejido. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* 87:763-745.
48. Castro, J., and R. A. Farley. 1979. Proteolytic fragmentation in the catalytic subunit of the sodium, potassium adenosine triphosphatase. *J. Biol. Chem.* 254:2221-2228.
49. Greep, E. B., W. J. Dolan, E. S. Robbins, and D. D. Sabatini. 1983. Participation of plasma membrane proteins in the formation of tight junctions by cultured epithelial cells. *J. Cell Biol.* 96:693-702.
50. U. H. S., and M. Evans-Layng. 1983. Polar distribution of Na⁺-K⁺ATPase in aggregating MDCK cells. *Exp. Cell Res.* 146:192-198.
51. Rodriguez-Boulan, E., and D. D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. *Proc. Natl. Acad. Sci. USA.* 75:5071-5075.
52. Rodriguez-Boulan, E., and M. Pendergast. 1980. Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. *Cell.* 20:45-54.
53. Rodriguez-Boulan, E., K. T. Paskiet, and D. D. Sabatini. 1983. Assembly of enveloped viruses in MDCK cells: polarized budding from single attached cells and from clusters of cells in suspension. *J. Cell Biol.* 96:866-874.
54. Marcus, P. 1962. Dynamics of surface modification in myxovirus-infected cells. *Cold Spring Harbor Symp. Quant. Biol.* 27:351-365.
55. Bergmann, J. E., A. Kupfer, and S. J. Singer. Membrane insertion at the leading edge of motile fibroblasts. *Proc. Natl. Acad. Sci. USA.* 80:1367-1371.
56. Roth, M. G., R. W. Compans, L. Giusti, A. R. Davis, D. P. Nayak, M. J. Gething, and J. S. Sambrook. 1983. Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV40 viruses carrying cloned hemagglutinin DNA. *Cell.* 33:435-443.
57. Rabito, C. A., and R. Tchao. 1980. (³H)Ouabain binding during the monolayer organization and cell cycle in MDCK cells. *Am. J. Physiol.* 238:C43-C48.
58. Matlin, K., D. F. Bainton, M. Pesonen, D. Louvard, N. Genty, and K. Simons. 1983. Transepithelial transport of a viral membrane glycoprotein implanted into the apical plasma membrane of Madin-Darby canine kidney cells. I. Morphological evidence. *J. Cell Biol.* 97:627-637.
59. Pesonen, M., and K. Simons. 1983. Transepithelial transport of a viral membrane glycoprotein implanted into the apical plasma membrane of Madin-Darby canine kidney cells. II. Immunological quantitation. *J. Cell Biol.* 97:638-643.