The Polarized Distribution of an Apical Cell Surface Glycoprotein Is Maintained by Interactions With the Cytoskeleton of Madin-Darby Canine Kidney Cells

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Abstract. A monoclonal antibody made against a 135-kD glycoprotein (gp135) on the plasma membrane of Madin-Darby canine kidney (MDCK) cells was used to study the development and maintenance of epithelial cell surface polarity. Immunofluorescence microscopy and immunogold electron microscopy of confluent monolayers demonstrated that gp135 had a polarized cell surface distribution and was only localized on the apical surface. The role of membrane contacts in establishing gp135 polarity was determined by plating cells in low Ca^{++}-medium to prevent the formation of intercellular junctions. Quantitative immunogold electron microscopy demonstrated that gp135 had a polarized distribution on cells lacking membrane contacts and was observed on the apical surface at a density 24 times that of the basal membrane contacting the substratum. The possibility that gp135 was associated with components of the apical cytoskeleton was investigated using cytoskeleton-disrupting drugs. Incubation in cytochalasin D produced a clustering of both actin and gp135, and double-label fluorescence microscopy demonstrated that these proteins were colocalized. Experiments using nocodazole had no effect, suggesting that gp135 could be interacting with actin microfilaments, but not microtubules. Treatment with Triton X-100 extracted ~50% of the gp135 and immunofluorescence microscopy indicated that the gp135 which remained associated with the detergent-insoluble cytoskeleton had a distribution identical to that of control cells. Experiments demonstrating that gp23, a nonpolarized glycoprotein, was preferentially extracted from the apical membrane suggested that the improperly sorted apical gp23 did not interact with the cytoskeleton. These results provided evidence that the polarized cell surface distribution of gp135 was maintained through its interaction with actin in the apical cytoskeleton.

One of the primary functions of epithelia in a variety of organs and tissues is to form a barrier between two compartments that will allow the vectorial transport of ions, sugars, amino acids, hormones, and proteins from one compartment to the other. Epithelial cells are able to carry out these specialized functions due to the presence of tight junctions which seal the intercellular spaces between individual cells and prevent passive transepithelial diffusion (11). Tight junctions are usually located at the boundary between the apical plasma membrane which borders the epithelial lumen and the basolateral membrane which contacts the basal lamina (11, 39, 44). The apical and basolateral membranes are each composed of distinctly different lipids and proteins and it is this polarized distribution of enzymes and transport systems that enables epithelia to carry out transepithelial transport (reviewed in references 39, 44).

Since native epithelia usually contain more than one cell type, recent work on cell polarity has been done in culture with homogenous cell lines. Madin-Darby canine kidney (MDCK)[1] cells have been extensively used for these studies because they have retained many of the differentiated properties of epithelia including the presence of functional tight junctions (7, 26, 36), the ability to carry out vectorial ion transport (6, 7, 36), expression of enzymes and glycoproteins characteristic of the distal nephron (16, 34, 38), and polarized distributions of plasma membrane lipids (14, 31), glycoproteins (4, 15, 21, 48), and cytoskeletal proteins (28, 29). Earlier work on both native epithelia and MDCK cells has provided some evidence that cell polarity is maintained by tight junctions which form an intramembrane barrier to the lateral diffusion of apical and basolateral membrane proteins and prevent their intermixing (17, 35, 49).

When MDCK cells are infected with lipid envelope vi-

1. *Abbreviations used in this paper*: CMEM, Eagle's minimum essential medium containing fetal bovine serum; CSK buffer, cytoskeleton buffer; GAM, goat anti-mouse IgG; GAR, goat anti-rabbit IgG; LC medium, low calcium culture medium; MDCK, Madin-Darby canine kidney; PA-gold, protein A-gold label; TX-100, Triton X-100; TX-114, Triton X-114.

ruses, the mature virions are assembled and released from the cell surface in a polarized manner (40); further studies have provided important new data on the subcellular and molecular mechanisms involved in the biogenesis of membranes and cell polarity (reviewed in references 22, 39, 44). Of considerable importance was the observation that polarized viral budding could occur in sparse cultures lacking cell–cell contacts (41), suggesting that tight junctions were not always required for the maintenance of epithelial cell surface polarity. Subsequent work using monoclonal antibodies provided evidence that basolateral membrane glycoproteins appeared to be randomly distributed over the entire cell surface in MDCK monolayers lacking tight junctions (15). This was confirmed by quantitative immunogold EM (33). However, after the formation of cell–cell contacts and the assembly of tight junctions, a continuous development of polarity was observed for the basolateral glycoproteins (15), results which have been supported by those of other workers (17, 28). More recently, Vega-Salas et al. (48) have obtained quantitative immunofluorescence data confirming that development of basolateral membrane polarity in MDCK cells occurs in a continuous manner after tight junctions have assembled. They also demonstrated that an 184-kD apical protein became polarized shortly after cell–substratum attachment in the absence of cell–cell contact formation and proposed that the maintenance of cell surface polarity for this protein did not require the presence of tight junctions (48).

In this report, we demonstrate that an apical membrane glycoprotein with a molecular mass of 135 kD (gp135) is polarized on MDCK cells lacking cell–cell contacts. We also provide morphological and biochemical evidence that gp135 is associated with the cytoskeleton and propose that maintenance of gp135 cell surface polarity is mediated through either a direct or indirect linkage to actin associated with the apical plasma membrane.

Materials and Methods

Cell Culture

MDCK cells obtained from Dr. J. Leighton, Medical College of Pennsylvania (Philadelphia, PA), were grown in MEM containing 10% FBS and gentamycin (CMEM) in a 95% air/5% CO_2 atmosphere at 37°C. Trypsin-EDTA was used for cell passage as described (32, 34) and experiments were done on cells of 110–130 passages. MDCK cells were plated at 1.5×10^4 cells/cm^2 in 96-well microtiter plates for RIA, on coverglasses (12-mm-diam) for immunofluorescence microscopy of monolayers, or on micropore filters (0.45-μm or 3-μm pores; Millipore Corp., Bedford, MA) for either semithin frozen or ultrathin sections. The MDCK cells were then grown for the time indicated with daily medium changes. For some experiments, cells were removed from culture dishes by incubation in 0.15 M NaCl, 20 mM Na_2HPO_4, 5 mM EGTA (pH 7.4) for 30 min, then plated at 2.5×10^3 cells/cm^2 in low Ca^{++} culture medium (Gibco Laboratories, Grand Island, NY) containing 10% dialysed FBS (LC medium), according to published procedures (13), on collagen-coated dishes and incubated for 24 h at 37°C. Hybridoma cell lines secreting monoclonal antibodies (19) against MDCK cell surface glycoproteins were isolated and cultured as described previously (15, 16). Medium components were purchased from Gibco Laboratories and sterile plasticware from Falcon Labware (Oxnard, CA).

Transepithelial Electrical Resistance Measurement

To determine tight-junctional permeability (6), MDCK cells were grown on micropore filters for 3 d, and transepithelial electrical resistances measured across 0.71 cm^2 of cell monolayer in a modified Ussing chamber by passing 10 μA of current through Hg/HgCl electrodes connected to the chamber by agar bridges containing 3 M KCl. Voltage deflections were measured on an electrometer (model 401; Keithley Instruments, Inc., Cleveland, OH) and transepithelial electrical resistances calculated after subtracting the background contributed by a blank filter.

Semithin Frozen Sections

After measuring transepithelial electrical resistance, cell monolayers on micropore filters were rinsed with PBS at 4°C, then fixed in 4% paraformaldehyde/0.1% glutaraldehyde PBS at 4°C for 30 min. Free aldehyde groups were quenched by incubating in 0.9 M sucrose containing 50 mM NH_4Cl for 1 h at 4°C. The cells were infiltrated with 1.5 M sucrose for 16 h at 4°C, mounted on specimen holders, and rapidly frozen by immersion in liquid nitrogen. Semithin frozen sections (0.5 μm) cut at −80°C (46), on either an ultramicrotome (model MT-2; Sorvall Instruments, Newton, CT) equipped with a FT5000 cryoattachment or a Cryonova ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD), were picked up on glass slides coated with 0.01% poly-L-lysine and stained immediately.

Treatment of MDCK Monolayers With Cytoskeleton-disrupting Drugs

Stock solutions of both cytochalasin D (1 mM) and nocodazole (33 mM) were made in DMSO and stored at −20°C. Just before use, these drugs were added to CMEM (1:1,000 dilution) to give final concentrations of 1 μM cytochalasin D and 33 μM nocodazole. Cell monolayers grown on coverglasses were incubated in CMEM containing 0.1% DMSO and either drug or CMEM containing only 0.1% DMSO (as a control) in a 5% CO_2 atmosphere for 4 h at 37°C. Cytochalasin D, nocodazole, and DMSO were purchased from the Sigma Chemical Co., St. Louis, MO.

Immunofluorescence Microscopy

The distribution of cell surface glycoproteins was determined by immunofluorescence staining of both semithin frozen sections and cell monolayers on coverglasses. All incubations were done for 30 min at 4°C and all antibodies were diluted in 2% BSA-PBS. To prevent nonspecific binding, the samples were first incubated in BSA-PBS, then sequentially in monoclonal supernatant (1:2 dilution), rabbit anti-mouse IgG, and goat anti-rabbit (GAR)-rhodamine with intervening BSA-PBS washes. Except where noted, antibodies were purchased as affinity-purified reagents (Cappel Laboratories, Cochranville, PA) and used at a 1:10 dilution. The samples were mounted in 90% glycerol-PBS and photographed on Tri-X film (Eastman Kodak Co., Rochester, NY) in a microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics. For double-labeling experiments, monolayers incubated in CMEM ± cytochalasin D were fixed as described above, permeabilized in acetone for 5 min at −20°C, and stained for gp135 as described above except that GAR-fluorescein was used instead of GAR-rhodamine. Actin microfilaments were then localized by incubating with phalloidin-rhodamine (1:20 dilution in PBS; Molecular Probes, Inc., Junction City, Oregon) for 30 min at 24°C (25). For localizing microtubules, MDCK cells incubated in CMEM ± nocodazole were incubated in a microtubule-stabilizing buffer for 15 s at 37°C followed by this buffer containing 0.5% Triton X-100 (TX-100) for 15 s at 37°C (3). The cells were then fixed with aldehydes and the microtubules localized with mouse monoclonal antitubulin (1:50 dilution; Amersham Corp., Arlington Heights, IL) followed by goat anti-mouse (GAM)-rhodamine (1:10 dilution). For comparative purposes, all immunofluorescence micrographs of membrane glycoprotein distribution were photographed and printed with identical exposure times.

Immunoelectron Microscopy

For localization of gp135 on confluent MDCK cells, monolayers were fixed as described above, dehydrated in graded ethanols, and then embedded in LR White resin (Polysciences Inc., Warrington, PA) according to published procedures (45) except that polymerization was done for 16 h at −20°C using an accelerator. Thin sections were cut on a diamond knife and picked up on 200-mesh grids coated with formvar films. Localization of gp135 was done by floating these grids on sequential drops of monoclonal supernatant (diluted 1:2 with BSA-PBS-0.025% Tween 20), rabbit anti-mouse IgG (1:10 dilution), and protein A-10-nm colloidal gold (PA-gold; Sigma Chemical Co.; 1:25 dilution) with intervening BSA-PBS-Tween washes for 1 h each at 24°C. Localization of gp135 on cells plated in LC medium was done by sequential incubation of fixed cells in monoclonal supernatant and rabbit anti-mouse IgG for 1 h each at 24°C, followed by PA-gold for either 2 or

16 h on a rotating platform to insure that the antibodies had access to the entire cell surface. After a postfixation in 2% glutaraldehyde–PBS for 30 min, the cells were dehydrated in ethanol and embedded in Epon 812. Thin sections were observed and photographed both with and without heavy metal poststaining in an electron microscope (model 100C; JEOL USA, Peabody, MA) operating at 60 kV.

Quantitative determination of membrane glycoprotein density on cells plated in LC medium was done on electron micrographs (25,000×) by measuring the length of cross sectioned apical (defined as the noncontacted surface) and basolateral (the surface in contact with the substratum) membranes, then counting the number of PA-gold particles for each membrane domain. Cell surface polarity was expressed as the apical particle density/basolateral particle density for each individual cell before calculation of the mean.

Preparation of MDCK Cytoskeletons

MDCK cells plated in 96-well microtiter plates were rinsed twice with a cytoskeleton buffer (CSK buffer) containing 10 mM Pipes (pH 6.8), 3 mM $MgCl_2$, 0.1 M NaCl, 0.3 M sucrose, and 1.2 mM PMSF; then extracted in CSK buffer containing 0.5% TX-100 for 15 min at 4°C according to Fey et al. (12). The detergent-extracted cells were rinsed twice with CSK buffer and the TX-100 cytoskeleton remaining in the well was fixed as described above. In some experiments, the TX-100–extracted cells were incubated for an additional 1 h at 24°C in CSK buffer.

Radioimmunoassay of gp135 and Cytoskeletal Proteins

The levels of gp135 on both the MDCK cell surface and the TX-100 cytoskeleton were determined at 4°C by a solid phase RIA. All steps were done at 4°C for 30 min each. Fixed cells or cytoskeletons were incubated in BSA–PBS to reduce nonspecific antibody binding followed by 100 μl of monoclonal supernatant (1:2 dilution). The cells were then washed with BSA–PBS, incubated in 100 μl of ^{125}I-GAM (2×10^5 cpm/well), and solubilized with 1% TX-100/0.5% sodium deoxycholate for ≥30 min at 24°C. The detergent extract was completely removed from the well with a cotton swab, placed in a plastic tube, and the levels of ^{125}I-GAM bound determined in an LKB Instruments, Inc. gamma counter. Measurement of actin in TX-100 cytoskeletons was identical except that rabbit polyclonal antiserum (1:100 dilution) against actin (a generous gift of Dr. W. J. Nelson, Fox Chase Cancer Center, Philadelphia, PA; see reference 29) and ^{125}I-GAR were used. The radioisotopes used in these assays were purchased from New England Nuclear, Boston, MA.

Immunoprecipitation and Immunoblotting Procedures

The molecular mass of gp135 was determined by two methods. For immunoprecipitation, MDCK cells were metabolically labeled 16 h in CMEM containing 50% normal glucose and [^{3}H]glucosamine (50 μCi/ml) or in CMEM containing [^{35}S]methionine (50 μCi/ml). The cells were solubilized in 1% TX-100, 0.5% sodium deoxycholate, 10 TIU/ml aprotinin, 1 mM PMSF, and 50 mM Tris-HCl (pH 7.2) for 1 h at 4°C. Isotopically labeled gp135 was immunoprecipitated from 100 μl of detergent extract by incubation at 4°C in 100 μl of monoclonal supernatant for 16–18 h followed by the addition of 50 μl of a 50% slurry of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) coupled to GAM F(ab')$_2$. After a 4-h incubation, the beads were washed five times in 0.25% TX-100, 150 mM NaCl, 1 mM PMSF, 5 mM EDTA, 10 TIU/ml aprotinin, 1 mg/ml ovalbumin, and 50 mM Tris-HCl (pH 7.2). Then they were pelleted in a microcentrifuge, and the immunopurified glycoprotein eluted with 1% SDS, 1 mM PMSF, 0.2 mg/ml ovalbumin, 1% β-mercaptoethanol, and 50 mM Tris-HCl (pH 6.7) by placing the tube in a boiling H_2O bath for 3 min.

The immunoprecipitated gp135 was identified by SDS-PAGE using a discontinuous buffer system (20). The gels were fixed and stained in 0.1% Coomassie Blue, 50% ethanol, 7% acetic acid, and 1% TCA then prepared for fluorography (1) using Enlightening (New England Nuclear). For immunoblotting, confluent MDCK cells were extracted with 1% TX-100, 10 TIU/ml aprotinin, 1 mM PMSF, and 65 mM Tris-HCl (pH 6.8) for 1 h at 4°C; then the extract was centrifuged in a microcentrifuge for 15 s at 4°C. In some experiments, MDCK cells were extracted in Triton X-114 (TX-114) and integral membrane proteins separated into the detergent phase according to the procedure of Bordier (2). 1 vol of cell extract was added to 2 vol of 2% SDS in 65 mM Tris-HCl (pH 6.8) for 10 min at 24°C before SDS-PAGE. MDCK proteins were transferred from unfixed gels to nitrocellulose filters (47) using procedures described previously (34), then the filters were incubated sequentially in monoclonal supernatant (1:10 dilution) followed by ^{125}I-GAM (10^5 cpm/ml) for 1 h each at 24°C. Identification of gp135 was done by exposing dried SDS gels or electroblotted nitrocellulose filters (using enhancing screens) to X-AR film (Eastman Kodak Co.) at −70°C. Apparent molecular mass of immunoprecipitated or immunoblotted gp135 was determined by comparison to ^{14}C molecular mass markers ranging from 14 to 200 kD (Bethesda Research Laboratories, Gaithersburg, MD).

Results

Identification and Characterization of a 135-kD Glycoprotein on the MDCK Cell Surface

A hybridoma cell line (designated 3F2) was isolated which secreted monoclonal antibodies that bound to antigens on the surface of confluent MDCK cells (Fig. 1 *a*). The antigen recognized by antibody 3F2 was identified by SDS-PAGE after immunoblotting (Fig. 1 *b*) or by immunoprecipitation after metabolically labeling with [^{3}H]glucosamine (data not shown), and it was determined to be a 135-kD glycoprotein (gp135). Similar results were also obtained from [^{35}S]methionine-labeled cells and by immunoblotting after detergent extraction with TX-114 (data not shown). The observation that gp135 partitioned into the TX-114 detergent phase strongly suggests that it is an integral membrane glycoprotein (2). Since the immunoblotting procedures were done in the absence of reducing agents (34), it appears that gp135 does not form higher molecular mass complexes requiring disulfide bonding. The levels of gp135 on the MDCK cell surface were determined during growth after plating at a subconfluent density and demonstrated to be on both subconfluent (24 h) and confluent (48–96 h) cells (Fig. 1 *a*). We chose the 72-h time interval for the majority of our studies on confluent cells since gp135 levels were high (Fig. 1 *a*);

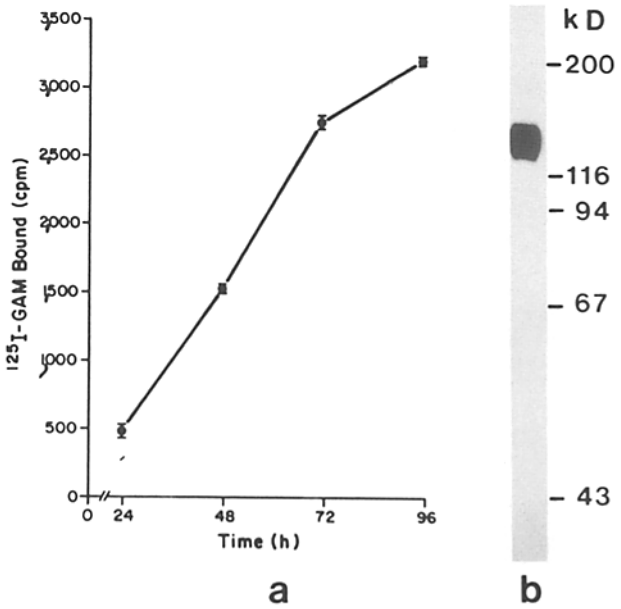

Figure 1. Identification of an apical cell surface antigen on MDCK cells. Cells were plated in microtiter wells at a subconfluent density, grown as described (see Materials and Methods), and fixed at 24-h intervals. (*a*) The levels of antigen recognized by monoclonal supernatant 3F2 were measured by RIA and expressed as ^{125}I-GAM bound (cpm/well ± SEM). (*b*) SDS-PAGE and immunoblotting demonstrated that the antigen was a polypeptide with an M_r of 135 kD when compared to ^{14}C-labeled mol mass standards.

and previous work had demonstrated that tight junctions were fully assembled and basolateral glycoproteins were polarized (15, 34).

Localization of gp135 on MDCK Cells

The cell surface distribution of gp135 was determined on semithin frozen sections of confluent monolayers. Immunofluorescence microscopy demonstrated that gp135 had a polarized distribution and was localized only to the apical cell surface (Fig. 2). These results were confirmed by immunoelectron microscopy on LR White–embedded MDCK cells. Ultrastructural observations of thin sections stained with monoclonal antibody 3F2 and PA–gold demonstrated that gp135 was distributed over the entire apical cell surface including the microvilli and was not detected on the basolateral membrane (Fig. 3).

Since another laboratory had provided evidence that a 184-kD protein was localized to the apical surface on MDCK cells lacking cell contacts (48), we decided to determine if gp135 could also develop a polarized distribution under similar culture conditions. For these studies, gp135 was localized by immunogold EM on cells maintained in LC medium for 24 h to prevent the formation of tight junctions (13) and other intercellular junctions (28, 29, 48). Ultrastructural examination demonstrated that cell junctions were not present under our culture conditions and that gp135 was localized primarily to the apical surface with only low levels being observed on the basolateral membrane contacting the substratum (Fig. 4 *a*). Quantitative measurement of PA–gold particle density demonstrated that the apical/basolateral ratio of gp135 was 24:1 (Table I). A monoclonal antibody to gp60, a basolateral glycoprotein with a nonpolarized distribution on subconfluent MDCK cells (15), was used in control experiments. Immunostaining of gp60 was observed on both the apical and basolateral membranes (Fig. 4 *b*) and quantitative determination of the apical/basolateral ratio was determined to be 0.98 (Table I) indicating that gp60 had a random distribution under these culture conditions.

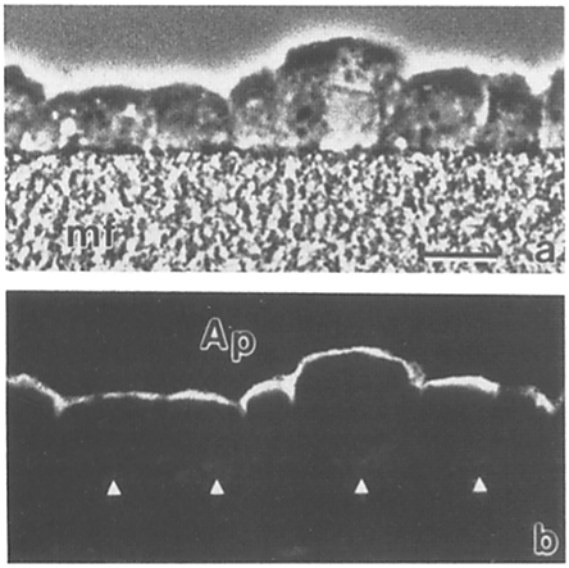

Figure 2. Immunofluorescence localization of gp135 on semithin frozen sections of MDCK cells. (*a*) Phase-contrast micrograph of a confluent MDCK monolayer grown on a micropore filter (*mf*). Immunofluorescence microscopy of the same section (*b*) demonstrated that gp135 was localized to the apical cell surface (*Ap*) and could not be detected on the basolateral membrane (*arrowheads*). Bar, 12.5 μm.

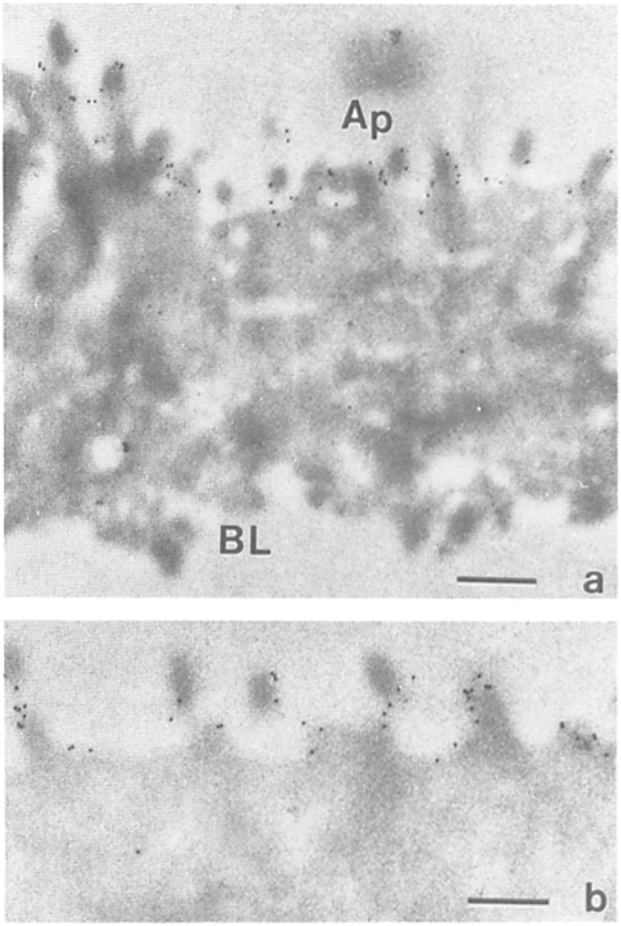

Figure 3. Ultrastructural localization of gp135 on confluent MDCK cells. Thin sections of cells embedded in LR White resin were incubated in monoclonal antibody and PA–gold as described in Materials and Methods. (*a*) Staining of gp135 was observed only on the apical cell surface (*Ap*) and not on the basolateral membrane (*BL*). At higher magnification (*b*), PA–gold particles were observed on microvilli and adjacent regions of the apical plasma membrane. Bars, 0.38 μm (*a*); 0.22 μm (*b*).

Effect of Cytoskeleton-disrupting Drugs on gp135 Cell Surface Distribution

One explanation for the polarized gp135 distribution on cells maintained in LC medium is that this glycoprotein was interacting with components of the apical cytoskeleton which prevented its lateral mobility in the membrane. This possibility was tested by studying the effects of drugs which can disrupt the organization of the cytoskeleton. Incubation in cytochalasin D caused the formation of membrane blebs and cell retraction (Fig. 5, *a* and *d*) similar to that described for cytochalasin B–treated MDCK cells (24). Under these conditions, there was an extensive disruption in cytoskeletal organization and large actin aggregates were observed (Fig. 5 *b*) confirming results published by other workers (6, 23, 25, 43). In double-labeling experiments, gp135 was observed in clusters (Fig. 5 *c*) that were colocalized with the actin ag-

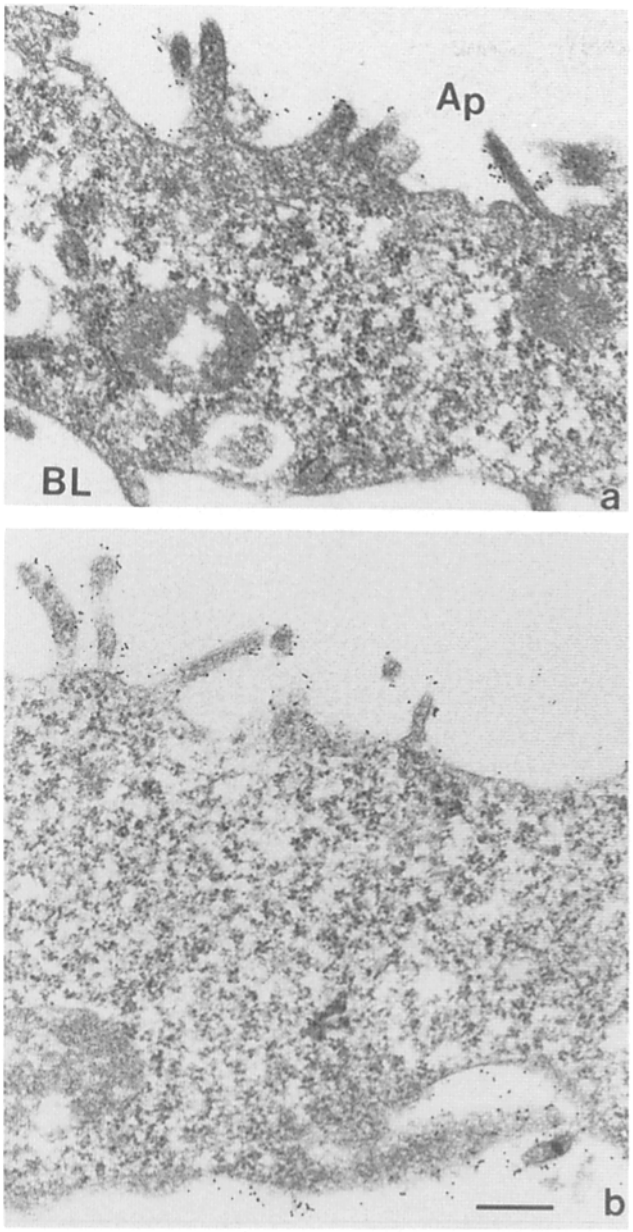

Figure 4. Ultrastructural localization of membrane glycoproteins on cells plated in LC medium. Immunogold EM demonstrated that (*a*) gp135 was distributed over the apical cell surface (*Ap*) including the microvilli and only small amounts were observed on the basolateral (*BL*) surface. As a control (*b*), gp60 was localized on cells cultured under identical conditions and determined to be on both the apical and basolateral membranes. Bar, 0.29 μm.

gregates (Fig. 5 *b*). Furthermore, gp135 was not observed on cell extensions (Fig. 5 *c*) which lacked actin (Fig. 5 *b*). In control experiments, it was determined that gp60 was found on membrane blebs as well as cell extensions (Fig. 5 *e*), demonstrating that this glycoprotein did not appear to aggregate with actin in cytochalasin D–treated cells. The cell surface distributions of gp135 and gp60 were determined by immunogold EM to confirm the immunofluorescence results. These data demonstrated that gp135 was confined to localized regions of the cell surface primarily on apical membrane extensions and was not detected on the basolateral membrane (Fig. 6 *a*). These groups of membrane extensions probably represented the gp135 aggregates observed by immunofluorescence microscopy (Fig. 5 *c*). It is possible that the formation of these aggregates was due either to the lateral movement of gp135 into the membrane extensions or to the clustering of microvillar and intermicrovillar membrane regions containing gp135. In contrast, gp60 was found on both the apical cell surface extensions as well as on basolateral processes extending into the pores of the micropore filter substratum (Fig. 6 *b*).

The association of cytoplasmic microtubules with gp135 was investigated using nocodazole, a drug that disassembles microtubules in MDCK cells (3, 48). When control cells were viewed by immunofluorescence microscopy after antitubulin staining, numerous microtubules were observed throughout the cytoplasm (Fig. 7 *a*) while, after nocodazole treatment, the majority of microtubules were disassembled (Fig. 7 *b*) confirming the results of other investigators (3, 43). Despite this loss of microtubules, the gp135 apical surface distribution did not change in the presence of nocodazole (Fig. 7 *c*).

Association of gp135 With the Cytoskeleton After Detergent Extraction

The data presented in the preceding section suggested that gp135 could be interacting with actin microfilaments in the cytoskeleton. To test this possibility further, MDCK cells were extracted with 0.5% TX-100 in CSK buffer under conditions that had been previously shown to produce an MDCK cytoskeleton (12, 24, 29). Determination of gp135 levels by RIA demonstrated that ~66% of the gp135 remained associated with the TX-100 cytoskeleton of 48-h cultures and that cytoskeletal-associated gp135 dropped to ~53% of apical membrane levels on 72-h cultures, even though the amount of actin remaining in the cytoskeleton increased (Table II). However, calculation of the gp135/actin ratio (4.45:1) indicated that the levels of gp135 bound to the cytoskeleton

Table I. Quantitative Measurement of gp135 and gp60 on MDCK Cells Lacking Cell–Cell Contacts

	Apical surface		Basolateral surface		
	Length	Particles per μm	Length	Particles per μm	Ap/BL*
	μm		*μm*		
gp135	2.53 ± 0.2	43.3 ± 3.8	1.87 ± 0.1	2.38 ± 0.4	24.1 ± 3.0
gp60	2.51 ± 0.2	37.7 ± 2.7	2.03 ± 0.4	38.3 ± 2.4	0.98 ± 0.04

MDCK cells plated in LC medium for 24 h were fixed and membrane glycoproteins localized by immunogold EM (see Materials and Methods).
* The ratio of Apical surface label to Basolateral surface label (*Ap/BL*) of PA-gold density was determined on 22 cells each for gp135 and gp60, calculated for each cell, and presented as the mean ± SEM.

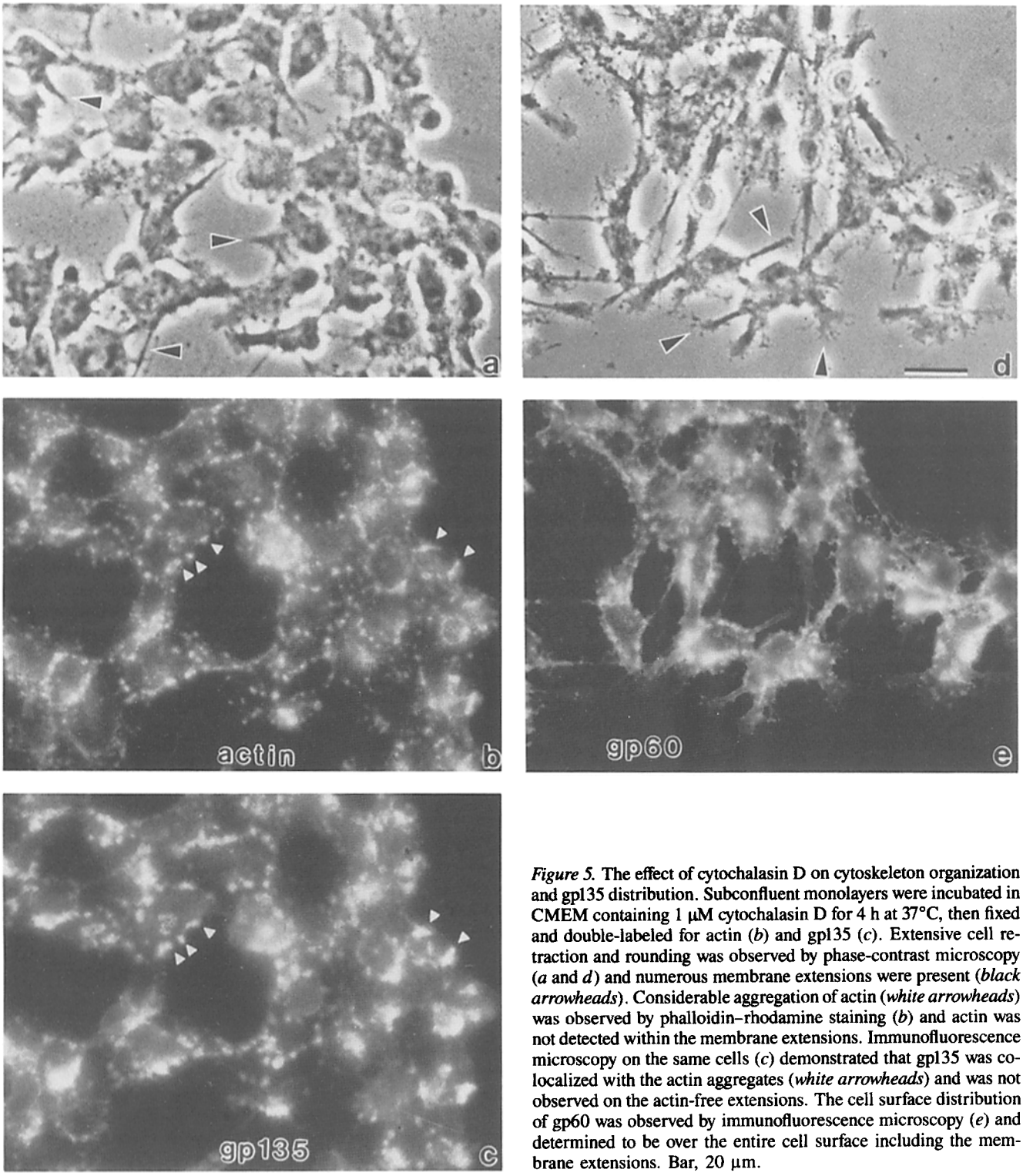

Figure 5. The effect of cytochalasin D on cytoskeleton organization and gp135 distribution. Subconfluent monolayers were incubated in CMEM containing 1 μM cytochalasin D for 4 h at 37°C, then fixed and double-labeled for actin (*b*) and gp135 (*c*). Extensive cell retraction and rounding was observed by phase-contrast microscopy (*a* and *d*) and numerous membrane extensions were present (*black arrowheads*). Considerable aggregation of actin (*white arrowheads*) was observed by phalloidin–rhodamine staining (*b*) and actin was not detected within the membrane extensions. Immunofluorescence microscopy on the same cells (*c*) demonstrated that gp135 was colocalized with the actin aggregates (*white arrowheads*) and was not observed on the actin-free extensions. The cell surface distribution of gp60 was observed by immunofluorescence microscopy (*e*) and determined to be over the entire cell surface including the membrane extensions. Bar, 20 μm.

remained constant. Incubation of TX-100–extracted cells (72 h) in CSK buffer for an additional 1 h had no effect on the amount of gp135 associated with the cytoskeletal fraction (53.8 ± 5.9%).

The distribution of gp135 on TX-100 cytoskeletons was studied further by immunofluorescence microscopy. After detergent extraction, gp135 was observed on all cells in the monolayer in a punctate staining pattern (Fig. 8 *b*) similar to that of untreated monolayers (Fig. 8 *a*) except that the staining intensity was less in the TX-100–treated cells. In control experiments, detergent-extracted monolayers were stained with a monoclonal antibody against a 23-kD cell surface glycoprotein (gp23), a glycoprotein which has a nonpolarized cell surface distribution on confluent MDCK monolayers (34). On intact cells, gp23 was observed on the apical surface in a punctate pattern (Fig. 9 *a*) that represented

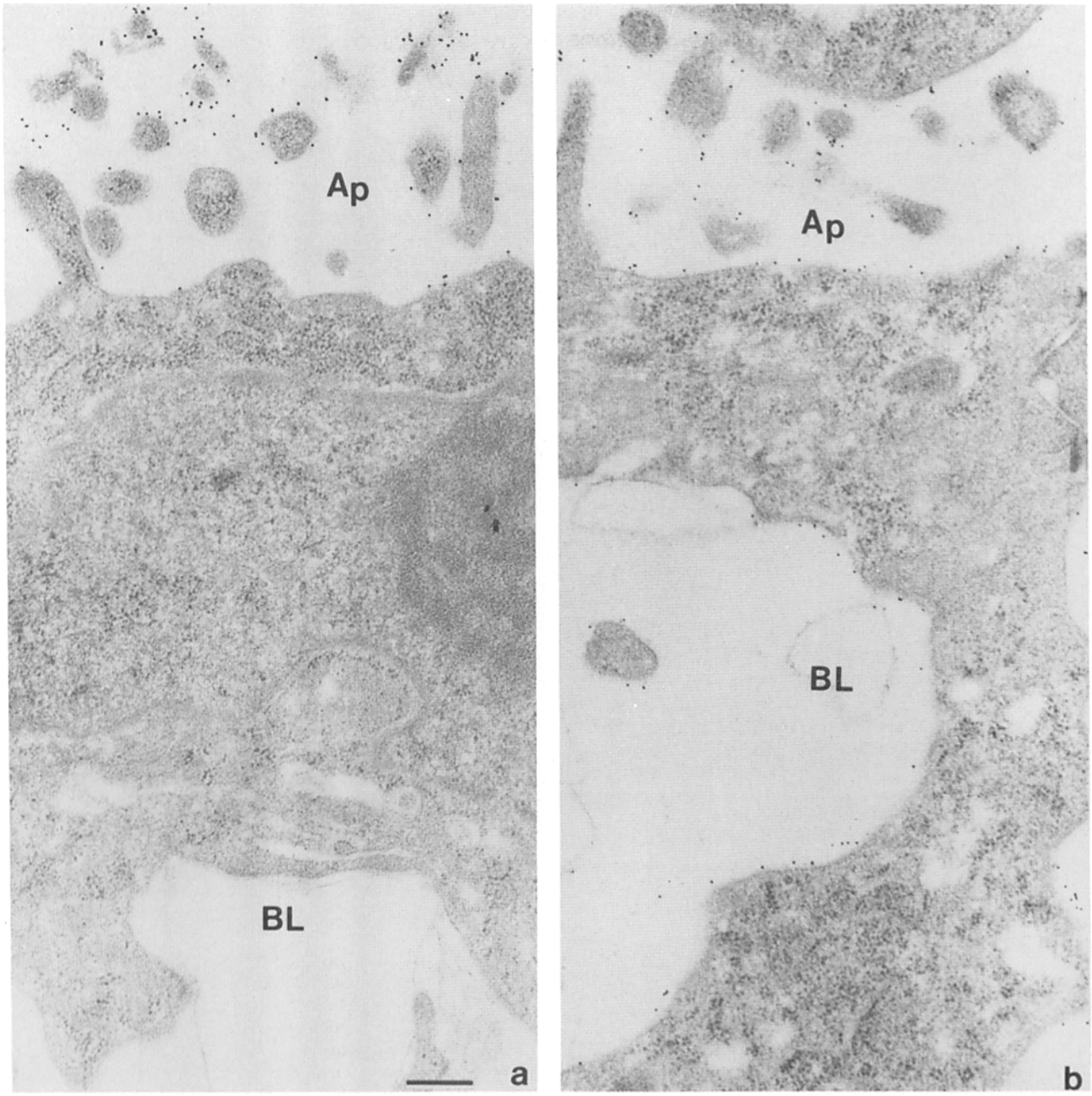

Figure 6. Ultrastructural localization of gp135 and gp60 after cytochalasin D treatment. Subconfluent cells grown on micropore filters were incubated in cytochalasin D as described above, then fixed and prepared for immunogold EM. Despite dramatic cell shape changes, gp135 (*a*) was localized primarily to numerous extensions of the apical (*Ap*) plasma membrane and was rarely observed on the adjacent cell surface or the basolateral membrane (*BL*). However, gp60 (*b*) was observed on both the apical and basolateral membranes. Bar, 0.29 μm.

microvillar staining (34). However, after TX-100 treatment, the majority of the apical gp23 was extracted (Fig. 9 *b*) while basolateral gp23 was still observed at high levels (Fig. 9, *b* and *c*).

Discussion

A monoclonal antibody recognizing a 135-kD glycoprotein on MDCK cells was used to determine gp135 cell surface distribution and characterize its possible interaction with the cytoskeleton. On confluent monolayers, gp135 had a polarized distribution and was localized only to the apical cell surface. In addition, immunogold EM demonstrated that gp135 was confined primarily to the apical (or noncontacted) surface of cells in LC medium lacking intercellular junctions (13, 28, 48) and was rarely observed on the basolateral surface contacting the substratum. Quantitative immunogold EM demonstrated that the apical/basolateral ratio of gp135 was 24:1 after 24 h in LC medium. These results support those previously made on a 184-kD protein which developed an ~12:1 polarity ratio on MDCK cells under similar conditions (48). Our results also support the proposal of Vega-Salas et al. (48)

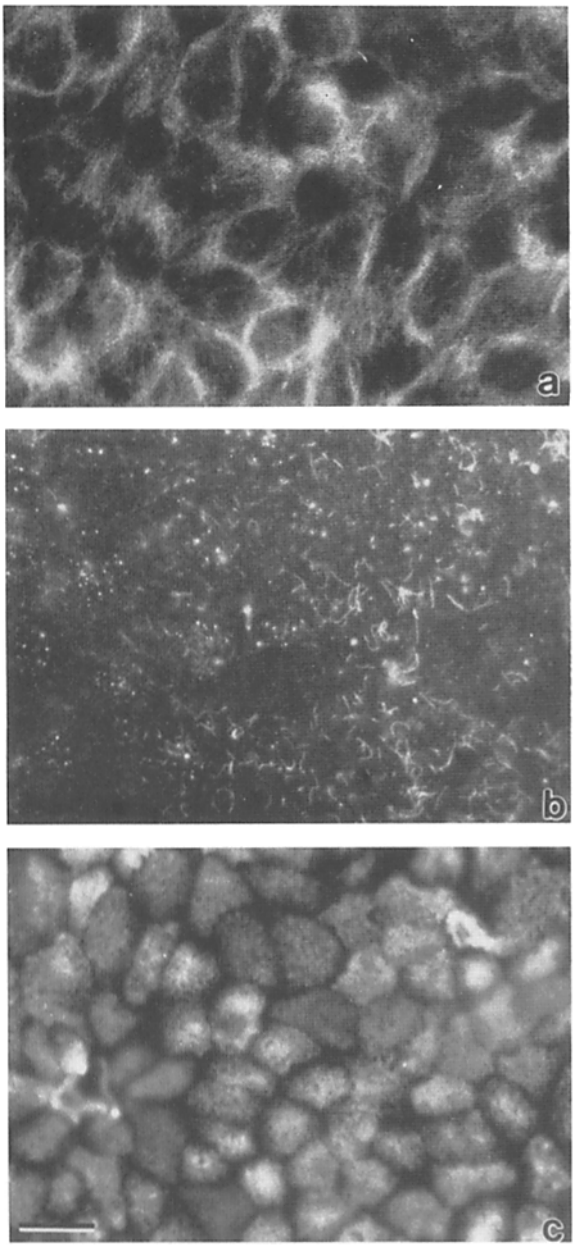

Figure 7. The effect of nocodazole on microtubule structure and gp135 cell surface distribution. Confluent MDCK monolayers were incubated in CMEM (*a*) or CMEM containing 33 μM nocodazole (*b* and *c*) for 4 h at 37°C. The cells were then fixed and stained with either antitubulin (*a* and *b*) or monoclonal antibody 3F2 (*c*). In control cells (*a*), immunofluorescence microscopy demonstrated the presence of numerous cytoplasmic tubules as well as intense staining associated with the lateral membranes. After nocodazole treatment, the majority of microtubules were disassembled (*b*), but the cell surface distribution of gp135 (*c*) appeared unaffected. Bar, 20 μm.

Table II. The Association of gp135 With the Cytoskeleton

Time	gp135		gp135 remaining (%)	Actin
	Intact cells	Cytoskeleton		
h				
48	1,765 ± 169	1,164 ± 122	66.2 ± 5.2	5,206 ± 87
72	2,444 ± 173	1,291 ± 94	53.4 ± 3.1	5,748 ± 632

MDCK cells cultured in microtiter wells for the indicated times were incubated in either CSK buffer, or CSK buffer containing 0.5% TX-100 for 15 min at 4°C before fixation. The levels of cell surface gp135 were determined by RIA (see Materials and Methods) on either intact cells or TX-100 cytoskeletons for five experiments (in triplicate) and presented as ^{125}I-GAM bound (cpm ± SEM/well). Actin levels were determined on TX-100 cytoskeletons and presented as ^{125}I-GAR bound (cpm/well ± SEM).

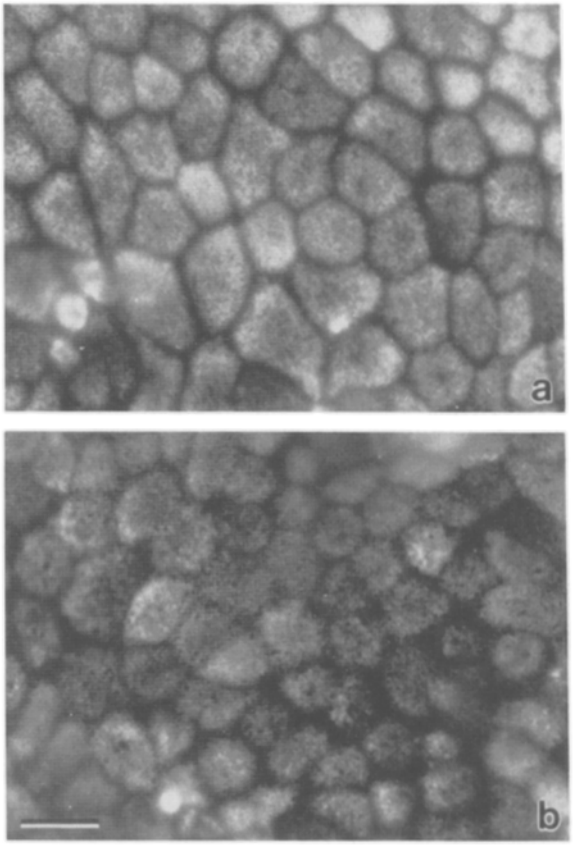

Figure 8. Localization of gp135 on the MDCK TX-100 cytoskeleton. MDCK cells were incubated in either CSK buffer (*a*) or CSK buffer containing 0.5% TX-100 (*b*) for 15 min at 4°C. After washing with CSK buffer, the cells were fixed and cell surface gp135 localized by immunofluorescence microscopy. Since these micrographs were photographed and printed with the identical exposure times, the reduced fluorescence after TX-100 treatment probably reflects gp135 extraction. Bar, 20 μm.

that cell contact with the substratum can produce a polarized distribution of apical membrane glycoproteins in the absence of tight junctions. Although the mechanism of polarity development has not been determined under these conditions, it is possible that the interaction of the plasma membrane with the substratum initiates the organization of the cytoskeleton into distinct apical and basolateral regions. This proposal is supported by numerous studies demonstrating that the apical brush border of both kidney and intestine contain unique cytoskeletal proteins (reviewed in reference 27). Furthermore, the membrane skeleton proteins fodrin and ankyrin have been localized to the basolateral membrane of both

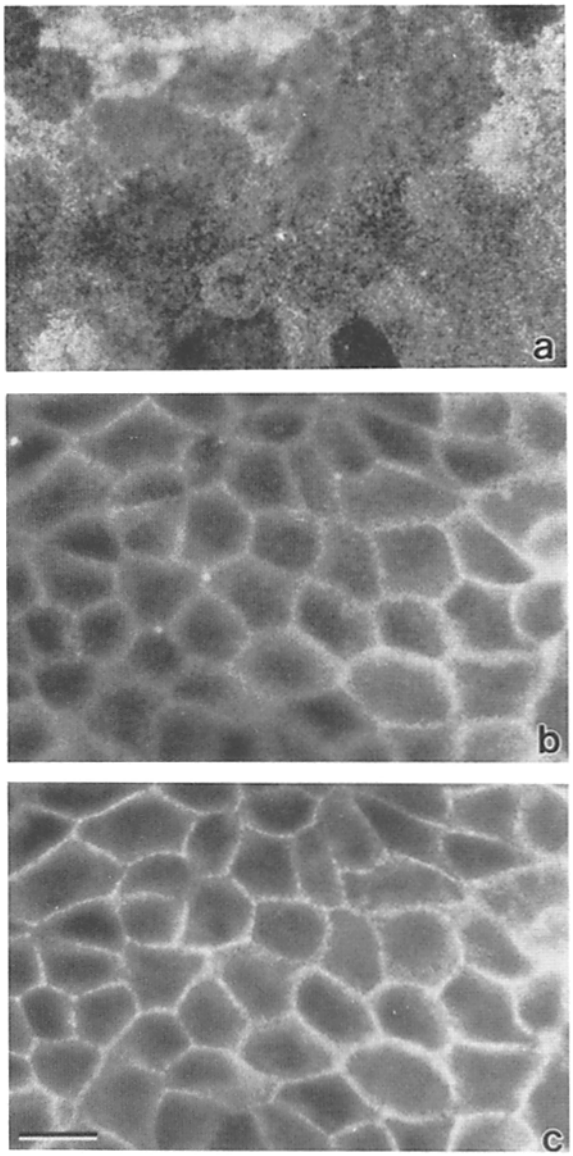

Figure 9. Localization of gp23 on the MDCK TX-100 cytoskeleton. Confluent cells were incubated in either CSK buffer (*a*) or CSK buffer containing 0.5% TX-100 (*b* and *c*) for 15 min at 4°C and gp23 distribution was determined by immunofluorescence microscopy. After CSK buffer treatment (*a*), intense staining of gp23 was demonstrated on the apical cell surface. On TX-100 cytoskeletons (*b*), gp23 staining was greatly reduced at the apical cell surface. However, when the same monolayer was viewed with the focal plane in the center of the cell (*c*), intense gp23 was observed on the basolateral membranes. Bar, 20 μm.

MDCK and native epithelial cells (9, 10, 28, 29), and it has been demonstrated that cell–cell contact is required for the association of fodrin with the membrane (28, 29).

From the data presented in this paper, there are several lines of evidence supporting the possibility that gp135 is interacting with the apical cytoskeleton of MDCK cells. The observation that gp135 is confined to the apical membrane of subconfluent cells suggests that this glycoprotein is partially immobilized and cannot diffuse laterally to the basolateral membrane. These results are supported by the observation that lectin binding sites do not freely diffuse on the apical surface of an amphibian epithelial cell line (8). However, determination of the lateral mobility of two apical membrane proteins after disruption of intestine tight junctions has demonstrated that these proteins have diffusion constants similar to freely mobile membrane proteins (49). These data do not appear to be inconsistent with those obtained for the lectin binding sites (8), since the latter probably comprise a variety of membrane glycoproteins and it is also likely that not all apical cell surface proteins interact with the cytoskeleton.

TX-100 extraction of MDCK cells produces an insoluble fraction which has been operationally defined as the cytoskeleton (12, 24, 29) and scanning EM has demonstrated that the apical microvillar structure remained intact under these extraction conditions (12). Using an identical extraction procedure, we have obtained immunogold evidence that ~50% of the gp135 was associated with the TX-100 cytoskeleton. These results are similar to those of Meza et al. (24) who demonstrated that ~36% of ^{125}I-labeled cell surface proteins remained with the cytoskeleton after TX-100 extraction. In addition, Rapraeger et al. (37) were unable to release ~20% of an integral membrane proteoglycan and demonstrated that this represented a fraction that was bound to the basolateral cytoskeleton. Based on our structural studies, there is the possibility that gp135 was interacting either directly or indirectly with actin microfilaments that have been identified in MDCK cells (23–25, 43). This proposal is supported by double-labeling fluorescence microscopy demonstrating a colocalization of actin and gp135 after cytochalasin D treatment, indicating that disorganization and aggregation of actin microfilaments was accompanied by a clustering of gp135. Furthermore, examination of the MDCK cytoskeleton by immunofluorescence microscopy demonstrated that the detergent-insoluble gp135 retained a punctate-staining pattern similar to that of unextracted cells, suggesting that this glycoprotein was associated with cytoskeletal components of the apical microvilli.

It is not clear if the gp135 fraction extracted with TX-100 represented gp135 that was unassociated with cytoskeletal or other membrane components. One possibility is that gp135 was coupled to actin filaments which were not fully incorporated into the cytoskeleton and that this loosely associated fraction was extracted from the cells with TX-100. This suggestion is supported by data from two other studies on MDCK cells demonstrating that a considerable amount of actin can be extracted by TX-100 (24) and that 25% of total cellular actin was F-actin, which was not associated with the detergent-insoluble cytoskeleton (18). It is further supported by the observation that a membrane protein–cytoskeletal complex containing actin could be extracted from mammary epithelial cell microvilli by TX-100 and that the amount of complex varied according to the stability of the microvillar actin core (5).

In kidney and toad bladder epithelium, the codistribution of the anion transporter with fodrin and ankyrin on the basolateral membrane has been proposed as evidence for the possible interactions of these proteins (9, 10). This work has been extended by the observation that fodrin becomes associated with the MDCK basolateral membrane concominant with the formation of cell–cell contacts and the development of (Na^+,K^+)ATPase polarity (28, 29). Further studies demonstrating that ankyrin can bind directly to (Na^+,K^+)ATP-

ase (30) have provided strong evidence for the interaction of this membrane protein with the basolateral cytoskeleton (28, 29). Even though data have been provided which suggest an interaction between apical surface actin and gp135, they do not demonstrate whether this was a direct interaction or if additional linking proteins could also be involved.

A 23-kD glycoprotein (gp23) has been observed on both the apical and basolateral membranes of the MDCK cell line used for the studies described in this paper (34). However, since gp23 was only localized on the basolateral membrane of both high electrical resistance MDCK cell lines and dog kidney epithelium, it was proposed that the nonpolarized gp23 lacked a basolateral targeting signal (34). From the work described in the present study, it appears that the gp23 which resided in the apical surface could be selectively extracted with TX-100. These results suggest the interesting possibility that basolateral gp23 could be interacting with cytoskeletal components associated with the basolateral membrane that are lacking from the apical membrane, allowing the gp23 which was mistargeted to the apical surface to be detergent extracted. Another laboratory has shown that ~80% of a 36-kD nonpolarized glycoprotein could be also extracted from MDCK cells by TX-100 (42) but morphological evidence determining whether this glycoprotein was also preferentially localized to the apical or basolateral cytoskeleton after detergent treatment was not presented. One interpretation of our results is that another protein(s) was involved in the binding of gp135 to the actin cytoskeleton. Based on the observations that ankyrin and fodrin only appear to be localized to the basolateral membrane of epithelial cells (9, 10, 28, 29), it seems unlikely they could be responsible for linking gp135 to the MDCK apical cytoskeleton leaving open the possibility that other proteins may be involved. At the present time, there is only a single report describing the direct interaction of a cytoskeletal protein with an epithelial membrane glycoprotein (30). We are presently purifying gp135 from MDCK cells and our future goal will be to determine if this glycoprotein is directly linked to actin microfilaments or if independent anchoring proteins are also involved.

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