Actin Microfilaments Play a Critical Role in Endocytosis at the Apical but not the Basolateral Surface of Polarized Epithelial Cells

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Abstract. Treatment with cytochalasin D, a drug that acts by inducing the depolymerization of the actin cytoskeleton, selectively blocked endocytosis of membrane bound and fluid phase markers from the apical surface of polarized MDCK cells without affecting the uptake from the basolateral surface. Thus, in MDCK cell transformants that express the VSV G protein, cytochalasin blocked the internalization of an anti-G mAb bound to apical G molecules, but did not reduce the uptake of antibody bound to the basolateral surface. The selective effect of cytochalasin D on apical endocytosis was also demonstrated by the failure of the drug to reduce the uptake of ^{125}I-labeled transferrin, which occurs by receptor-mediated endocytosis, via clathrin-coated pits, almost exclusively from the basolateral surface. The actin cytoskeleton appears to play a critical role in adsorptive as well as fluid phase apical endocytic events, since treatment with cytochalasin D prevented the apical uptake of cationized ferritin, that occurs after the marker binds to the cell surface, as well as uptake of Lucifer yellow, a fluorescent soluble dye. Moreover, the drug efficiently blocked infection of the cells with influenza virus, when the viral inoculum was applied to the apical surface. On the other hand, it did not inhibit the basolateral uptake of Lucifer yellow, nor did it prevent infection with VSV from the basolateral surface, or with influenza when this virus was applied to monolayers in which the formation of tight junctions had been prevented by depletion of calcium ions.

EM demonstrated that cytochalasin D leads to an increase in the number of coated pits in the apical surface where it suppresses the pinching off of coated vesicles. In addition, in drug-treated cells cationized ferritin molecules that were bound to microvilli were not cleared from the microvillar surface, as is observed in untreated cells. These findings indicate that there is a fundamental difference in the process by which endocytic vesicles are formed at the two surfaces of polarized epithelial cells and that the integrity and/or the polymerization of actin filaments are required at the apical surface. Actin filaments in microvilli may be part of a mechanochemical motor that moves membrane components along the microvillar surface towards intermicrovillar spaces, or provides the force required for converting a membrane invagination or pit into an endocytic vesicle within the cytoplasm.

F LUID phase and receptor-mediated endocytosis are processes that enable eukaryotic cells to take up both large and small molecules from their environment (see Anderson, 1991). The internalization of surface components that occurs during endocytosis also permits cells to modulate the protein composition of their plasma membranes since, to varying degrees, the interiorized proteins may be rapidly returned to the cell surface, or targeted to lysosomes for degradation (see Goldstein et al., 1985; Rodman et al., 1990). In polarized epithelial cells, which have two distinct plasma membrane domains facing different physiological compartments, endocytosis can take place from both surfaces. In such cells, molecules interiorized at one surface may also undergo transcytotic transfer to the opposite one (see Rodman et al., 1990). In fact, in some epithelial cells the transcytotic pathway is an essential part of the route used by newly synthesized plasma membrane protein molecules to reach their site of function (Bartles et al., 1987; Mostov and Deitcher, 1986).

The nature of the mechanisms that lead to the formation of endocytic vesicles is under active investigation. Receptor-mediated endocytosis takes place in clathrin-coated vesicles and it has been proposed that assembly of the coat drives the process of vesiculation (see Steer and Heuser, 1991; Anderson, 1991). Although the uptake of extracellular fluid (pinocytosis) can take place within clathrin-coated vesicles, several recent studies indicate that noncoated vesicles or "caveolae", formed by an as yet obscure mechanism, may play an important role in pinocytosis, as well as in the receptor-mediated uptake of low molecular weight ligands (Rothberg et al., 1992; Anderson et al., 1992).

The fact that many integral plasma membrane proteins are linked to cytoskeletal elements that may control their location and mobility (see Luna, 1991) raises the possibility that

the cytoskeleton also plays a role in the formation of endocytic vesicles. The participation of the actin-containing cytoskeletal network in phagocytosis and endocytosis has been extensively studied by examining the effect on these processes of the cytochalasins, drugs which lead to disassembly of actin filaments. Cytochalasins completely abolish phagocytosis by macrophages, a process which requires a major reorganization of the plasma membrane to engulf large particles (Axline and Reaven, 1974). On the other hand, there are conflicting reports on the extent of inhibition of endocytosis by these drugs. Using different cell types, several groups have reported that they inhibit the endocytic uptake of fluid phase markers, such as HRP and tritiated sucrose (Wagner et al., 1971; Pratten and Lloyd, 1979; Blok et al., 1982), but others (Willis et al., 1972; Klaus, 1973; Haigler et al., 1979; Piasek and Thyberg, 1980; Kyle et al., 1988) failed to observe an inhibitory effect. A recent study showed that in one cell type (African Green Monkey Kidney cells, VERO), cytochalasin D selectively inhibited the endocytic uptake of ricin and Lucifer yellow from nonclathrin-coated areas of the plasma membrane, while having no effect on the internalization of transferrin that occurs via clathrin-coated vesicles (Sandvig and Van Deurs, 1990). On the other hand, several authors have found an inhibitory effect of cytochalasins on the uptake of various ligands by receptor-dependent processes (Salisbury et al., 1980; Kaufman et al., 1990).

In polarized epithelial cells, the cytoskeleton plays an important role in the generation and maintenance of the polarized phenotype. In the basolateral surface of such cells actin microfilaments reach the plasma membrane, through their indirect interactions with cadherins or integrins at zonulae adherens or focal adhesion sites, respectively (see Geiger, 1989). In addition, at this surface an ankyrin/fodrin-based submembraneous scaffolding (see Nelson, 1989) appears to restrict the movement of specific integral membrane proteins, such as the Na^+,K^+ATPase, and thus determines their basolateral segregation (Nelson and Hammerton, 1989). On the apical surface of intestinal epithelial cells, the actin-containing cytoskeleton is highly developed, extending from the terminal web into the core of microvilli and towards regions of intercellular contacts (see Louvard, 1989). Moreover, apical actin has been shown to play a role in maintaining the apical polarization of certain proteins in MDCK cells, even in the absence of cell–cell contacts (Ojakian and Schwimmer, 1988).

We have previously shown that viral envelope glycoproteins serve as useful probes for studying the biogenesis of epithelial cell polarity and plasma membrane protein trafficking in polarized epithelial cells (Rodriguez-Boulan and Sabatini, 1978; Rindler et al., 1984, 1985, 1987; Gottlieb et al., 1986). When expressed in transfected MDCK cells grown on a permeable support, the VSV G glycoprotein accumulates almost exclusively in the basolateral plasma membrane domains of the cells (Compton et al., 1989). On the other hand, when confluent monolayers of transfected cells are formed on a solid support, a condition which restricts the access of nutrients from the medium to the basolateral surface and leads to the apical relocation of a substantial portion of basolateral markers, considerable amounts of the G protein also accumulate on the apical surface (Gottlieb et al., 1986). We have taken advantage of this

system to study the endocytic behavior of the apically and basolaterally located G protein molecules. This led us to discover a basic difference between the endocytic mechanisms that operate at the two surfaces of polarized epithelial cells. Thus, the integrity of the actin cytoskeleton was found to be required for apical but not for basolateral endocytosis of the G protein, since only the former was blocked by cytochalasin D. Using a variety of other probes we found that the intactness of the actin cytoskeleton is required for all apical endocytic events, whether they take place by the adsorptive pathway or by fluid phase pinocytosis.

Materials and Methods

Cell Culture and Materials

Low-resistance (strain II) MDCK cells (originally obtained from Dr. J. Leighton, Medical College of Pennsylvania, Philadelphia, PA) and the higher-resistance strain I (provided by Dr. G. Ojakian, SUNY Downstate Medical Center, Brooklyn, NY) were grown in MEM containing 10% FBS (Hyclone Laboratories, Logan, UT) and antibiotics, as previously described (Gonzalez et al., 1987).

The hybridoma producing mAbs to VSV G was a gift of Dr. J. Lewis (SUNY Downstate Medical Center, Brooklyn, NY). A rabbit polyclonal anti–HA antibody was obtained from Dr. M. Rindler (New York University Medical Center). A stock (25 mg/ml) solution of cytochalasin D (Sigma Chemical Co., St. Louis, MO) in DMSO was prepared and used by dilution to a final concentration of 25 µg/ml in culture medium. Controls were always treated with an equal concentration of DMSO.

For immunofluorescence experiments, 1.5×10^5 MDCK cell transformants expressing the VSV G protein (MDCK-G cells) were plated onto coverslips which had been placed into 24-well dishes. The next day, the cultures were placed in medium containing 20 mM sodium butyrate to induce synthesis of the viral glycoprotein (Gottlieb et al., 1986). After a 24-h incubation, the medium containing butyrate was changed and the incubation continued for another 24-h period. At this time the monolayers on coverslips were used to follow the endocytotic uptake of anti-G immunecomplexes.

Viral Infection

MDCK cells (strain I) were plated into Transwell filter chambers (1.5×10^6 cells/chamber) containing a large pore (3.0 µm) filter, which allows VSV penetration and infection from the basolateral surface. Strain II MDCK cells are unable to form a tight monolayer on such large pore size filters. The filter chambers were maintained in 6-well culture dishes for 4 d with daily changes of medium before infection. Only monolayers displaying a resistance >1,000 ohms cm^2 were used. When cells grown on coverslips were infected, the cells were first grown to confluency on the coverslips and then maintained for 3 d with daily changes of media. To infect cells with influenza virus in low calcium medium that prevents the formation of tight junctions, 24 h before infection the coverslips were washed several times in HBSS and then incubated in spinner MEM (lacking calcium) containing 5% dialyzed FCS (CF-MEM). Cells grown on coverslips, 35-mm dishes, or filter chambers were washed three times with serum-free medium before the addition of the viral inoculum, which was always in a medium containing calcium. Coverslips received influenza A/WSW virus in 0.2 ml of serum-free medium at a multiplicity of infection (moi)[1] of 20 pfu/cell. Cultures on 35-mm dishes and filters that received virus only from the apical side were infected at an moi of 10 pfu/cell in 0.5 ml of serum-free medium. Filter-grown cells were infected with VSV from the basolateral surface at 5 pfu/cell in 0.2 ml of serum-free medium. To achieve this, 0.5 ml of MEM was added to the apical compartment and the Transwell chamber was placed over the 0.2-ml viral inoculum placed on a piece of parafilm. A similar protocol was used for infection with influenza, but in this case the virus was present in the medium bathing the apical surface. In all cases, cultures were exposed to the viral inoculum for 45 min, after which time the inoculum was removed and the cells cooled on ice. At this time the Transwell chambers were returned to the 6-well dish in which they were normally maintained. The following treatments were applied to the surface

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1. *Abbreviations used in this paper:* CF, cationized ferritin; moi, multiplicity of infection; SFV, Semliki Forest Virus.

from which infection had been carried out: the monolayers were washed three times with cold PBS-Ca over a 15-min period and incubated with cold pronase (0.2 mg/ml) for 10 min to inactivate adherent virions. They were then washed twice in cold PBS-Ca and incubated in the cold for 15 min with two changes of MEM containing 30% horse serum. Cultures were then placed in MEM containing 30% FBS and incubated at 37°C for 6 h to allow infection to proceed. Monolayers on coverslips that had been incubated in CF-MEM 24 h before infection were returned to that medium for this incubation.

Transfection of MDCK Cells

Cultures of strain II MDCK cells growing in T-75 flasks (Corning Glass Works, Corning, NY) were transfected with 75 μg of the expression plasmid using a modified calcium phosphate method and exposure to the DNA for 7 h, as previously described (Shen et al., 1982). The next day, the cultures were trypsinized and 1/100th or 1/50th of the total cell population was seeded into 10-cm plates containing 7 ml of culture medium supplemented with 400 μg/ml Geneticin sulfate (Gibco Laboratories, Grand Island, NY). The medium was changed every 3-4 d and, after 2-3 wk, drug-resistant colonies were picked for expansion and screening by immunofluorescence microscopy.

Immunofluorescence

Cells on coverslips were fixed in 4% paraformaldehyde in PBS-Ca for at least 1 h at room temperature, or overnight at 4°C. Coverslips were washed once with PBS-Ca at room temperature before incubation with antibodies. To visualize antigens at the apical surface of MDCK cells, coverslips were inverted on top of a 25-μl drop of first antibody and incubated at 37°C for 1 h. They were then washed for 1 h at room temperature with PBS-Ca containing 0.2% gelatin and the second, rhodamine-conjugated, antibody (Cappel Laboratories, Malvern, PA) was then applied, exactly as the first. The coverslips were then washed again for 1 h and then mounted on slides using Elvatol (Monsanto Chemical Co., St. Louis, MO.). When internalized and basolateral molecules were to be visualized, coverslips were incubated with 0.2% TX-100 in PBS-Ca for 10 min before incubation with antibody. When the distribution of viral glycoproteins was analyzed in infected monolayers grown on Transwell filters, after fixation, 0.4 ml of the appropriate antibody was added to the apical surface or, to label the basolateral surface, 0.1-0.2 ml of antibody was applied to a piece of parafilm, and the Transwell placed directly on it. The filters were then washed and immersed in 100% ethanol before air drying. The excised filters were mounted on a glass side with Eukit (Calibrated Instruments, Ardsley, NY), a xylene-based medium that helps to clarify the filter. Specimens were examined by epifluorescence using a Leitz fluorescent microscope (E. Leitz, Rockleigh, NJ) or by laser scanning microscopy using a Sarastro System with Phoibos Software. The scanning line was 0.2-μm wide and the stage was raised in 0.5-μm steps.

Metabolic Labeling

Cells on 35-mm dishes or on filters were labeled in methionine-deficient RPMI (Gibco Laboratories) supplemented with [^{35}S]methionine (208 μCi/ml; sp act 1,000 ci/mmole) for the times indicated in the figure legends. After labeling, the medium was removed and the cells lysed in 0.25 ml of 10 mM Tris-HCl, pH 8.0, containing 2% SDS. The solubilized cell extract was sonicated, boiled for 5 min and mixed with 1.2 ml of solution A (2.5% TX-100, 50 mM Tris, pH 7.5, 0.2 M NaCl, 1 mM EDTA) before centrifugation for 10 min in a microfuge. The appropriate antibody (2 μl) was added to the supernatant and the mixture was incubated overnight in the cold. Protein A-Sepharose beads (Sigma Chemical Co.) were then added and incubation continued overnight in the cold with continuous gentle agitation. The beads carrying immunecomplexes were recovered by sedimentation, washed six times by resuspension in solution A containing 0.2% SDS, agitation for 15 min, and resedimentation. They were then processed to analyze the immunecomplexes by SDS-PAGE and fluorography.

Uptake of ^{125}I-labeled Transferrin

For these experiments, confluent monolayers of MDCK cells were formed by plating 1.5 × 10^6 cells into a Transwell filter unit of pore size 0.4 μm. The medium was changed daily for three consecutive days, and the cells were used for endocytosis studies on the fourth day after plating. Human transferrin (Sigma Chemical Co.) was iodinated using the Iodogen reagent (Pierce Chemical Co., Rockford, IL).

Approximately 24 h before the experiment, the monolayers were rinsed three times with serum-free DMEM, and incubated overnight in DME containing 1% FBS. The next morning, cells were rinsed twice with serum-free DME and further incubated in this medium for 1 h. To determine the specific uptake of ^{125}I-labeled transferrin it was necessary to measure and subtract the background of nonspecifically bound radioactivity recovered with monolayers incubated with the labeled ligand in the presence of a 500-fold excess of nonradioactive transferrin. Therefore, the filters were divided in two groups, one was incubated with ^{125}I-labeled transferrin (1 μg/ml, sp act 6 × 10^6 dpm/μg) in DME containing 500 μg/ml each of the nonspecific competitors, BSA, and ovalbumin. The other group was incubated with ^{125}I-labeled transferrin in DME containing 500 μg/ml each of BSA and human transferrin. Both sets of filters were preincubated for 60 min in the respective protein solution before addition of the ^{125}I-labeled transferrin. For each time point, duplicate filters from each group were removed and washed extensively at 4°C with several changes of PBS-Ca^{2+} for 1 h. The filters were then treated twice for 15 min each with an acidic stripping solution (0.2 M acetic acid and 2 M NaCl) and then washed several times in PBS-Ca^{2+}. They were then excised from the Transwell chamber with a scalpel and their radioactivity measured in an Auto Gamma Spectrometer (Hewlett-Packard Co., Palo Alto, CA). At each time point, specific endocytosis of ^{125}I-labeled transferrin was calculated as the difference between the average radioactivities associated with the two groups. Using this protocol no detectable endocytosis of ^{125}I-labeled transferrin occurred in filters incubated with the probe at 4°C. In experiments using cytochalasin D, cells were incubated with the drug for 15 min at 37°C before the addition of labeled transferrin.

Endocytosis of Iodinated mAbs

The antibody was iodinated using the Iodogen reagent (Pierce Chemical Co.) to specific activities of ~10^7 cpm/μg. MDCK-G cells (4 × 10^5) were plated onto 14-mm-diam Transwell filter inserts (Costar, Cambridge, MA). The medium was changed three times at 24-h intervals and 20 mM sodium butyrate was present during the last two intervals. To begin the experiments, filters were incubated with or without cytochalasin D for 15 min at 37°C and then immediately chilled to 4°C by replacing the medium with cold PBS. They were then incubated for 2 h at 4°C with 0.5 ml of iodinated monoclonal anti-G containing 1.0 μg/ml of the IgG and 10 mg/ml of BSA applied either to the apical or basolateral surface. After the incubation with antibody, the filters were washed twice in cold PBS containing 10 mg/ml BSA and the uptake experiment was initiated by incubation in serum-free medium with or without cytochalasin D at 37°C for the times indicated. Filters removed at the different time points were transferred to PBS at 4°C, washed at least five times with cold PBS for 30 min, and then incubated with an acidic stripping solution (0.2 M acetic acid and 2 M NaCl) at 4°C for 10 min. The monolayers were incubated for 15 min with cold PBS, and then twice for 15 min periods with proteinase K (0.4 mg/ml in PBS; Sigma Chemical Co.). Finally, they were washed five times with cold PBS before measuring the radioactivity in the Gamma counter. The background of cell-associated radioactive antibody bound at "zero" time was determined in monolayers that, after receiving the labeled antibody at 4°C, were maintained throughout the cold, and were washed and treated for acid and protease stripping as were those incubated at 37°C. The total amount of ^{125}I-antibody bound at time zero was determined from filters that were washed, but not treated by the stripping procedure. Generally 10-15 times more antibody bound to the basolateral surface (~10-20 × 10^3 cpm/filter) than to the apical.

Endocytosis of Cationized Ferritin

MDCK cells grown in 14-mm Transwell chambers were incubated at 37°C for 15 min in DME serum-free medium with or without cytochalasin D (25 μg/ml). Two different types of uptake experiments were then carried out in the presence or absence of the drug. In the first, a continuous-labeling protocol, the cationized ferritin was present during a 55-min incubation at 37°C. In the second, a cold binding-chase protocol, the cells were chilled to 4°C, and incubated for 60 min at that temperature with 1 ml cationized ferritin (1 mg/m in PBS Ca^{2+}; Molecular Probes, Eugene, OR) added to the apical or basolateral side. The solution containing the probe was then removed and the filters were incubated at 37°C for a chase period of 45 min in serum-free DME. In all cases, after the incubations the monolayers were chilled in cold PBS Ca^{2+}, and fixed in 2% glutaraldehyde followed by 1% OsO$_4$ in 0.1 M cacodylate buffer. The filters were then excised and processed for conventional EM. Specimens were viewed and photographed using a JEM-1200EXII electron microscope.

-TX-100   +TX-100
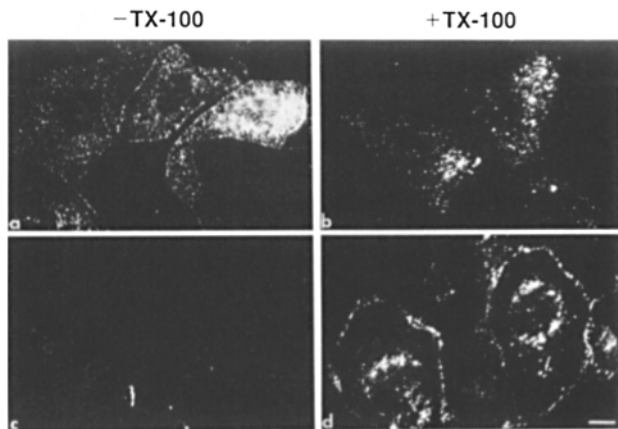
Figure 1. Endocytosis and transcytosis of immunecomplexes containing apical G molecules. MDCK-G cells grown on coverslips were incubated for 30 min at 4°C with a hybridoma supernatant containing a monoclonal anti–G immunoglobulin. After removal of the medium, coverslips were washed with cold PBS-Ca^{2+} and either kept on ice (*a*), or incubated for 15 min (*b*), or 60 min (*c* and *d*) at 37°C. The specimens were fixed and incubated with rhodamine-labeled goat anti–mouse immunoglobulin, without (*a* and *c*) or with (*b* and *d*) prior permeabilization with Triton X-100 to allow access of the antibodies to the cell interior and the basolateral membrane surface. Bar, 10 μm.

Results

The presence of G protein on the apical surface of most cells within monolayers of permanent MDCK-G transformants grown on coverslips was easily detected by indirect immunofluorescence using an anti-G mouse mAb followed by a rhodamine-labeled goat anti–mouse IgG (Fig. 1 *a*). Using such monolayers, the endocytosis and transcytosis to the basolateral surface of G protein–mAb complexes formed on the apical surface was observed when, following binding of the mAb at 4°C, the cells were incubated at 37°C. Thus, when after 15 min at this temperature the second antibody was applied to fixed monolayers that were permeabilized with detergent (Fig. 1 *b*), internalized anti-G mAb immunecomplexes were detected in many cells in what appear to be vesicular structures within the cytoplasm. After 60 min, the immunecomplexes had largely disappeared from the apical surfaces (Fig. 1 *c*) and transcytosis was extensive, since they could be visualized after detergent permeabilization as clearly marking the outline of the basolateral surfaces of many cells (Fig. 1 *d*).

It appears from these observations that the internalized, apically formed, immunecomplexes containing G protein molecules are preferentially delivered to the basolateral surface. This is in contrast to the behavior of immunecomplexes formed with antibody to an endogenous apical protein, leucine–aminopeptidase, which after interiorization preferentially returned to the apical surface (Louvard, 1980). When we applied to the apical surface polyclonal antibodies to the G protein, however, the immunecomplexes that formed were internalized but most failed to be delivered to the basolateral surface (Fig. 2). They were instead, retained intracellularly over long periods of time, probably for degradation in lysosomes (Anderson et al., 1982; Mellman and Plutner, 1984). The transcytosis of a large fraction of the G protein complexed to mAb would seem to reflect the operation of a sort-

Monoclonal
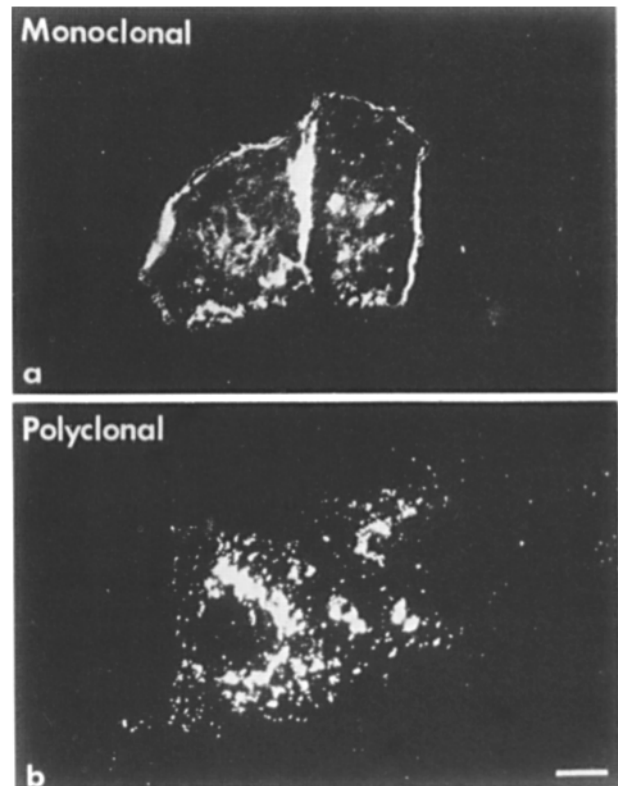
Polyclonal

Figure 2. Apical immunecomplexes of G protein with polyclonal antibodies are internalized but not transferred to the basolateral surface. Confluent MDCK-G monolayers on coverslips were cooled to 4°C and incubated with either monoclonal (*a*) or polyclonal anti-G antibodies (*b*). After removal of the antibody and washing, the coverslips were incubated for 60 min at 37°C, and then fixed and treated with Triton X-100 to allow the detection of intracellular and basolateral antibodies by indirect immunofluorescence. Bar, 10 μm.

ing mechanism that normally recognizes the interiorized glycoprotein and returns it to the basolateral surface, to maintain its predominantly basolateral segregation in the face of intense membrane protein recycling (Matlin et al., 1983). On the other hand, G-protein immunecomplexes formed with polyclonal antibodies are probably extensively cross-linked, which would lead to their targeting to lysosomes.

Considerable evidence obtained with different systems indicates that transport of membrane vesicles through the cytoplasm involves the participation of cytoskeletal elements (see Vale 1987; Kelly, 1990). We, therefore, examined the effect of cytoskeletal inhibitors on the transcellular transfer of apical G molecules complexed to mAbs. In these experiments, the binding of the fluorescent second antibody to residual non-interiorized apical G mAb immunecomplexes was blocked by first incubating the intact cells with unlabeled second antibody. Thus, only internalized and basolaterally located complexes were detected when the fluorescent goat antibody was applied after detergent permeabilization. It was found that the transcellular transport of apically formed immunecomplexes containing G protein was not affected by microtubule inhibitors, such as colchicine (not shown), but that cytochalasin D, an agent that leads to the depolymerization of actin filaments (see Cooper, 1987),
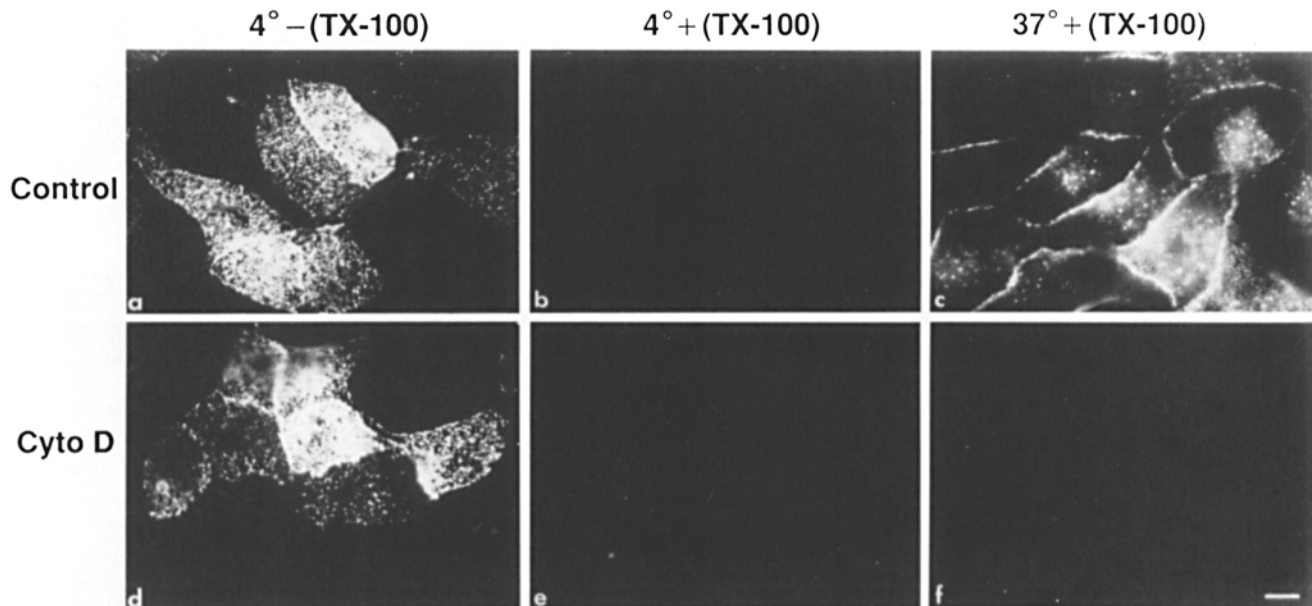
Figure 3. Inhibition of apical endocytosis and transcytosis of anti-G immunecomplexes by cytochalasin D. Confluent monolayers of MDCK-G cells on coverslips were preincubated at 37°C for 15 min without (a–c) or with cytochalasin D (d–f) and, after chilling to 4°C, incubated for 60 min with monoclonal anti–G immunoglobulin. The monolayers were then washed with cold PBS-Ca^{+2} and fixed, either immediately (a, b, c, and e) or after a 30-min incubation at 37°C (c and f). Immunecomplexes on the apical surface were visualized by incubation with rhodamine-labeled goat anti–mouse IgG without prior permeabilization with Triton X-100 (a and d). To visualize endocytosed and transcytosed immunecomplexes (b, c, e, and f), the monolayers were first incubated with an excess of non-fluorescent goat anti–mouse IgG to block the subsequent binding of rhodamine-labeled anti–mouse immunoglobulin to the cell surface. They were then permeabilized with 0.2% TX-100 and processed for indirect immunofluorescence. Bar, 10 μm.

completely abolished it (Fig. 3). Thus, when the mAbs were applied to monolayers incubated with cytochalasin D, no immunecomplexes were found either intracellularly or on the basolateral membrane of any cell, even after a 30-min incubation at 37°C (Fig. 3 f). These observations, therefore, suggested that cytochalasin D totally prevented endocytosis of the immunecomplexes.

Since several investigators have reported that cytochalasins have no effect on receptor-mediated endocytosis in nonpolarized cells (Sandvig and Van Deurs, 1990; Mellman, 1984) and the basolateral surface of epithelial cells may be regarded as functionally equivalent to the surface of nonpolarized cells, we considered the possibility that cytochalasin D only blocks apical endocytosis. The effect of the drug on endocytosis from the two surfaces of MDCK-G cells was, therefore, examined quantitatively on filter grown monolayers which were incubated with ^{125}I-labeled monoclonal IgG applied apically or basolaterally. As shown in Fig. 4, treatment with cytochalasin D effectively prevented the uptake of antibodies bound to the apical surface, but had no effect on the uptake of immunecomplexes formed on the basolateral surface. The insensitivity of basolateral endocytosis to the microfilament disassembling drug was also demonstrated in monolayers incubated with ^{125}I-labeled transferrin. Previous studies have shown that transferrin uptake, which is mediated by the transferrin receptor and occurs by clathrin-coated vesicles (Hopkins, 1983; Wileman et al., 1988), takes place almost exclusively at the basolateral surface of MDCK monolayers grown on a permeable support (Fuller and Simons, 1986) and Fig. 5 demonstrates that cytochalasin D had no effect on the basolateral uptake of ^{125}I-labeled transferrin.
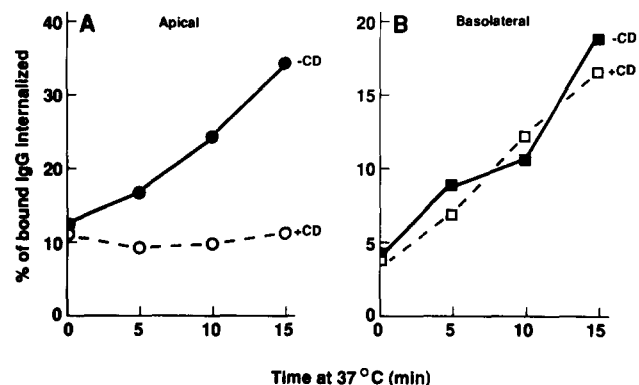
Figure 4. Cytochalasin D inhibits the endocytosis of ^{125}I- labeled monoclonal anti–G immunoglobulin molecules bound to the apical surface of confluent MDCK-G monolayers, but not of those bound to the basolateral surface. Monolayers of MDCK-G cells grown on Transwell filters (0.45-μm pore size) that displayed a transepithelial resistance of 200–300 ohms cm^2 were assayed, as described in Materials and Methods for their capacity to internalize, in the presence (○, □) or absence (●, ■) of cytochalasin D (25 μg/ml), anti-G immunecomplexes containing ^{125}I-labeled mAb bound to the apical (A) or basolateral (B) surfaces. The uptake values plotted are not corrected for the nonspecifically adsorbed radioactivity that, after the stripping procedure, remained cell associated in monolayers that received the antibody at 4°C and were maintained at this temperature throughout the experiment. The amounts of ^{125}I-labeled mAb bound at zero time to each of the two surfaces of MDCK-G monolayers were ~1,500 cpm/filter and ~10,500 cpm/filter, for the apical and basolateral surfaces, respectively. The specificity of this binding is demonstrated by the fact that the corresponding values for nontransfected MDCK monolayers that do not express the G protein were ~10-fold lower.
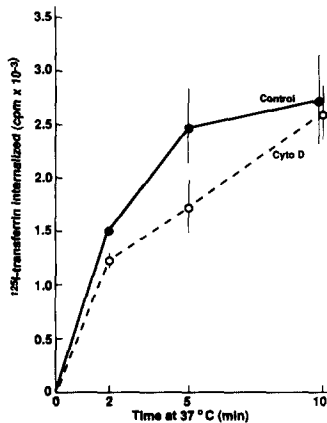
Figure 5. Cytochalasin D does not inhibit the endocytosis of ^{125}I-labeled transferrin bound to the basolateral surface of MDCK-G monolayers. Filter-grown MDCK-G monolayers were preincubated for 30 min at 37°C in serum-free DME, with or without cytochalasin D. The uptake, in the presence or absence of the drug, of ^{125}I-labeled transferrin bound at 4°C to the basolateral surface was then measured as described in Materials and Methods. The background level of radioactivity associated with monolayers incubated with ^{125}I-labeled transferrin in the presence of a 500× molar excess of nonradioactive transferrin was subtracted from each point. This amounted to an average of 224 cpm/filter.

To determine whether cytochalasin D inhibits other coated vesicle-mediated endocytic events from the apical surface we attempted to infect MDCK monolayers with influenza virus in the presence of this drug. Normally, many enveloped viruses, including influenza use the endocytic route to enter the host cell (Helenius and Marsh, 1982; Matlin et al., 1981, 1982), and MDCK monolayers are highly susceptible to infection with influenza from the apical surface (Rodriguez-Boulan and Sabatini, 1978; Fuller et al., 1984). However, we found that influenza infection was very inefficient when MDCK cells grown on coverslips and pretreated with cytochalasin D for 15 min were incubated with the viral inoculum for 45 min in the presence of the drug. Thus, in control cultures, virally encoded newly synthesized HA molecules were abundant on the apical surfaces of almost all cells 5 h after removal of the inoculum (Fig. 6 *a*) and (Fig. 7 *A*, *a*), but when infection was attempted in the presence of cytochalasin D, only rare single cells, or small groups of cells, displayed surface HA molecules (Figs. 6 *b*; and 7 *A*, *b*). This was not due to interference with the processes of viral replication, protein synthesis, or HA transport to the cell surface, since cytochalasin D had no effect when it was added only after the inoculum was removed (Fig. 6 *c*). The extent of inhibition of viral infection was also monitored by comparing the levels of [35S]methionine HA synthesized in filter or culture dish-grown monolayers which were incubated with apically added virus in the absence or presence of cytochalasin D. As shown in Fig. 7 *B* (lanes *a* and *b*) for filter grown cultures, cytochalasin D led to an almost complete inhibition of the production of labeled HA, assessed 4-6 h after incubation with the virus.

That the effect of cytochalasin D on preventing viral infection was restricted to situations in which the virus was allowed to enter the cell only from the apical surface, was apparent from the finding that the drug had no effect on influenza virus infection (Fig. 8) when the inoculum was added to cells that had been previously cultured in calcium-free medium, in which formation of tight junctions and the differentiation of the two cell surface domains does not occur (Vega Salas et al., 1987). This definitively established that the drug neither interferes with binding of the virus to the
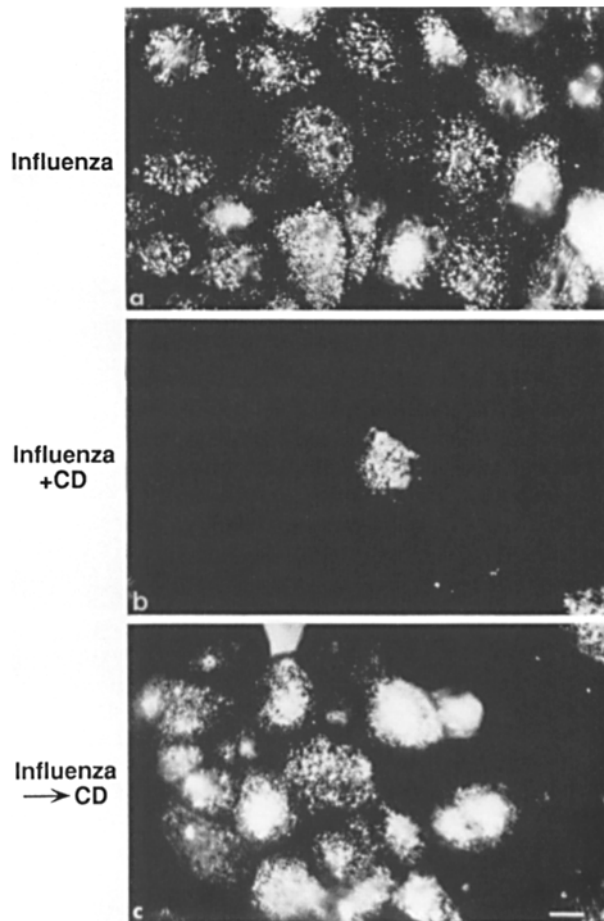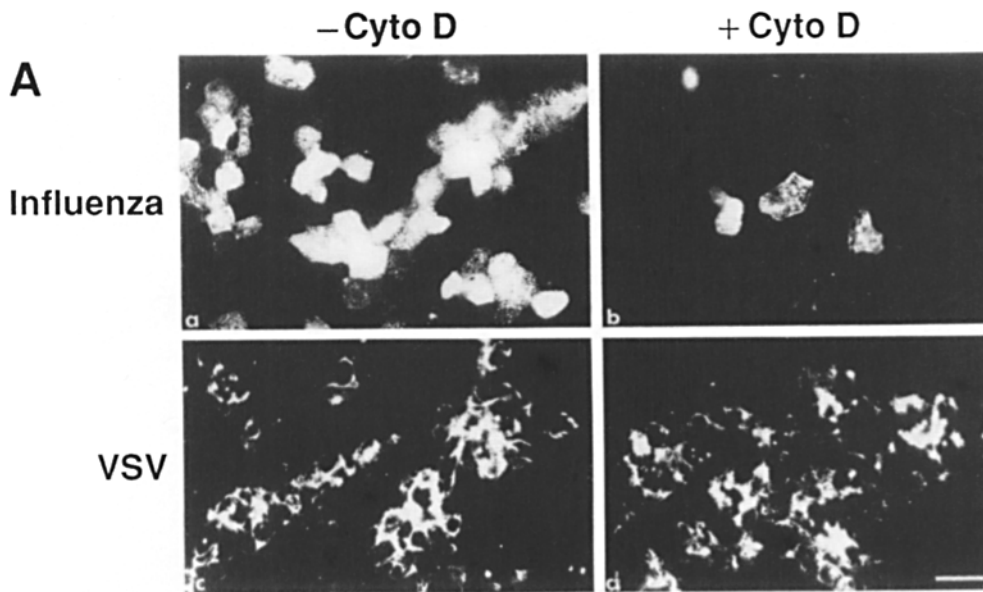

Figure 6. Cytochalasin D prevents influenza virus infection of confluent MDCK cell monolayers grown on an impermeable support. MDCK cell monolayers formed on coverslips were maintained as controls (*a* and *c*) or pretreated (*b*) with cytochalasin D (25 μg/ml) for 15 min at 37°C before incubation for 45 min at this temperature with the influenza viral inoculum (20 pfu/cell). After removal of the inoculum, the monolayers were incubated for 6 h and then fixed and analyzed for apically, located hemagglutinin (*HA*) by indirect immunofluorescence using a rabbit anti-HA antibody. In *c* cytochalasin was only applied to the monolayer for a 45-min period, after removal of the viral inoculum. Bar, 10 μm.

cell surface nor abolishes the infectivity of the inoculum. Furthermore, the drug did not impair infection by VSV when this virus was applied to the basolateral side of filter-grown monolayers, as monitored by either metabolic labeling (Fig. 7 *B*, lanes *c* and *d*) or immunofluorescence detection of accumulated G protein molecules (Fig. 7 *A*, *c* and *d*).

The effect of cytochalasin D on apical endocytosis was also examined by electron microscopy using as a probe cationized ferritin (CF), which is known to bind to anionic sites on the cell surface and to be interiorized in clathrin-coated vesicles (Farquhar, 1978; Skutelsky and Anon, 1976). Cells that were pretreated or not with cytochalasin D for 15 min at 37°C were then incubated with cationized ferritin for an additional 45-min period at 37°C in the presence or absence of the drug. It was found (Fig. 9) that, irrespective of the presence or absence of cytochalasin D, after this continuous labeling procedure the entire apical surfaces of the cells, including microvilli and intermicrovillar spaces, were
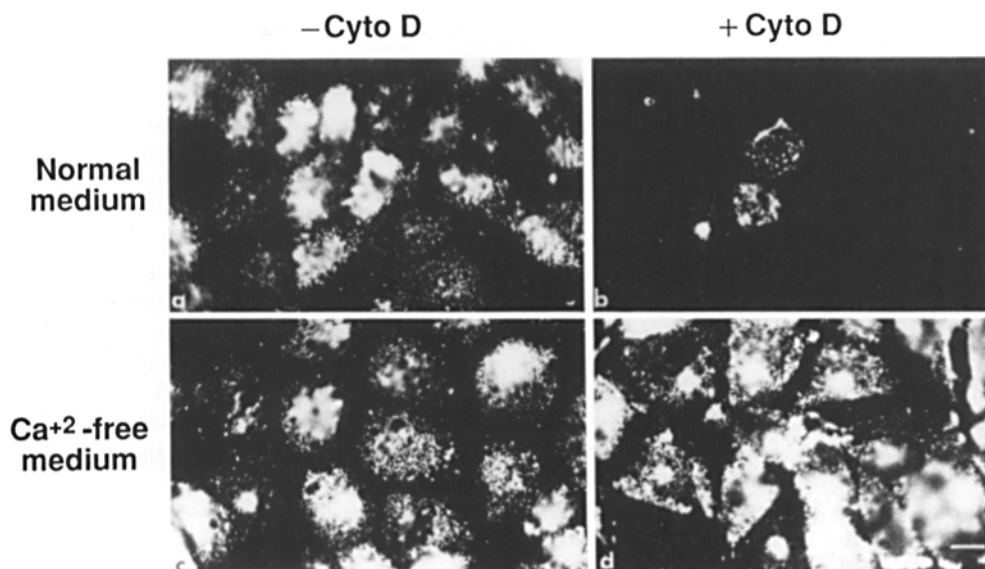
Figure 7. Cytochalasin D inhibits influenza virus infection from the apical surface but not VSV infection from the basolateral surface of filter-grown MDCK cells. Strain I MDCK cells grown on Transwell filter chambers and pretreated (*A, b* and *d*; *B,* lanes *b* and *d*) or not (*A, a* and *c*; *B,* lanes *a* and *c*) with cytochalasin D were infected from either the apical side with influenza virus (*A, a* and *b*; *B,* lanes *a* and *b*) or the basolateral side with VSV (*A, c* and *d*; *B,* lanes *c* and *d*), as described in Materials and Methods. In *A,* 6 h after the inoculum was removed the monolayers were fixed and processed for indirect immunofluorescence after applying anti-HA (*a* and *b*) or anti-G (*c* and *d*) to the apical or basolateral side, respectively. In *B,* 4.5 h after removal of the inoculum the infected cultures were labeled with [³⁵S]methionine for 1.5 h. Cell lysates were prepared and analyzed by immunoprecipitation with antibodies to HA (*a* and *b*) or VSV G (*c* and *d*), followed by SDS electrophoresis. Bar, 20 μm.
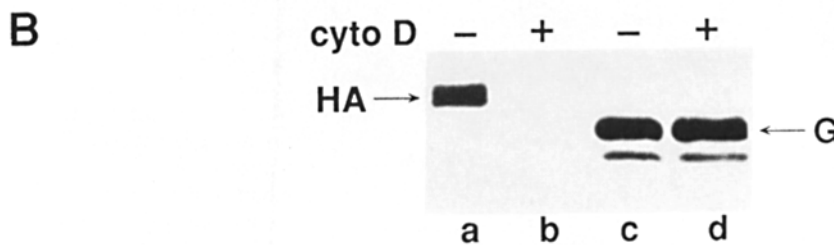
covered by ferritin, which was also frequently found within coated pits. As expected, in control cells (Fig. 9 *a*) the label was interiorized and was present within numerous cytoplasmic structures of varying sizes and morphology, such as endosomes and lysosomes. In cells treated with cytochalasin D (Fig. 9, *b* and *c*), however, almost no ferritin was found in intracellular organelles except for some structures that ap-

pear to be coated vesicles, immediately below the apical surface. Very strikingly, the number of apical-coated pits that showed clear continuity with the apical plasma membrane was greatly increased after cytochalasin treatment. Thus, in control cells there were 23 ± 5 coated pits for 100 μm of apical contour length (*n* = 5), whereas in cytochalasin treated cells the number was 2.3-times larger (54 ± 10). In

Figure 8. Cytochalasin D does not inhibit influenza virus infection of MDCK monolayers lacking tight junctions. Confluent MDCK cell monolayers grown on coverslips were incubated for 24 h in low calcium medium (CF-MEM, see Materials and Methods) to disassemble tight junctions (*c* and *d*) or maintained in normal DME medium (*a* and *b*). After the viral inoculum was removed, incubation was continued in CF-MEM (*c* and *d*) or DME (*a* and *b*) for 6 h before fixation and processing for indirect immunofluorescence with anti-HA antibodies. Bar, 10 μm.
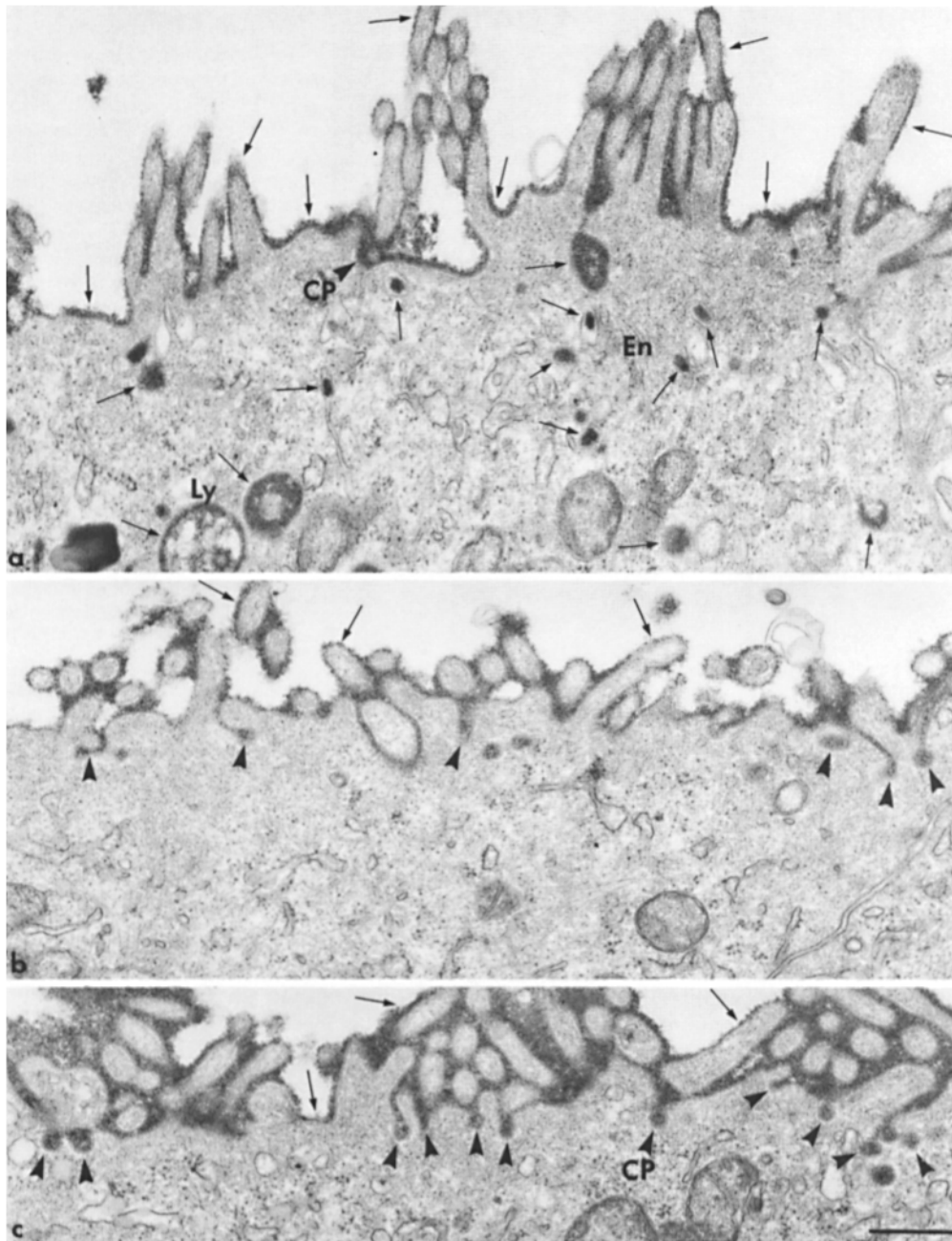
Figure 9. Cytochalasin D inhibits the uptake of cationized ferritin from the apical surface of MDCK cells and increases the frequency of coated pits on that surface. Control (*a*) and cytochalasin D pretreated (*b* and *c*) MDCK cell monolayers were incubated in serum-free MEM containing cationized ferritin (*CF*), in the continuing absence or presence of the drug, for 45 min at 37°C before fixation. The arrows point to CF either on the apical surface of the cells or in the cytoplasm within endosomes (*En*) and lysosomes (*Ly*). The arrowheads point to coated pits (*CP*) at the apical surface, which in cytochalasin D–treated cells (*b* and *c*) are frequently connected to the plasma membrane by long narrow necks. Bar, 0.5 μm.

the treated cells almost all the coated pits contained ferritin and many pits were connected to the apical surface by long narrow necks. It is, therefore, quite likely that what ostensibly were coated vesicles just beneath the plasma membrane represented profiles of obliquely sectioned coated pits. Cytochalasin D, therefore, appeared to block apical endocytosis by preventing the pinching off of the coated pits from the plasma membrane to form coated vesicles.

In other experiments, the cationized ferritin was allowed to bind to control and cytochalasin D treated cells during a 1-h incubation at 4°C, which was followed by a subsequent chase incubation of 45 min at 37°C in the absence of the probe. These experiments also showed (Fig. 10) the complete inhibition of endocytosis and an increase (2.5×) in the number of coated pits in the apical surface (from an average of 13 ± 4 to 32 ± 5 coated pits for 100 μm of apical contour

length) in cytochalasin D–treated cells. Similar experiments (not shown) in which ferritin was applied to the basolateral surface of monolayers grown on filters showed that, although ferritin labeling of the basolateral surface was quite sparse, cytochalasin D affected neither the internalization of the marker, nor the frequency of coated pits (which was 6 ± 2 and 9 ± 1 coated pits/100 μm of contour length in control and drug treated cells, respectively). In these experiments, an additional effect of cytochalasin became apparent. Thus, in control cells, during the incubation at 37°C a lateral displacement of bound cationized ferritin molecules seemed to occur on the apical plasma membrane, from the microvilli, which were initially almost completely covered with ferritin particles (Fig. 10 *a*), to the intermicrovillar spaces, where endocytosis takes place (Fig. 10 *c*). In cells treated with cytochalasin D, in which endocytosis was inhibited, how-
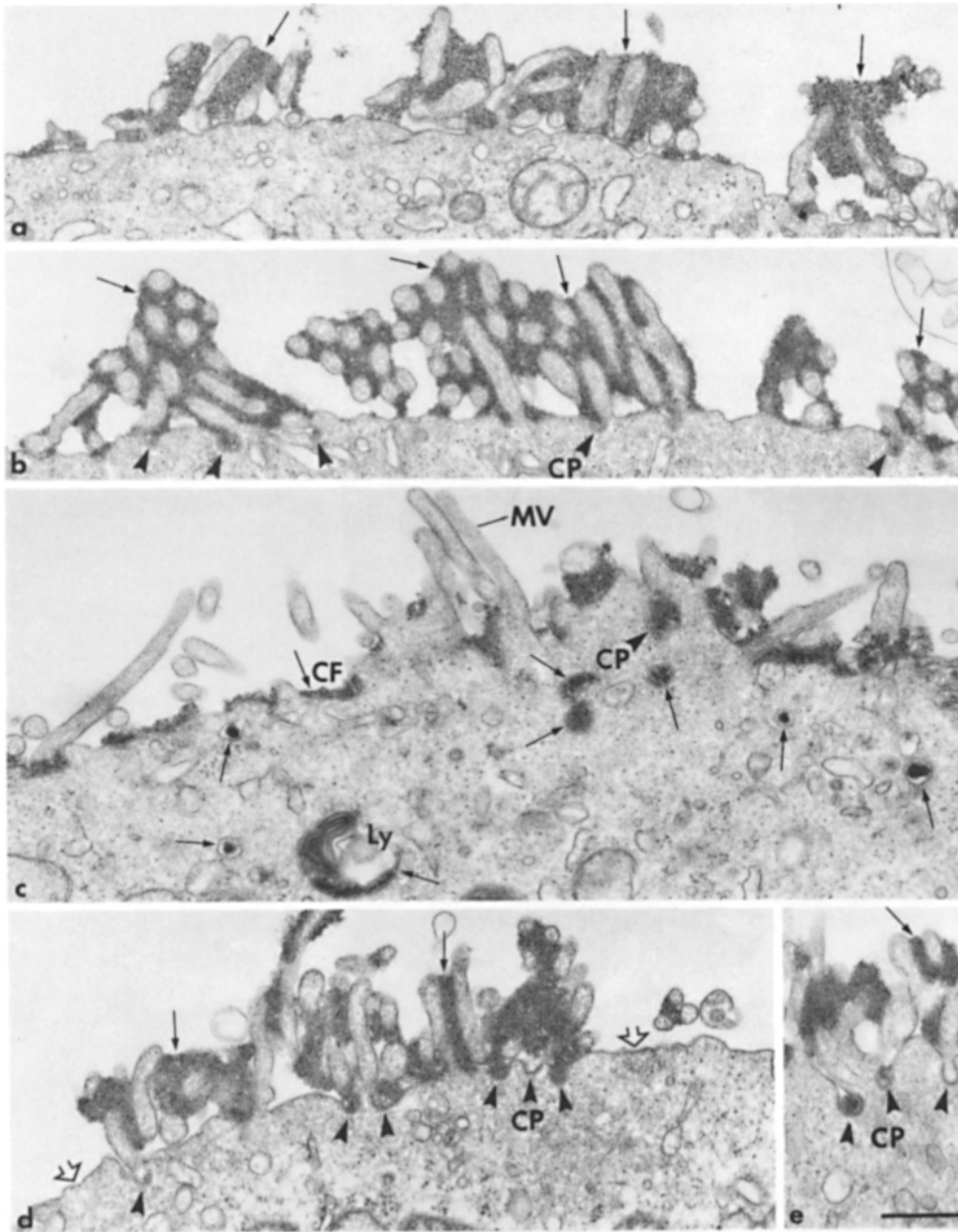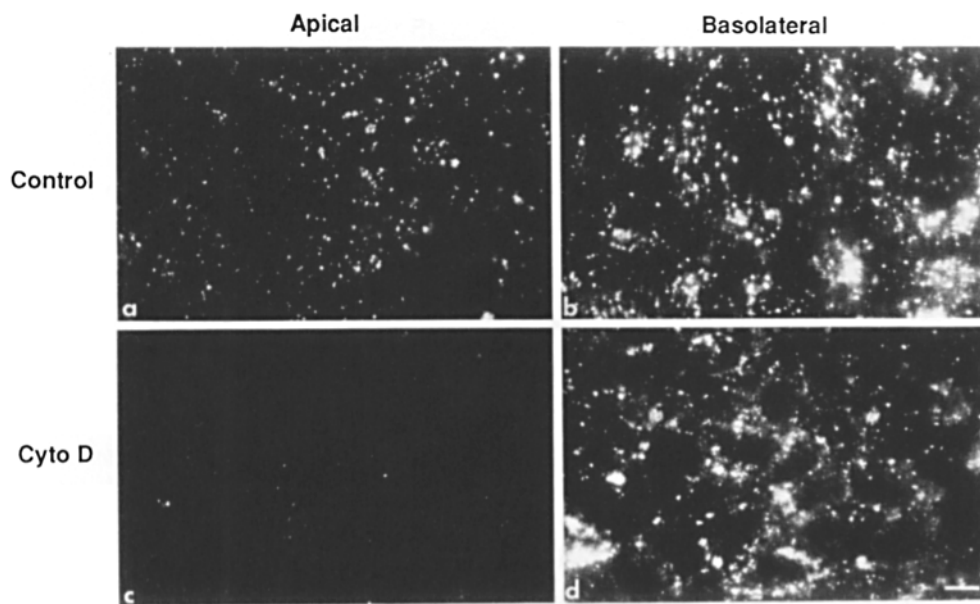
Figure 10. Cytochalasin D inhibits apical endocytosis and the clearance of cationized ferritin from microvilli. Control (*a* and *c*) or cytochalasin D pretreated (*b*, *d*, and *e*) monolayers were incubated in the continuing absence or presence of the drug with cationized ferritin (*CF*) for 1 h at 4°C in PBS-Ca^{2+} and then either fixed immediately (*a* and *b*) or after further incubation for 45 min without CF at 37°C in serum-free DME without (*c*) or with (*d* and *e*) cytochalasin D. Note that during the chase period in the absence of cytochalasin D, previously bound CF is interiorized (*c, arrows*) and that many of the microvilli (*MV*), which were initially covered by CF (*a, arrows*), are cleared of ferritin particles (*c*). In the presence of cytochalasin D (*b, d,* and *e*), endocytosis of CF does not take place and coated pits (*CP*) (*arrowheads, d, e*) accumulate near the base of the microvilli, which remain coated by CF (*d, e, arrows*). In addition, after the chase incubation in the presence of cytochalasin D large areas of the apical surface are frequently observed which are not covered by CF (*open arrows, d*). Bar, 0.5 μm.

ever, the microvilli retained their ferritin coat throughout the incubation (Fig. 10, *d* and *e*). This observation raises the possibility that cytochalasin D also has a primary effect on the displacement of the surface molecules that carry ferritin from the microvillar portions of the plasma membrane towards the coated pits. In the pulse chase experiments, it was also striking that in cytochalasin–treated cells large areas of plasma membrane lacking microvilli and free of ferritin appeared in the apical plasma membrane during the course of the chase incubation (Fig. 10 *d*). It seems likely that those regions of the plasma membrane devoid of ferritin contain membrane components that reach the cell surface by exocytosis during the incubation with cytochalasin D, since this drug is known not to inhibit the arrival of new proteins to the plasma membrane (Salas et al., 1986).

The inhibitory effect of cytochalasin D on apical endocytosis was also demonstrated by the finding (Fig. 11) that the

drug almost completely blocked the apical uptake of Lucifer yellow, a soluble marker for fluid phase endocytosis whose entrance into endosomes does not require binding to the plasma membrane (Swanson et al., 1985) (Figs. 11, *a* and *b*, and 12, *a* and *b*). On the other hand, the basolateral uptake of Lucifer yellow observed in monolayers grown on filters, appeared to be unaffected by cytochalasin D treatment (Fig. 11, *c* and *d*). It is noteworthy that the apical uptake of this dye was much more intense in cells grown on coverslips (compare Fig. 11 *a* with Fig. 12 *a*) and that in control cells, after 2 min of uptake, Lucifer yellow was highly concentrated in the apical cytoplasm near the tight junctions (Fig. 12 *a*). In addition to MDCK cells, the colon derived Caco-2 (Fig. 12, *c* and *d*) cells and T-84 cells (Fig. 12, *e* and *f*) both showed reduced apical uptake of Lucifer yellow as a result of treatment with cytochalasin D. This indicates that this effect of cytochalasin D is not unique to MDCK cells.

Apical | Basolateral

Control

Cyto D

Figure 11. Cytochalasin D inhibits apical but not basolateral endocytosis of Lucifer yellow by filter-grown MDCK cells. Confluent monolayers of MDCK cells grown and maintained on Transwell filters were incubated without (*a* and *b*) or with (*c* and *d*) cytochalasin D for 15 min at 37°C. Medium containing Lucifer yellow (10 mg/ml) was then added to either the apical (*a* and *c*) or basolateral (*b* and *d*) chambers and incubation continued for 10 min at 37°C. The chambers were than washed for 30 min with several changes of cold PBS-Ca²⁺ using a Pasteur pipette to rinse the surfaces. The filters were removed with a scalpel, mounted on a large coverslip, and immediately examined without fixation. Bar, 10 μm.

Control | Cyto D

MDCK

Caco2

T84
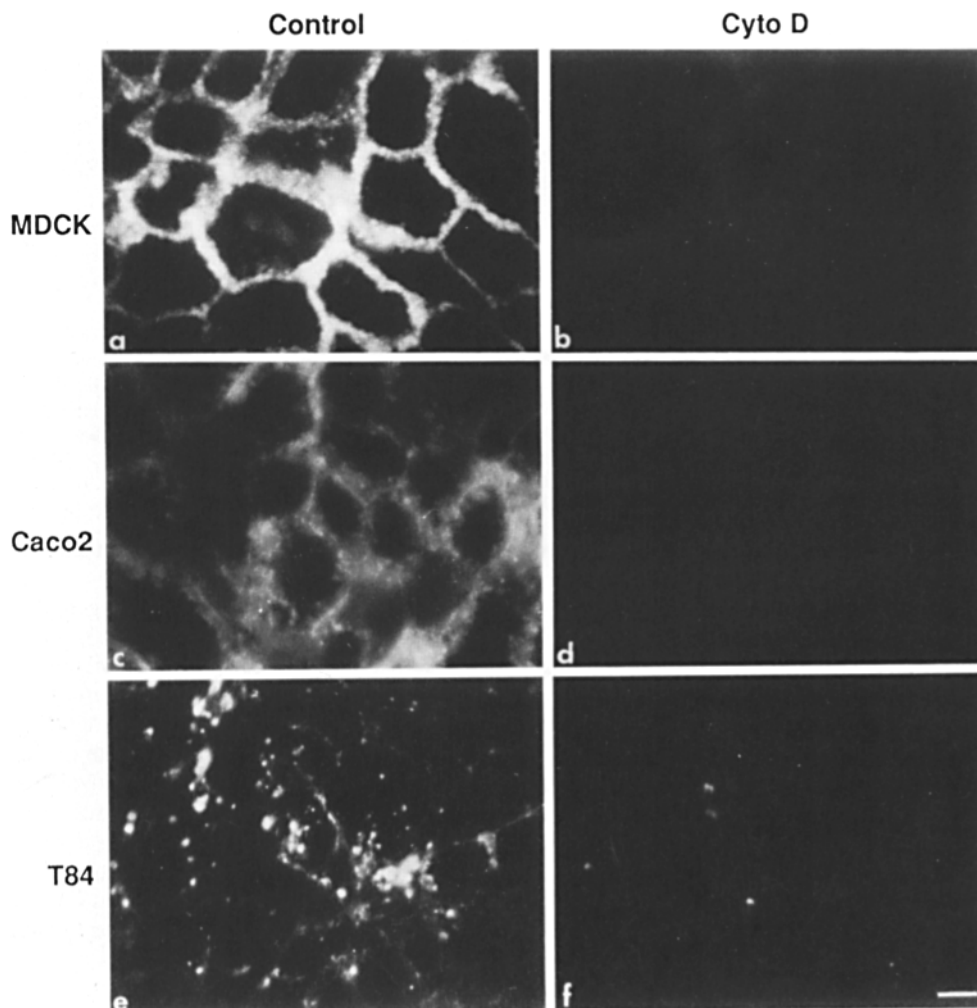
Figure 12. Cytochalasin D inhibits the fluid phase uptake of Lucifer yellow from the apical surface of several epithelial cell lines grown on coverslips. Confluent monolayers of MDCK (*a* and *b*), Caco-2 (*c* and *d*), and T-84 cells (*e* and *f*) were maintained for 3 d on coverslips. To initiate the experiment, the monolayers were incubated in the absence (*a, c,* and *e*) or presence (*b, d,* and *f*) of cytochalasin D for 15 min. They were then incubated with Lucifer yellow (*a* and *b,* 2 mg/ml; *c–f,* 10 mg/ml) for 5 min, chilled, and examined without fixation, as in Fig. 11. Bar, 10 μm.

Control　　**Cyto D**
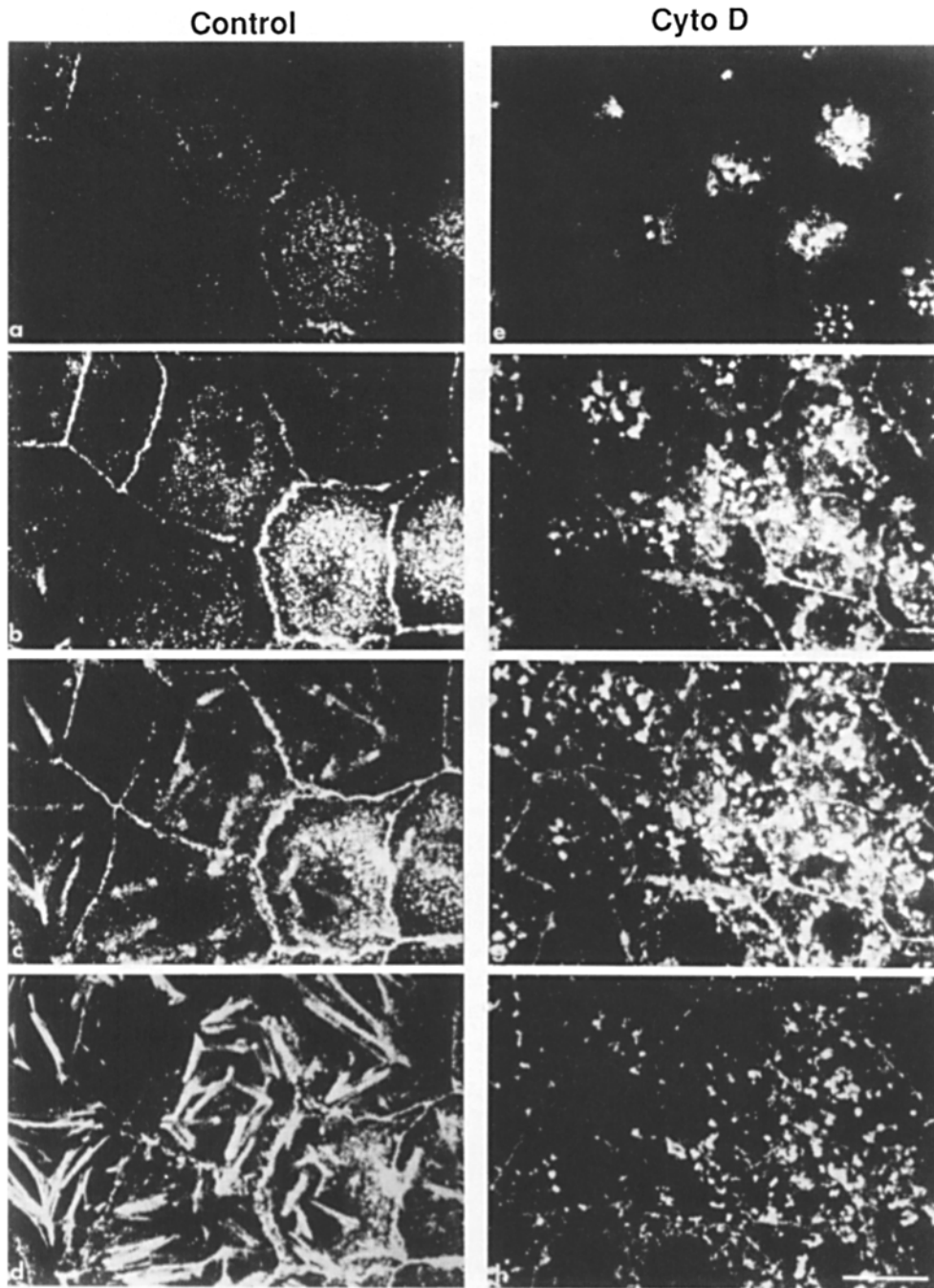
Figure 13. Cytochalasin D leads to the disorganization of actin microfilaments in apical as well as basolateral regions of MDCK cells. Confluent monolayers of MDCK cells grown on coverslips were incubated without (*a–d*) or with (*e–h*) cytochalasin D (25 μg/ml) for 60 min at 37°C. The fixed and permeabilized monolayers were incubated with rhodamine-conjugated phalloidin (6 μg/ml) for 30 mins at room temperature and examined by laser scanning confocal microscopy. Micrographs corresponding to optical sections at the apical (*a, e*), subapical (*b, f*), middle (*c, g*) and basal (*d, h*) regions of the cells from control (left side) and cytochalasin (right side) treated monolayers are shown side by side. Bar, 5 μm.

Cytochalasin D Alters the Organization of Both Apical and Basolateral Actin Filaments in MDCK Cells

The differential effect of cytochalasin D on apical and basolateral endocytosis led us to investigate whether the drug preferentially disassembled actin filaments in the apical region of the cell. We, therefore, examined by laser scanning confocal microscopy (Fig. 13) the distribution of rhodamine-labeled phalloidin—a probe that binds to polymerized but not monomeric actin (Cooper, 1987)—in control (Fig. 13, *a–d*) and cytochalasin D–treated (Fig. 13, *e–h*) monolayers. Analysis of pairs of fluorescent micrographs representing optical sections at the apical (Fig. 13, *a* and *e*), subapical (*b* and *f*), middle (*c* and *g*), and basal regions (*d* and *h*) of the cells revealed that cytochalasin profoundly affected actin or-

ganization throughout the cell. After cytochalasin treatment, stress fibers visible in the basal regions of control cells (Fig. 13 *d*), were no longer present (Fig. 13 *h*). Similarly, in the middle and subapical regions of the cell the circumferential bundles of actin that are associated with the lateral membranes in control cells (Fig. 13, *b* and *c*) were largely disorganized after cytochalasin treatment (Fig. 13, *f* and *g*). Finally, actin bundles within microvilli, which in control cells give rise to a fine punctate pattern in the apical and subapical regions (Fig. 13, *a* and *b*), after drug treatment became aggregated and clumped away from the cell periphery. We, therefore, conclude that the differential effect of cytochalasin D on apical endocytosis reflects a more prominent role of actin filaments in this process and not a preferential sparing of basolateral microfilament integrity.

Discussion

We have presented a series of experiments demonstrating that treatment of polarized MDCK cell monolayers with cytochalasin D, a drug that acts by interfering with the function of actin-containing microfilaments (see Cooper, 1987), selectively inhibits the capacity of the cells to endocytose membrane-bound and fluid-phase markers applied to the apical surface, without affecting endocytosis from the basolateral surface. Thus, cytochalasin D prevented the apical uptake of a mAb bound to surface VSV G molecules and of soluble Lucifer yellow, but did not impair the uptake of the same markers or of labeled transferrin when they were applied to the basolateral surface. Moreover, the drug prevented infection with influenza virus only when the inoculum was applied to the apical surface of intact monolayers, while infection from the basolateral surface was unaffected. In addition, the drug completely abolished the interiorization of cationized ferritin bound to anionic sites on that surface. Since sialic acid residues, which serve as receptors for influenza virus (Rogers and D'Souza, 1989), are present in many plasma membrane glycoproteins and glycolipids and the anionic sites that bind cationized ferritin are distributed over a wide variety of cell surface components, one can conclude that cytochalasin D blocks a critical step common to many, if not all apical endocytic events.

Cytochalasin D Prevents the Pinching Off of Coated Vesicles and the Displacement of Microvillar Surface Components to the Intermicrovillar Spaces

An electron microscopic analysis of the effect of cytochalasin D on the interiorization of cationized ferritin applied to the apical surface was particularly informative with respect to the endocytic steps blocked by the drug. Cytochalasin D did not affect the binding and distribution of the cationized ferritin, which initially covered the microvilli and a large fraction of intermicrovillar spaces in the apical membrane, but completely blocked the interiorization of the marker during the subsequent incubation. This seemed to result from the failure of coated pits to pinch off from the apical surface, since the number of coated pits containing ferritin substantially increased after cytochalasin treatment, and many of the coated pits observed in the drug-treated cells were connected to the surface by long necks. When the cationized ferritin uptake was analyzed under pulse–chase conditions, it was apparent that in control cells endocytosis was accompanied by clearance of the microvillar surface reflecting a displacement of ferritin molecules towards the intermicrovillar spaces, where coated pits are formed. A similar displacement of a bound ligand from the microvilli to the intermicrovillar regions of the plasma membrane was observed during the endocytic uptake of Semliki Forest Virus (SFV) in BHK cells (Helenius et al., 1980). Strikingly, we found that cytochalasin D not only blocked interiorization but also prevented the clearance of ferritin molecules from the microvillar surface. This could result from a direct block of the displacement of ferritin along the microvilli, although the same effect would also be expected if the initial action of cytochalasin were to inhibit the pinching off of coated vesicles in the regions between microvilli, which would be required to maintain the flow of proteins of the microvillar membrane into the intermicrovillar space.

The Differential Inhibitory Effect of Cytochalasin D on Apical Endocytosis Is Probably a Consequence of the Distinct Subcortical Cytoskeletal Structures Associated with the Two Plasma Membrane Domains

What molecular processes must be affected by cytochalasin D for it to interfere with apical endocytosis? It should first be noted that molecular motors are thought to be required in endocytosis (Anderson, 1991). Such motors may either participate in the transport of proteins in the plane of the membrane (Sheetz et al., 1990) to the site of vesicle formation or in the actual pinching off of the plasma membrane vesicle into the cytoplasm (Anderson, 1991). Indeed, a mechanochemical function of the latter type has been proposed (Shpetner and Vallee, 1992) for dynamin, a GTPase that is defective in the *Drosophila shibire* mutant, which is incapable of endocytosis at the nonpermissive temperature (Kosaka and Ikeda, 1983).

The inhibitory effects of cytochalasins on cell motility have long been recognized and it is clear that these drugs decrease the rate of addition of monomeric actin molecules to the barbed, fast growing, ends of actin filaments (MacLean-Fletcher and Pollard, 1980; Bonder and Mooseker, 1983), thus reducing the final state of actin polymerization within the cell. In addition, it has been shown that cytochalasins may also sever actin filaments (Schliwa, 1982), as well as inhibit interfilament interactions which contribute to the formation of a cytoskeletal network (MacLean-Fletcher and Pollard, 1980). The specificity of cytochalasin action on actin-containing microfilaments is well established (see Cooper, 1987) and recently, rigorous proof was provided that cytochalasin acts directly on actin molecules, since it was shown that expression in cytochalasin-sensitive cells of a mutant form of actin present in cytochalasin-resistant cells confers drug resistance to the transfected cells (Ohmori et al., 1992). Our observations can, therefore, be taken to directly demonstrate the participation of the actin-based microfilament system in apical endocytosis in MDCK cells and, hence, to reveal the existence of a relationship between actin filaments and membranous components that participate in endocytosis at the apical surface.

The selective effect of the drug on apical endocytosis could either imply that actin filaments do not associate with the sites of endocytosis that occur in the basolateral membrane, where an alternative motor could operate, or that the microfilaments at the basolateral surface are less sensitive to the depolymerizing action of cytochalasin D. The latter possibility is unlikely, given the apparent disaggregation of actin microfilaments throughout the cell that we observed after drug treatment. With respect to the former possibility it is important to recognize that in polarized epithelial cells, the two plasma membrane domains differ substantially in their mode of association with the actin cytoskeleton. At the basolateral surface microfilaments are primarily associated with zonula adherens junctions and focal adhesion sites, probably via the complex interaction of actin with several of its associated proteins, including vinculin, talin, paxillin, and α-actinin (see Luna, 1990). Accordingly, in fully developed MDCK cell monolayers there is a high concentration of actin near limited areas of the lateral membrane (Nelson and Veshnock, 1986). On the other hand, throughout the basolateral domain, it is the fodrin/spectrin cytoskeleton that is intimately associated with the membrane, playing a major

role in establishing and maintaining the basolateral segregation of some integral membrane proteins, such as the NA^+, $K^+ATPase$, to which it is linked via an association with ankyrin (Nelson et al., 1990; Nelson and Veshnock, 1987). In fact, the ankyrin-mediated linkage of some basolateral membrane proteins to the underlying cytoskeleton, which is triggered by the establishment of cell–cell contacts, appears to prevent the endocytosis of those proteins and thus contribute to their metabolic stabilization (Hammerton et al., 1991).

A stabilizing fodrin/spectrin submembranous cytoskeleton is not present directly under the apical membrane, except in epithelia with inverted polarity, such as the retinal pigmentary epithelium in which the Na^+, K^+ ATPase is apically located (Gundersen et al., 1991). Rather, in the apical plasma membrane domain an actin-containing submembranous cytoskeleton is well developed. In the best studied example, that of the intestinal epithelium, actin filaments constitute the core of microvilli and together with other proteins from the subapical filamentous network known as the terminal web (see D. Louvard, 1989). Within the microvilli, actin filaments—which have their barbed ends directed towards the microvillar tips (Mooseker and Tilney, 1976)—are bundled by villin and fimbrin and are linked to the microvillar membrane by a complex of calmodulin and a 110-kD myosin I molecule which is believed to be able to associate directly with membrane phospholipids (Adams and Pollard, 1989; Zot et al., 1992) and to mediate membrane displacements because of its capacity, when activated by actin, to serve as a mechanochemical energy transducer (Mooseker and Coleman, 1989). The actin-dependent movement of membrane-associated myosin I molecules that would be expected to be inhibited by cytochalasin D would, however, be directed towards the barbed end of microfilaments and, hence, towards the microvillar tip. Nevertheless, because the microvillar surface may be unexpandable, the upward migration of membrane embedded myosin I molecules may itself determine the countercurrent downward displacement of other membrane components of the microvillar surface. This displacement would be inhibited by cytochalasin D which, hence, would arrest endocytosis by limiting the availability of membrane components at the intermicrovillar sites where coated pits are formed.

It is also possible that microfilaments play a direct role in the generation of endocytic vesicles only at the apical surface. Although it is thought that assembly of a protein coat on the cytoplasmic surface of a membrane patch induces the vesicle curvature, a provocative report has indicated that in coated vesicles obtained from brain tissue specific actin-binding proteins may be part of the clathrin coat itself (Kohtz et al., 1990). In fact, evidence was presented that such proteins are capable of mediating the association of clathrin-coated vesicles with assembling actin filaments in vitro. It, therefore, seems possible that only in certain cell types and, perhaps only at the apical surface of epithelial cells, closure of a coated pit to form a coated vesicle depends on the assembly of actin monomers into microfilaments, with which cytochalasin D interferes. This raises the possibility that there are compositional differences in the coats of pits forming at the two surfaces of polarized epithelial cells.

A previous study with human lymphoblastoma cells has also provided morphological evidence for the association of microfilaments with coated pits (Salisbury et al., 1980). In this case, it was found that cytochalasin B blocked the internalization of ferritin-conjugated anti–IgM antibody bound to the surface of the cells and the tracer accumulated in coated pits, which led to the proposal that the actin filaments serve to pull the membrane inwards during coated vesicle formation. Since a cytochalasin inhibition of endocytosis has not been generally observed with other cell types (see below), it would seem possible that a specialized actin filament-dependent mechanism for endocytic vesicle formation operates in both lymphoid cells and at the apical surface of polarized epithelial cells.

The Fluid Phase Uptake at the Apical Surface of MDCK Cells That Is Inhibited by Cytochalasin D Is Likely To Occur Primarily in Non-clathrin–coated Vesicles

MDCK cells are known to take up fluid phase markers, such as Lucifer yellow, at equal rates from both surfaces (Bomsel et al., 1989). Using fluorescence microscopy, we found that the uptake of this dye from the apical surface of confluent monolayers was completely inhibited by cytochalasin D, whereas basolateral uptake seemed unaffected. Fluid phase markers, however, can be taken up by inclusion in coated vesicles involved in receptor-mediated endocytosis, as well as within non-clathrin–coated vesicles. The mechanism for the latter form of uptake is as yet unknown, but it is not blocked (Sandvig and Van Deurs, 1990) under conditions that lead to acidification of the cytosol and interfere with the formation of coated vesicles (Sandvig et al., 1987; Heuser, 1989). The extent to which each of these endocytic pathways contributes to fluid phase uptake in MDCK cells has not been determined, but it is worth noting that in cells grown on coverslips we found that uptake of Lucifer yellow from the apical surface was intense and took place almost exclusively near the zones of intercellular contact, whereas when monolayers were grown on filters, the apical uptake of the dye was markedly reduced, even though under the same conditions intense apical interiorization of cationized ferritin in coated vesicles took place. These observations suggest that Lucifer yellow, at least in the coverslip-grown MDCK cells, is primarily interiorized by nonclathrin–coated vesicles and, therefore, that cytochalasin D also inhibits this type of endocytic process at the apical surface. On the other hand, the basolateral uptake of Lucifer yellow, which was observed in filter grown monolayers, was unaffected by cytochalasin D, again showing the specificity of the inhibitory effect of the drug on apical endocytosis.

The Cytochalasin Inhibition of Apical Endocytosis Is Likely To be a General Feature of Polarized Epithelial Cells

It seems likely that the inhibitory effect of cytochalasin D on apical endocytosis is a general one for all transporting epithelia. In a previous study (Blok et al., 1982) it was found that cytochalasin D substantially reduced the apical uptake of the fluid phase marker HRP by epithelial cells in explants of human jejunum, although the basolateral uptake was not studied in this system. In addition, we have observed that cytochalasin D blocks the apical uptake of Lucifer yellow by monolayers of polarized Caco-2 and T-84 cells, which are

both derived from human colonic carcinomas. Moreover, the uptake of cationized ferritin from the apical surface of the epithelial cells in neonatal rat ileum explants (Gonnella and Neutra, 1984), and in a polarized pig kidney epithelial cell line LLC-PK1, was also inhibited by the drug (our unpublished results). In light of these observations it seems somewhat surprising that in short term suspension cultures of primary hepatocytes the interiorization of asialoorosomucoid bound to the asialoglycoprotein receptor, which is normally present on the basolateral surface of the cells, is inhibited by cytochalasin D (Kaufman et al., 1990). In hepatocytes, however, the basolateral surface consists of several subdomains and the one facing the perisinusoidal space of Disse, where the asialoglycoprotein receptor may be expected to be concentrated, is characterized by the presence of abundant microvilli. Therefore, in that region of the basolateral surface the subplasmalemmal actin cytoskeleton may very well bear a resemblance to the apical cytoskeleton of other epithelial cells, thus accounting for the sensitivity of the uptake of asialoorosomucoid to cytochalasin D.

The selective effect of cytochalasin D on apical endocytosis should be taken into account in considering the conflicting reports in the literature on the capacity of cytochalasins to inhibit receptor-mediated and fluid phase endocytosis in different cell types. A study with African green monkey kidney cells (VERO; Sandvig and Van Deurs, 1990) is of particular interest since it demonstrated that the endocytic uptake of transferrin, which occurs by clathrin-coated pits, was completely insensitive to cytochalasin D, whereas in the same cells the uptake of the fluid phase markers Lucifer yellow and tritiated sucrose, and of ricin, which binds to cell surface carbohydrates, was inhibited by ~60%. Furthermore, it was shown that the bulk of the ricin uptake was insensitive to acidification of the cytoplasm and, therefore, did not take place in clathrin-coated vesicles. This led the authors to conclude that in the cells they studied only uptake by non clathrin-coated vesicles is sensitive to cytochalasin D. Given the fact that VERO cells are at least partially polarized (Srinivas et al., 1986; Clayson et al., 1989) it seems quite possible that their transferrin receptors, whose internalization is insensitive to cytochalasin D, are primarily localized in basolateral-like plasma membrane domains, whereas ricin-binding sites and the sites for fluid phase uptake are found in both basolateral- and apical-like regions.

Cytochalasin D Inhibits Infection by Viruses That Enter Cells by Apical Endocytosis

The strikingly different effects of cytochalasin D on endocytosis from the two cell surfaces were also manifested in its selective capacity to essentially completely prevent infection of intact monolayers with influenza virus, when the viral inoculum was applied to the apical surface, and in its inability to inhibit infection with VSV when the inoculum was applied to the basolateral surface of monolayers established on permeable filters. Extensive previous surveys of the effect of cytochalasins on infection of cultured cells by various viruses indicated that, in most cases, the drug enhanced the infectability of the cells (Koch and Koch, 1978), which could possibly be attributed to the disappearance of a cortical cytoskeletal barrier to the endocytic uptake of the virions. To our knowledge, however, no previous studies have been carried out on the effect of the drug on viral infection of pola-

rized epithelial cells. In our work it was apparent that cytochalasin D acted by preventing an early step in virus infection from the apical surface and not by inhibiting some essential step in the virus development cycle, since the drug had no effect when added immediately after the phase of viral adsorption had been completed. Moreover, cytochalasin D had no effect on influenza virus infection when the inoculum was applied to monolayers that lacked tight junctions as a consequence of their maintenance in Ca^{2+}-free medium. This finding not only demonstrates that the drug does not inhibit influenza virus replication, but that it only blocks endocytic uptake from an intact apical surface. In this regard, it should be noted that influenza virus receptors (which are molecules bearing exposed sialic acid residues) (Rogers and D'Souza, 1989) under normal conditions are largely restricted to the apical surface of MDCK cells (Matlin et al., 1981). It, therefore, appears that in cells lacking tight junctions at least some receptor molecules are displaced to basolateral domains where they can undergo endocytosis and mediate viral entrance into the cells by the cytochalasin-resistant mechanism that operates in those domains. On the other hand, it is also possible that in MDCK cells grown in low calcium medium, the virus still undergoes internalization through "apical domains" but the process is no longer sensitive to the cytochalasin. Indeed, it is well known that the polarized organization of epithelial cells is not totally abolished in the absence of tight junctions. Thus, the polarized budding of influenza and VSV is maintained in single cells attached to a substratum (Rodriguez-Boulan et al., 1983) and, in cells lacking intercellular contacts, some proteins still maintain their apical or basolateral localization (Ojakian and Schwimer, 1988; Salas et al., 1988), although disruption of tight junctions by Ca^{2+} depletion leads to the redistribution of some previously segregated markers (Herzlinger and Ojakian, 1984; Pisam and Ripoche, 1976; Ziomek et al., 1980).

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