Apical and Basolateral Coated Pits of MDCK Cells Differ in Their Rates of Maturation into Coated Vesicles, but Not in the Ability to Distinguish between Mutant Hemagglutinin Proteins with Different Internalization Signals

Hussein Y. Naim, D'Nette T. Dodds, Colleen B. Brewer, and Michael G. Roth

Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9038

Abstract. In polarized epithelial MDCK cells, all known endogenous endocytic receptors are found on the basolateral domain. The influenza virus hemagglutinin (HA) which is normally sorted to the apical plasma membrane, can be converted to a basolateral protein by specific mutations in its short cytoplasmic domain that also create internalization signals. For some of these mutations, sorting to the basolateral surface is incomplete, allowing internalization of two proteins that differ by a single amino acid of the internalization signal to be compared at both the apical and basolateral surfaces of MDCK cells. The rates of internalization of HA-Y543 and HA-Y543,R546 from the basolateral surface of polarized MDCK cells resembled those in nonpolarized cells, whereas their rates of internalization from the apical cell surface were fivefold slower. However, HA-Y543,R546 was internalized approximately threefold faster than HA-Y543 at both membrane domains, indicating that apical endocytic pits in polarized MDCK cells retained the ability to discriminate between different internalization signals. Slower internalization from the apical surface could not be explained by a limiting number of coated pits; apical membrane contained 0.7 as many coated pits per cell cross-section as did basolateral membranes. 10–14% of HA-Y543 at the apical surface of polarized MDCK cells was found in coated pits, a percentage not significantly different from that observed in apical coated pits of nonpolarized MDCK cells, where internalization was fivefold faster. Thus, there was no lack of binding sites for HA-Y543 in apical coated pits of polarized cells. However, at the apical surface many more shallow pits, and fewer deep, mature pits, were observed than were seen at the basolateral. These results suggest that the slower internalization at the apical surface is due to slower maturation of coated pits, and not to a difference in recognition of internalization signals.

THE functions of many cells require that the plasma membrane be organized into distinct domains differing in their composition and function (Rodriquez-Boulan and Powell 1992; Nelson, 1992). The continuous line of MDCK epithelial cells has been one of the best studied model systems for the establishment and maintenance of cell polarity. The apical surface of these cells is enriched in glycolipids, contains a set of proteins distinct from those found at the basolateral domains, and lacks any known endocytic receptors. Proteins containing recognition signals for internalization through clathrin coated pits, including normally apically sorted proteins into which internalization signals have been inserted by mutagenesis (Brewer and Roth, 1991; Le Bivic et al., 1991), are transported to the basolateral surface of MDCK cells. One of these mutant proteins, the influenza virus hemagglutinin mutant, HA-Y543, contains a tyrosine in place of cysteine at position 543 in the short, 12-amino acid long, cytoplasmic domain. This mutation converts influenza virus hemagglutinin (HA)[1] from a protein that is internalized slower than the bulk rate of membrane uptake by clathrin coated pits into one internalized at rates between 5 and 16% per minute, depending upon the expression system and cell type (Lazarovits and Roth, 1988; Ktistakis et al., 1990; Brewer and Roth, 1991; Naim and Roth, 1994*a*). The same point mutation causes HA-Y543 to be expressed almost entirely at the basolateral surface of MDCK cells (Brewer and Roth, 1991). Recently we have shown that the rate of HA-Y543 internalization can be improved by second-site point mutations, allowing us to create a series of extremely similar proteins that differ in the rates at which they are internalized (Naim and Roth, 1994*a*), prob-

Address all correspondence to Michael G. Roth, Dept. of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9038. Tel.: (214) 648-3276. Fax: (214) 648-8856.

1. *Abbreviations used in this paper*: HA, influenza virus hemagglutinin; VSV, vesicular stomatitis.

ably due to differences in the affinity with which they bind to coated pits (Fire et al., 1991). One of the proteins, containing a tyrosine at position 543 and an arginine at position 546 of the HA sequence, was expressed at both the apical and basolateral surfaces of MDCK cells (Naim, Dodds, and Roth, manuscript in preparation). This protein, HA-Y543,R546, was internalized about threefold faster than was HA-Y543 in fibroblasts, at a rate of 35%/min. This internalization rate is similar to, or faster than, the internalization rate of the transferrin receptor (McGraw et al., 1991; Hansen et al., 1992; Naim and Roth, 1994*a*). Thus, HA-Y543,R546 was an excellent candidate protein to use to measure internalization from both surfaces. In addition, we have observed that certain mutant HAs, including HA-Y543, have sorting signals that do not function as efficiently when the expression of those proteins is stimulated by sodium butyrate, as others have reported for mutants of the LDL receptor (Matter et al., 1994). In the presence of butyrate, enough HA-Y543 and HA-Y543,R546 were transported to the apical membrane to allow us to investigate several aspects of the sorting of proteins into the apical endocytic pathway. In particular, we were interested in the question of whether clathrin-coated pits at the apical surface of polarized MDCK cells retained the ability to discriminate between HAs known to be internalized at very different rates both in fibroblasts and at the basolateral surface of MDCK cells.

Endocytosis of proteins (Le Bivic et al., 1991; Gottlieb et al., 1993), toxins (Prydz et al., 1992; Eker et al., 1994) and fluid (Bomsel et al., 1989; Melby et al., 1991) from the apical surface of MDCK has been observed to differ in several characteristics from that at the basolateral surface. Le Bivic and colleagues reported that wild-type and mutant nerve growth factor receptors internalized less efficiently from the apical than the basolateral surface of MDCK cells, but did not measure the rates at which this occurred, or by which pathway. Gottlieb et al. (1993) observed that the rates of internalization of the vesicular stomatitis (VSV) G protein were ~1%/min at both surfaces of MDCK cells and that internalization of G was inhibited by cytochalasin D only at the apical surface. Jackman et al. (1994) reported that apical endocytosis of ricin is also inhibited by cytochalasin D in polarized Caco 2 cells.

In the experiments presented below, we found that endocytosis of two HA mutant proteins from the apical surface was much slower than from the basolateral, but that the relative efficiency of recognition of the two proteins by coated pits, as reflected by their internalization rates, was not changed. We present evidence that the slower internalization at the apical surface is due to a slower maturation of endocytic pits. This difference is observed only in confluent, polarized MDCK cells. We confirm previous observations that apical, but not basolateral endocytosis is inhibited by cytochalasin D (Gottlieb et al., 1993; Jackman et al., 1994).

Materials and Methods

Site-directed Mutagenesis and Subcloning

HA-mutants were constructed by the site-directed mutagenesis protocol of (Kunkel, 1985) and have been previously reported (Naim and Roth, 1994*a*). These genes were subcloned into the expression vector pCB6 (Brewer, 1994) under the control of the cytomegalovirus immediate-early promoter, for expression in MDCK cells, or into a SV40 expression vector (Naim and Roth, 1994*b*), for experiments in CV-1 cells. All subcloning was performed according to established protocols (Sambrook et al., 1989) with reagents from Boehringer Mannheim Corp. (Indianapolis, IN) or Qiagen Inc. (Chatworth, CA). cDNAs were sequenced with Sequenase (United States Biochemical Corp., Cleveland, OH) according to instructions from the manufacturer. Reagents not otherwise identified were from Sigma Immunochemicals (St. Louis, MO).

Cell Culture, Cell Lines, and Labeling Procedures

Strain II MDCK cells were grown in DME medium containing 5% Serum plus (Biocell Laboratories, CA), 5% FCS (Intergene Co., Purchase, NY) and antibiotics. MDCK cell lines expressing HA, or HA-mutants were prepared and maintained according to established protocols (Brewer and Roth, 1991). Each cell line formed an electrically tight monolayer and properly localized methionine transporters at the basolateral surface, as measured by a standard methionine uptake assay (Brewer and Roth, 1991). For experiments where polarity and polarized endocytosis were investigated, cells were cultured on 24-mm Costar Transwell filters (Costar Corp., Cambridge, MA) with 3-μm pore size and fed daily for 5 d before the experiment. Monolayers were inspected by electron microscopy to establish that they formed monolayers only on one side of the filters. 16 h before the experiment, 10 mM sodium butyrate, (Sigma) in DME was added to the cells to increase protein expression.

HAs were labeled with ^{35}S-amino acids as described previously (Thomas et al., 1993). Cell monolayers on Transwell filters (Costar Corp.) were washed several times with PBS containing 1 mM Mg^{2+} and 0.1 mM Ca^{2+}, and then were incubated for 45–60 min at 37°C in media lacking methionine and cysteine. Trans^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, CA) was diluted to a concentration of 2.5 mCi/ml in this medium, and cells were labeled for 30 min from the basolateral side by placing the Transwell filters containing 400 μl of labeling medium lacking radioactive label in the apical compartment on to 140 μl of labeling medium that had been placed on a piece of parafilm. The cells were then incubated at 37°C for 30 min. For the chase, filters were transferred back to their dishes and prewarmed DME was added to both apical (2 ml) and basolateral (3 ml) chambers and cells were incubated at 37°C for 90 min.

Internalization Assay

Internalization of HA mutants expressed on subconfluent MDCK cells grown on plastic, to measure internalization when cells were not polarized, was performed exactly as described previously for CV-1 cells (Naim and Roth, 1994*a*). Briefly, MDCK cell lines expressing HA mutants were plated in 6-well dishes 1 d before the experiment so that the monolayer was subconfluent. Cells were then prestarved and labeled for the times described above, except that each culture received 400 μl labeling medium containing 500 μCi Trans^{35}S-label and chased at 37°C for 90 min in complete DME The chase was stopped with ice-cold DME and the cells were placed on ice. Rabbit anti-HA serum diluted 1/300 in DME was allowed to bind to HA at the cell surface for 45 min. Unbound antibody was washed away and cells were subsequently rinsed twice, 10 min each, with low-bicarbonate DME containing 10 mM Hepes and 0.5% BSA, then once with DME containing 10 mM Hepes for 10 min. The monolayers were then returned to 37°C in a circulating water bath for the times indicated. After each time point, cell cultures were chilled again and returned to ice. To determine the amount of antibody-bound HA that was internalized, 100 μg/ml trypsin was added to the cell monolayer to cleave HA at the cell surface into its HA1 and HA2 subunits, a treatment that does not remove the bound antibody (Lazarovits and Roth, 1988; Brewer and Roth, 1991). After 50 min, trypsin was removed and cells were washed once with DME containing 10% Serum Plus, to inactivate residual trypsin. Before lysis, 200 μl of Serum Plus containing 200 μg/ml soya bean trypsin inhibitor was added to each well as an additional precaution to avoid residual trypsin activity. Cells were then lysed in 1 ml lysis buffer, clarified by centrifugation at 11,000 *g* for 10 min. Half of each lysate was taken and mixed with an equal volume of NET/GEL (150 mM NaCl, 10 mM Tris HCl, pH 8.0, 5 mM EDTA, 0.5% NP-40, 0.25% gelatin) containing 6.5 μl of protein A–Sepharose gel to immunoprecipitate HA that was bound to the antibody. Samples were analyzed on 12.5% SDS-PAGE. The amount of cleaved HA (HA1 and HA2) and the uncleaved HA (HA0) in each sample was quantified by scanning with PhosphorImager (Molecular Dy-

namics, Sunnyvale, CA). The percent of HA that was internalized at any period of chase was calculated by dividing the optical density of the uncleaved (HA0) band by the sum of the optical densities of all of the HA bands in that sample: (HA0)/(HA0+HA1+HA2) × 100%. The percentage of HA that remained inaccessible to trypsin on cells left at 4°C was subtracted as background from the values measured after a chase at 37°C. For HA-Y543 expressed in MDCK cells, this background ranged between 5 and 8% of HA that had bound antibody, and for HA-Y543, R546, background ranged between 3 and 9%.

To measure the internalization rates from either apical of basolateral cell surfaces, 4–5 d after seeding MDCK cells on to Transwell filters monolayers were washed 2× with PBS containing 0.1 mM Ca^{2+} and 1 mM Mg^{2+}. Prewarmed starvation medium was added to both sides of the filter and the cells were incubated at 37°C for 60 min. Cells were labeled and chased as described above. Antibody binding and internalization was performed as described above, except that antibody was added to only one side of a monolayer. One ml of medium containing antibody was used to bind HA on the apical side and 2 ml of medium containing antibody was used for the basolateral side. To determine the surface population remaining at the end of each chase period, trypsin was added to the side where the antibody had bound, and on the opposite side DME containing 200 μg/ml STI was added. Cells were lysed in 1 ml lysis buffer and immunoprecipitated and analyzed as above.

Internalization in the Presence of Hypertonic Medium or Cytochalasin D

Cells were labeled as described and, after 70 min of chase, prewarmed hypertonic medium (DME containing 0.47 M sucrose) or medium containing 25 μg/ml cytochalasin D (from a stock of 25 mg/ml in DMSO) was added to both apical and basolateral sides and the chase was continued for 20–30 min. Cells were then chilled, antibody was bound to a single side of a monolayer, and then the internalization was performed in a prewarmed media that either contained sucrose or Cytochalasin D. Washing and the subsequent lysis and analysis were done as described above.

Fluid Phase Uptake

HRP type II, from Sigma, was used at a concentration of 10 mg/ml in DME. Prewarmed medium containing HRP was added to duplicate samples of cells from either the apical (1 ml) or basolateral (2 ml) sides and incubated at 37°C. For the zero time point, ice-cold medium containing HRP was added to cells held on ice for 5 or 15 min. At the end of each time point, HRP-containing medium was replaced with ice cold PBS containing 1% BSA and the cells were kept on ice. Cells were washed 3× with ice cold PBS containing 1% BSA followed by 3× with PBS. Cells were then lysed with 1 ml of 1% Triton X-100 in PBS. Nuclei and other large insoluble material were pelleted at 11,000 *g* for 10 min and supernatants were transferred to other microfuge tubes. Freshly prepared reaction buffer (0.9 ml of 14 mM KH_2PO_4; 0.1% Triton X-100; 0.02% H_2O_2; 0.007% O-Dianisidine) was added to 0.1 ml of lysate and the activity of HRP was directly measured as a function of time in a Cary 118 spectrophotometer. The HRP concentration in each extract was determined using a standard curve of HRP of a predetermined concentration whose absorbency was also measured. The volume of fluid internalized was then calculated from the known concentration of HRP in the medium.

Electron Microscopy and Immunogold Staining

Cells grown on Transwell filters were fixed, embedded and sectioned orthogonally to the filter according to published procedures (Lee et al., 1993). For immunoelectron microscopy, cells cultured on filters were washed 3× with PBS containing 1 mM Mg^{2+} and 0.1 mM Ca^{2+} and were fixed with 0.5% glutaraldehyde and 3.7% paraformaldehyde at 23°C. Filters were cut from their supports and immersed in DME containing ~1 μg/ml anti-Japan HA monoclonal antibody for 60 min with continual rocking. The filters were washed 3× with PBS containing 1% BSA. Rabbit anti–mouse IgG conjugated to gold (10 nm diam) was added in this solution at a final concentration of 1.6 μg/ml and samples were gently rocked for 1 h. The filters were then washed 3× for 5 min each with PBS containing 1% BSA. Subsequent fixation in 1% OsO_4 + 1.5% potassium ferrocyanide in 0.1 M sodium cacodylate, washings, dehydration, and embedding in Epon was according to published procedures (Hartfield et al., 1991). MDCK cells or CV-1 cells cultured on plastic were processed in a similar manner as polarized MDCK cells on filters and were embedded in situ on plastic dishes. The plastic-embedded cells were then removed from the plastic dishes and strips were cut and reembedded for ultra thin sectioning.

Measurement of Cell Surface Lengths and Counting of Coated Pits

Cells were visualized with a JEOL 1200 EX electron microscope at magnifications of 25,000× (at the microscope screen) and 250,000× (on a video monitor). Typical coated pits in the early or late stages of formation were counted separately for each cell cross-section for the apical or basolateral membranes. Each plastic section contained a cross section of monolayer containing ~20 cells. Only plasma membrane invaginations with a clearly recognizable electron-dense, cytoplasmic coat were counted. Two observers independently counted coated pits in five separate experiments. One of the observers, Dr. A. McDowall, director of our imaging facility, was not aware of the purpose of the experiments.

To determine the number of coated pits per unit membrane length of each domain, images of the cells were digitized and magnified 250,000× and then printed in sections on an Apple Pro600 laser printer. The sections were assembled and measured with a topographic Map measurer. An average ratio of basolateral to apical surfaces was calculated from the measurement of four cell cross-sections and found to be ~4 for MDCK type II cells. A ratio of 3.8 has been reported previously for type I MDCK cells (Parton et al., 1989).

Estimation of the Efficiency of Antibody Labeling for Biochemical Experiments and the Amount of HA-Y543 Present at the Apical Surface of Polarized MDCK Cells

To estimate the efficiency of antibody labeling on cells expressing radioactive HA-Y543 for internalization experiments, we compared the proportion of the radioactive population that could be bound by antibody added to the apical cell surface at 4°C and recovered after immunoprecipitation to the amount of radioactive HA-Y543 that was cleaved by trypsin added to the culture medium. Antibody bound 7.5% of the trypsin-accessible population of the mutant HA. Binding of antibody to radioactive HA-Y543 was not competed by a large excess of nonradioactive HA-Y543 added to the cell lysate, indicating that the amount of protein recovered after immunoprecipitation was equivalent to the amount that had bound antibody at the time that the cells were lysed.

To determine the efficiency of labeling with antibody and gold for immunoelectron microscopy, we estimated the number of HA-Y543 proteins that would be present at the apical cell surface by comparing the total amount of radioactive HA-Y543 recovered by immunoprecipitation after a pulse and short chase of MDCK cells expressing this protein to the amount recovered from a culture of similarly labeled CV-1 fibroblasts infected with a SV40 expression vector carrying the HA-Y543 cDNA, which produces much more protein. From this experiment we calculated the ratio of amounts of synthesis of the protein in the two cell types. From a parallel culture of the infected CV-1 fibroblasts we immunoprecipitated all of the HA-Y543. HA-Y543 and antibody in the immunoprecipitate were separated by electrophoresis on polyacrylamide gels and stained with Coomassie blue. Samples of known amounts of BSA were included on the gel. The optical density of the stained protein bands on the gel was measured by laser scanning densitometry, and the amount of HA-Y543 present was determined by comparison with the BSA standards. We had previously determined that the rates of the degradation of HA-Y543 in MDCK cells or CV-1 cells differ by less than twofold. From this information, and knowledge of the steady-state distribution of HA-Y543 in polarized MDCK cells treated overnight with sodium butyrate (40% at the cell surface and 15% of the surface population at the apical cell surface, manuscript in preparation), we calculated that the apical surface of each MDCK cell expressing HA-Y543 contains ~3,000 of the proteins.

Results

The Internalization of an HA Mutant from the Apical Cell Surface Is Less Efficient than from the Basolateral Cell Surface

In fibroblasts, HA-Y543 is internalized approximately

threefold slower than is HA-Y543,R546. Thus, we compared the internalization rates of the two mutant HAs at both the apical and basolateral surfaces of MDCK cells to determine if the apical surface retained the ability to discriminate between internalization signals with different affinities for coated pits. We cultured MDCK cells expressing either HA wt, HA-Y543 or HA-Y543,R546 in Transwell filters to establish polarized monolayers. In parallel, cultures of MDCK cells expressing each protein were grown as subconfluent, unpolarized cells on plastic dishes. The internalization of the proteins at both apical and basolateral membrane domains of polarized cells, or at the undifferentiated plasma membrane of nonpolarized cells, was then measured (Fig. 1). The initial rates of endocytosis of both proteins was fivefold slower from the apical membrane (Fig. 1 *a*) than from the basolateral membrane (Fig. 1 *b*), or the free surface of subconfluent MDCK cells (Fig. 1 *c*). However, the endocytic apparatus at the apical surface was still selective. HA wt was not internalized from the apical side during the interval of the experiment (there was insufficient signal to measure HA wt internalization from the basolateral surface) and the approximately threefold difference in internalization rates between the two mutant HAs was maintained at both membrane domains (Fig. 1, *a* and *b*). The fact that selectivity was maintained suggests that the slower internalization of membrane proteins at the apical surface was not due to a loss of high affinity binding sites for internalization signals in apical coated pits. In addition, inducing protein expression with butyrate had no effect on internalization rates in MDCK cells or in CV-1 fibroblasts under the growth conditions used in our experiments (not shown).

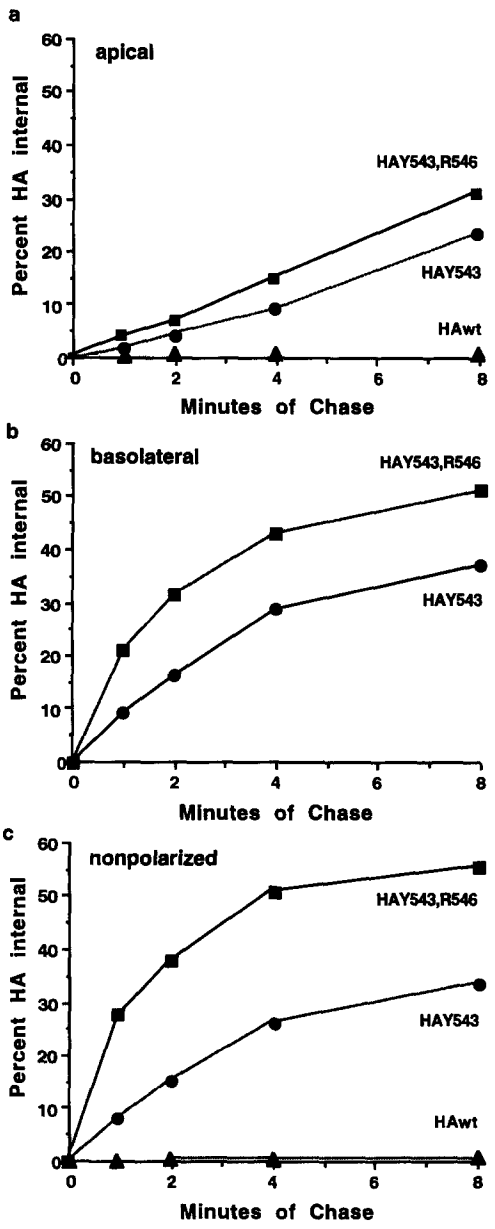

Figure 1. Internalization of HA wt, HA-Y543, or HA-Y543, R546 from the apical cell surface in polarized MDCK cells is slower than from the basolateral surface. Polarized MDCK cells expressing each protein were grown on Transwell filters for 4 or 5 d. Subconfluent, nonpolarized cells were grown on plastic dishes one day before the experiment. Cells were treated with 10 mM sodium butyrate for 16 h before internalization was measured. Internalization of HA mutants was measured at the (*a*) apical and (*b*) basolateral surface of polarized cells, or (*c*) at the free surface of nonpolarized cells. The values shown are averages from three experiments and the standard deviation did not exceed 5% of the value for any data point.

The Maturation of Endocytic Pits at the Apical Membrane Is Slower than at the Basolateral Surface

Several mechanisms might cause the slow internalization of HA mutants from the apical surface. Slower internalization would occur if the number of endocytic pits able to take up proteins at the apical side was limiting, which seemed unlikely due to the low levels of proteins expressed. Slower internalization at the apical surface of MDCK cells might also occur if the lateral diffusion rate of HA mutants was slower than at the basolateral surface. However, this would have to be a relatively large decrease, since the ability of HA-Y543 to diffuse into a coated pit is probably not rate-limiting. The mobility of HA-Y543 in CV-1 fibroblasts, where internalization occurs at rates equal to the basolateral surface of MDCK cells, is fast enough to allow an average HA-Y543 protein to encounter a coated pit every 4–8 s (Fire et al., 1991), which is an order of magnitude faster than is the internalization of coated pits in fibroblasts. Slower internalization might also occur if the lifetime of a coated pit on the apical surface was significantly longer than that at the basolateral surface or if the number of binding sites for endocytic receptors in apical coated pits was significantly reduced. All of these possibilities can be distinguished by electron microscopy. If apical endocytosis was slow because the number of coated pit binding sites for mutant HAs were limiting, either due to fewer coated pits, fewer binding sites within the pits, or if mutant HAs reached the coated pits more slowly, then only one-fifth as much of the mutant HA should be observed inside coated pits on the apical surface as was observed at membrane domains where internalization was fivefold faster. If coated pits themselves were limiting, the number of coated pits at the apical domain should be one-fifth the number at the basolateral domain of polarized cells. In contrast, slow budding of endocytic

pits at the apical surface should result in more shallow pits and fewer deeply invaginated pits being observed. A slow step in a later process, such as the pinching off of pits, could also result in a slower rate of internalization. In this case, more deep pits might be observed at the apical surface than at the basolateral.

To investigate these possibilities, cells expressing the mutant HAs were grown on filters to form polarized monolayers and, in some experiments, cells were fixed and the HAs were labeled at the apical surface with anti-HA and protein A–gold particles. The filter and cells were embedded and thin sections were cut orthogonal to the filter. After staining to improve contrast, samples were visualized by electron microscopy and photographed. Endocytic pits were identified as regular depressions of the cell surface that were ~70–100 nm in diameter and contained an electron dense cytosolic coat (Figs. 2 and 3), although occasionally this coat was difficult to see. Pits containing identifiable electron-dense coats were counted on both sides of each cell photographed. The basolateral side of the MDCK strain II cells used in these experiments contained 1.5× as many coated pits as the apical side (386 apical pits were counted and 602 basolateral), which was not enough to explain the difference in internalization rates that we observed for the two mutant proteins at the two surface domains.

To determine whether the slow internalization of HA-Y543 at the apical membrane was due to a difference in the number of binding sites in apical coated pits, we measured the fraction of HA-Y543 that was found in coated

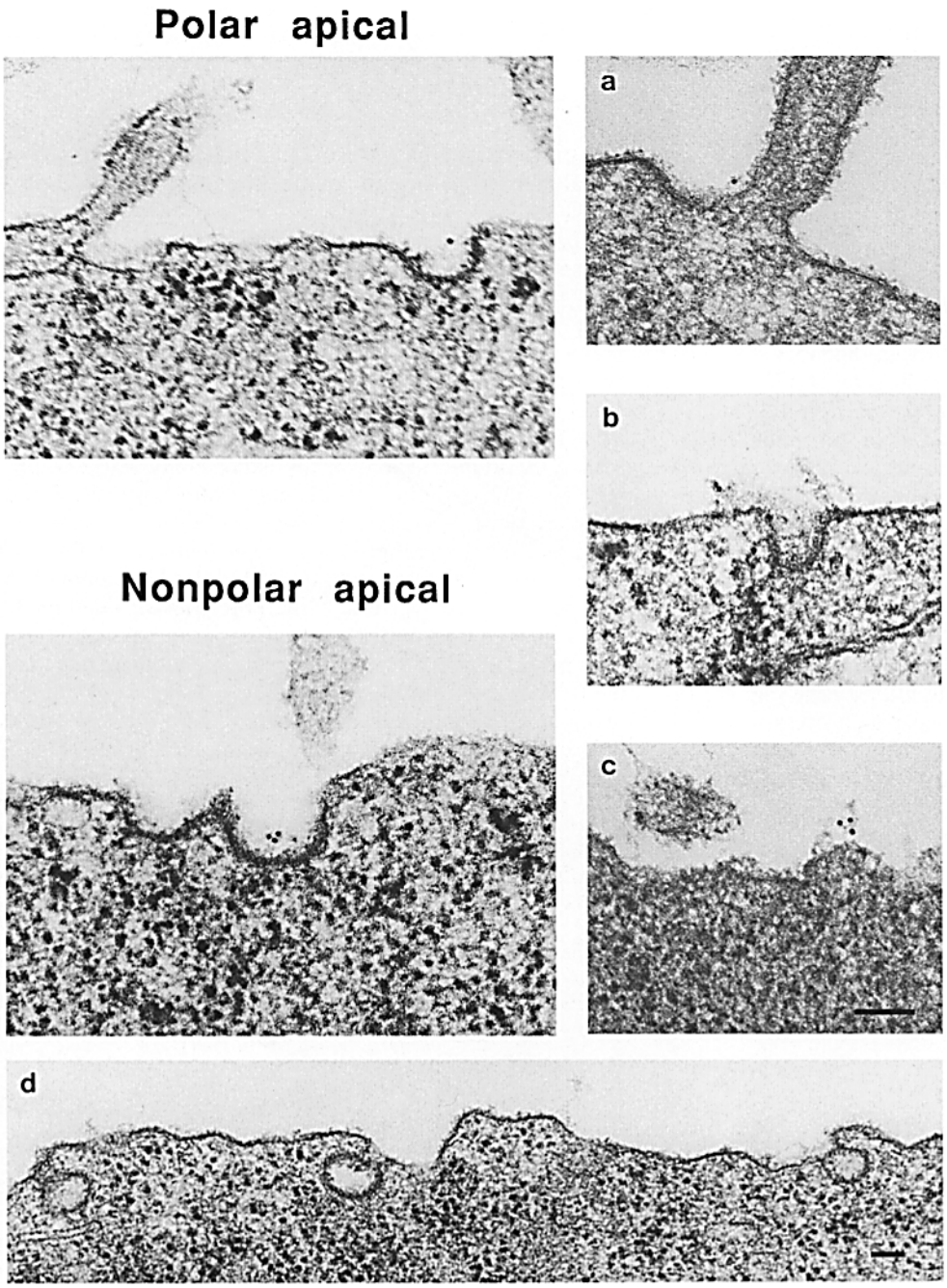

Figure 2. Localization of HA-Y543 in endocytic pits in polarized and nonpolarized cells. MDCK cells expressing HA-Y543 were grown on filters for 4 d, or were plated at low density on plastic for 1 d. The cells were fixed and then labeled with monoclonal anti-HA antibody applied to the apical cell surface, and then with rabbit anti-mouse IgG conjugated to 10 nm gold. (*a*) HA-Y543 outside coated pits in polarized MDCK cells. (*b*) HA-Y543 in the vicinity of a coated pit, typical for both polarized and unpolarized MDCK cells. (*c*) HA-Y543 on the cell surface of nonpolarized MDCK cells. (*d*) No gold labeling was detected on MDCK cells that did not express HA-Y543 and were treated identically to the cells shown in (*a–c*). Bar, 100 nm.

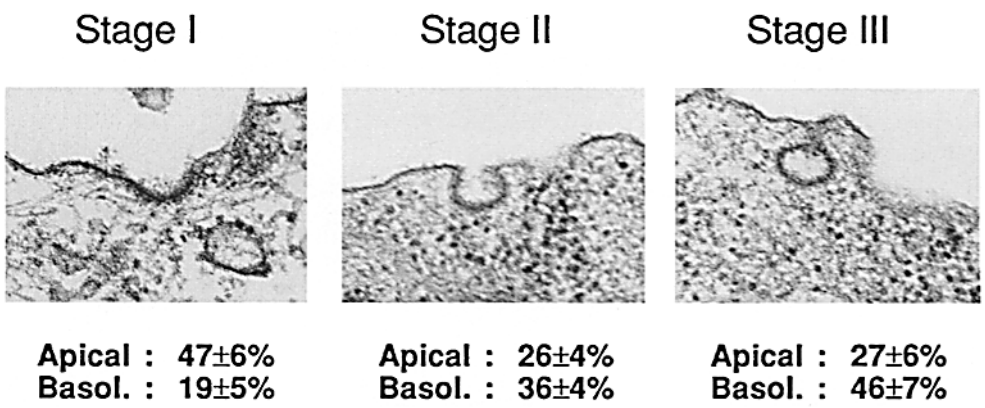

Figure 3. Analysis of clathrin coated pits from apical and basolateral cell surfaces in polarized MDCK cells. MDCK cells on filters were prepared for electron microscopy and coated pits were counted at the apical and basolateral surfaces. Three different stages in the maturation of coated pits were recorded separately in five experiments (Table II) and averaged. The percent of coated pits present at the apical or the basolateral surfaces that were in each stage are presented, with standard deviations, below micrographs showing examples of each stage.

pits on the apical surface of polarized MDCK cells at steady state. If there were fewer binding sites per coated pit and coated pits internalized with a $t_{1/2}$ of 1–2 min as measured in other cells (Anderson et al., 1977; Griffiths et al., 1989; Hansen et al., 1992), then from the internalization rates measured (Fig. 1) one would expect to find 1–3% of HA labeled with gold in coated pits at the apical surface of polarized cells and 7–14% of the label in coated pits under conditions where internalization was faster. For this experiment to be meaningful, we needed to establish two points: (*a*) that the background of gold labeling on cells not expressing HA-Y543 was sufficiently low and (*b*) that the labeling of HA-Y543 with the monoclonal antibody and gold was similar to the efficiency of labeling of radioactive HA-Y543 with rabbit antibodies (7.5% of the trypsin-accessible surface population, see Materials and Methods). We calculated that the apical surface of each MDCK cell expressing HA-Y543 contains ~3,000 of the proteins (see Materials and Methods). Dividing the surface area of the monolayer on the filter by the number of MDCK cells in the monolayer gave us a cross-sectional area containing 3,000 HA-Y543 proteins. Dividing this area by the number of 90-nm-thin sections required to cover the whole area, we estimated that each cell profile of apical membrane should contain about 40 HA-Y543 proteins.

Monolayers of polarized MDCK cells that either expressed no HA, or expressed HA-Y543, were grown on filters, fixed and then labeled with monoclonal anti-HA antibody and protein A conjugated to gold. Samples were prepared for electron microscopy and an equal number of profiles of cells either expressing or not expressing HA-Y543 were examined. We observed no binding of gold to MDCK cells that did not express HA-Y543 (Fig. 2 *d*), satisfying the first criterion for the experiment. After counting 420 gold particles on ~140 cell profiles, we calculated an average of ~3 gold particles labeled each apical cell profile (Table I and Fig. 2). This represented a labeling efficiency of 7.5% of the amount of HA-Y543 estimated to be present, which was the same efficiency of labeling as was achieved for internalization experiments. Of these gold particles, ~12% were observed in endocytic pits (Table I). Another 10–12% were within a distance of ~0.5 pit diameter of an endocytic pit (Fig. 2 *b*) and the rest of the gold label was dispersed on the plasma membrane (Fig. 2, *a* and *c*). Thus the percentage of the total HA-Y543 popu-

Table I. The Fraction of HA-Y543 in Endocytic Pits Does Not Vary Significantly under Conditions where Internalization Rates Differ by Fivefold

	Initial endocytic rate	Percent gold in endocytic pits	Percent gold beside endocytic pits	Number of gold particles	Number of cell profiles
HA-Y543 at the apical surface of polarized MDCK cells	1.7 ± 0.2	12.3 ± 3	11.9 ± 2.7	420 (n = 7)	140
HA-Y543 at the free surface of nonpolarized MDCK cells	9.0 ± 1.0	14.8 ± 3.5	7.7 ± 2.3	542 (n = 6)	90
HA wt at the free surface of MDCK cells	<0.5	0	0	500 (n = 3)	45

MDCK cells expressing HA-Y543 were grown on filters to form polarized monolayers, or were maintained for one day as non-polarized, subconfluent cultures on plastic. MDCK cells were treated with sodium butyrate overnight before labeling. All cells were fixed before they were labeled with anti-HA antibody and second antibody conjugated to colloidal gold. Gold particles were counted inside, within 50 nm of endocytic pits, and outside pits. Values are given with standard deviations. n, the number of monolayer profiles examined. Initial endocytic rate is the percent of protein internalized after 1 min.

lation that is present in coated pits at the moment the cells were fixed (which we assume to be the situation at steady-state) is five- to sevenfold greater than expected for coated pits internalizing HA-Y543 at the slow rate shown in Fig. 1, if one assumes that coated pits internalize as fast at the apical surface of MDCK cells as they do in other cell types where this rate has been estimated.

When this same analysis was performed on HA-Y543 expressed in subconfluent MDCK cells (Fig. 2), or in CV-1 cells where internalization was much faster, no significant difference in the percent of the protein in endocytic pits was observed (Table I and data not shown). Thus, the number of binding sites within endocytic pits at the apical surface of MDCK cells did not appear to be limiting, and could not explain the slower rate of internalization at the apical surface of polarized cells.

The distribution of HA-Y543 that we observed was that expected if the protein were binding to endocytic pits that took significantly longer to internalize. Two observations were consistent with that interpretation. We determined that the ratio of basolateral to apical membrane profiles of cells in thin sections was 4, but the basolateral membranes contained only 1.5, rather than 4, times as many coated pits as did the apical domain. Thus, the number of coated pits per unit area was actually greater at the apical surface, an observation that would be expected if apical coated pits had a longer lifetime at the apical surface and if recycling of clathrin back to the membrane occurred at similar rates at both membrane domains. In addition, most of the apical coated pits were either in an early phase or an intermediate phase of invagination (Table II and Fig. 3). Almost half of the coated pits at the apical cell surface were shallow, being invaginated less than half the diameter of the width of the pit at the cell surface (Fig. 3, *Stage I*). In contrast, almost half of the coated pits at the basolateral domain were deeply invaginated or closed (Fig. 3, *Stage III*). When compared by a two-tailed Student's *t* test, the average percent of apical coated pits in each stage was found to be significantly different than the average percent of basolateral coated pits in the same stage (the probability, *P*, that the averages for each stage of coated pit at the two membrane domains were the same was 0.001 for stage I, 0.003 for stage II, and 0.01 for stage III). These observations suggest that endocytic pits form more slowly at the apical than the basolateral surface, resulting in slower internalization of their content.

Internalization of HA Mutants from the Apical Surface Is Inhibited by Cytochalasin D

Recently, internalization of fluids and of the VSV G protein missorted to the apical surface of MDCK cells has been observed to be sensitive to cytochalasin D (Gottlieb et al, 1993). Since G protein lacks a high-affinity internalization signal for coated pits (Thomas et al., 1993; Thomas and Roth, 1994) and there is more than one apical endocytic pathway (Eker et al., 1994), we were interested in determining if cytochalasin D would also affect internalization of a protein with a reasonably good internalization signal for clathrin-coated pits. MDCK cells expressing HA-Y543,R546 were grown as polarized monolayers on filters and treated with 25 μg/ml cytochalasin D in DME, as previously reported by Gottlieb et al. (1993). As a control, cells were treated in parallel with hypertonic medium, which completely inhibits clathrin-mediated endocytosis by causing coated pits to disassemble (Heuser and Anderson, 1989) and release any trapped proteins (Henis and Roth, unpublished observations). After 30 min of this treatment, internalization from the apical or the basolateral domains was measured in the presence or absence of cytochalasin D or hypertonic medium. Treatment with hypertonic medium completely inhibited the internalization of HA-Y543,R546 from either side of the cell (Fig. 4, *a* and *b*). Cytochalasin D inhibited internalization from the apical side by 45–50% (Fig. 4 *a*) but did not affect the internalization of the HA mutant from the basolateral side (Fig. 4 *b*). The effect of cytochalasin D on apical but not the basolateral internalization of HA-Y543,R546 is consistent with the results reported by Gottlieb et al. (1993) suggesting that the internalization from the apical side is influenced in some way by actin. However, we were not able to detect complete inhibition of HA internalization by this drug.

To measure the effect of cytochalasin D on all internalization from apical or basolateral membranes, we measured the fluid-phase endocytosis of HRP from each side of MDCK cells grown as polarized monolayers on filters (Fig. 5). Duplicate samples were either treated with cy-

Table II. Shallow Coated Pits Are More Often Observed at the Apical than the Basolateral Surface in MDCK Cells

	Numbers of coated pits counted								Percent coated pits of each morphology					
	Stage I		Stage II		Stage III		Total		% stage 1		% stage 2		% stage 3	
exp.	ap	bl	ap	bl	ap	bl	ap	bl	ap	bl	ap	bl	ap	bl
1	29	16	23	50	22	68	74	134	39	12	31	37	30	51
2*	55	30	23	43	38	88	116	161	47	19	20	27	33	55
3*	39	31	20	43	29	41	88	115	44	27	23	37	33	36
4	27	17	13	36	10	44	50	97	54	18	26	37	20	45
5	31	18	16	38	11	39	58	95	53	19	28	40	19	41
Mean ± SD									47 ± 6	19 ± 5	26 ± 4	36 ± 4	27 ± 6	46 ± 7

Coated pits were counted at apical and basolateral membranes of MDCK cells grown on permeable supports in five separate experiments by two different observers, one of whom(*) was an experienced electron microscopist not associated with this project (see Materials and Methods). Only pits with electron-dense, cytoplasmic coats were counted. The morphological stages are those shown in Fig. 3. *ap*, apical; *bl*, basolateral.

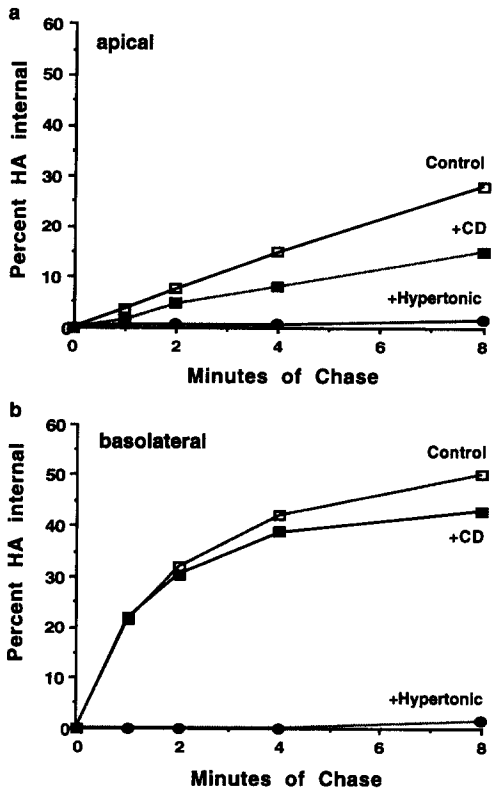

Figure 4. Effect of cytochalasin D and hypertonic medium on the endocytosis of HA-Y543,R546 from the apical and basolateral cell surface in MDCK cells. Internalization of HA-Y543,R546 was measured at both the (*A*) apical and (*B*) basolateral surfaces of polarized MDCK cells in monolayers on Transwell filters. Values plotted are averages of three experiments. The standard deviations of all data points were between 5 and 7% of each value.

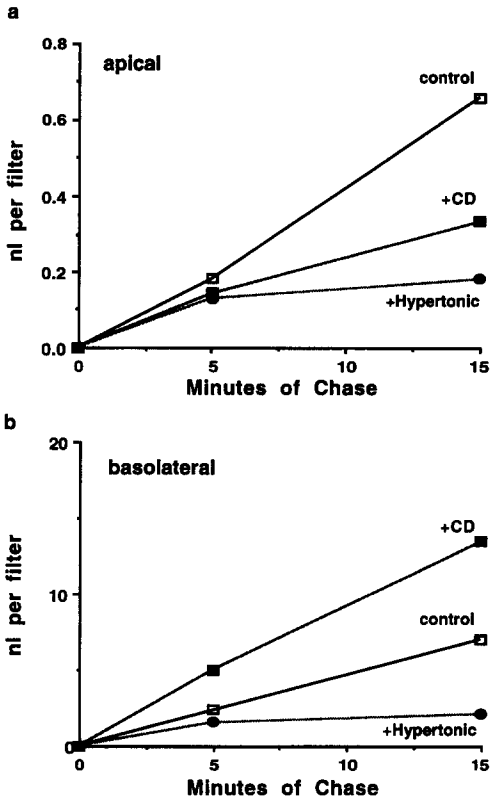

Figure 5. Fluid-phase uptake in MDCK cells. Fluid phase endocytosis of HRP was measured from the (*A*) apical or the (*B*) basolateral cell surfaces for cells in DME containing either no additives, or 25 μg/ml cytochalasin D, or 470 mM sucrose. Values were corrected for HRP that remained with cells incubated only on ice and each point in the graph represents an average of results from two experiments that varied by ~10%.

tochalasin D, hypertonic buffer, or left untreated for 30 min at 37°C and then the same medium containing HRP at a concentration of 10 mg/ml was added to the cells, either from the apical or the basolateral side. Samples were incubated at 37°C for 5 or 15 min. For the zero time point, medium containing HRP was added to the cells on ice. After the period for internalization, HRP activity was measured as described in Materials and Methods. Fluid uptake from the basolateral surface was 7 nl in 15 min per filter and internalization of fluid from the apical surface was 0.7 nl in 15 min (Fig. 5). Treatment with cytochalasin D stimulated fluid phase uptake from the basolateral side by twofold, a result which we have observed not only in MDCK cells (Fig. 5 *b*), but also in nonpolarized CV-1 cells (R.-H. Wang and M. G. Roth, unpublished observations). However, cytochalasin D inhibited apical uptake of HRP by 50% at 15 min. Hypertonic medium inhibited both apical and basolateral uptake by ~73 and 70% after 15 min, respectively, suggesting that the clathrin-mediated pathway contributed no more than about 70% of total endocytosis at each membrane domain.

Discussion

There are several reports that endocytosis from the apical surface of MDCK cells differs from that at the basolateral (Le Bivic et al., 1991; Melby et al., 1991; Prydz et al., 1992; Gottlieb et al., 1993; Eker et al., 1994). This raises the question of whether the internalization apparatus at the specialized apical surface, which apparently has no need to recognize proteins with high affinity internalization signals, nevertheless retains the mechanisms capable of this sorting event. Our results comparing the rates of internalization of HA-Y543 and HA-Y543,R546 at both the apical and basolateral domains of MDCK cells indicate that the ability of endocytic apparatus to discriminate between two proteins with internalization signals of different "strengths" was the same at the apical and basolateral surfaces of polarized epithelial cells. Since we observed our mutant proteins in apical coated pits by immunocytochemistry and could inhibit their internalization by hypertonic medium, we assume that most, if not all, of the internalization that we observed at the apical surface of MDCK cells was through a clathrin-mediated pathway. This internalization was approximately fivefold slower than that measured at the basolateral surface of polarized MDCK cells or in unpolarized MDCK cells or fibroblasts, and fluid-phase uptake of HRP from the apical medium was 10-fold slower. We observed by electron microscopy that 10–14% of HA-Y543 was located in endocytic pits at the apical surface of both polarized and nonpolarized MDCK cells. Thus, there was no evidence for lack of binding sites for

HA-Y543 within apical coated pits and differences in the fraction of the HA-Y543 population residing in coated pits at different membrane domains can not explain the differences in the rates of internalization measured there. Apical coated pits were significantly shallower, on average, than at the basolateral surface, and the number of endocytic pits per unit of apical membrane was actually greater than at the basolateral surface. Taken together, these observations indicate that the coated pits at the apical surface recognize proteins as efficiently as at the basolateral surface, but they mature into vesicles much more slowly. Comparing the percent of HA-Y543 seen in coated pits by electron microscopy (10–14%) and the percent internalized each minute at different membrane domains (1.7%/min apical and 9%/min basolateral), we estimate the lifetime of a coated pit on the polarized apical surface to be ~6–8 min and at basolateral or nonpolarized domains to be 1.1–1.5 min. This latter figure is essentially that measured previously in fibroblasts. An important conclusion from these results is that the rate of maturation of coated pits can vary at different places within the same cell type. Perhaps clathrin-mediated endocytosis at other specialized domains, such as synaptic membranes, is also regulated not only by the numbers of coated pits that form, but in the rate at which they mature.

In experiments using reconstituted systems, four distinct events in the maturation of coated pits have been identified: the binding of the AP-2 complex to the plasma membrane, the recruitment of clathrin to the AP-2 complex, the growth of a curved clathrin lattice into a deeply invaginated pit, and the pinching off of the coated vesicle (reviewed by Schmid, 1993). The observation that there are more coated pits per unit membrane at the apical surface and that they are significantly shallower suggests that the third step in maturation is slow at that domain. The coated pits at the apical surface of MDCK cells do not resemble the rather elongated coated pits seen in cells expressing mutant dynamins, which are also defective in the third step of internalization (Kosaka and Ikeda, 1983; Damke et al., 1994), suggesting that the slow step in coated pit formation at the apical domain occurs earlier than the function requiring dynamin. This would be most easily explained by a difference in the amount of a membrane-bound factor that either controls the addition of clathrin triskelions into the lattice, or regulates membrane curvature, perhaps by controlling the introduction of pentagons among the hexagons of the lattice, which is a requirement for attaining a curved clathrin surface (reviewed by Kirchhausen, 1993). The involvement of a membrane-bound factor in this process is further suggested by our observation that in a mutant MDCK cell line that forms electrically tight monolayers but missorts plasma membrane proteins, HA-Y543 is internalized at 7–9%/min from both the apical and basolateral domains (manuscript in preparation).

Previously Gottlieb and colleagues showed that cytochalasin D inhibited the internalization of VSV G protein from the apical, but not from the basolateral surface of MDCK cells. Since G lacks a tyrosine-dependent internalization signal for endocytosis, we investigated the effects of cytochalasin D on internalization of HA-Y543-R546, which contains a high affinity internalization signal for coated pits. We confirm that cytochalasin D specifically inhibited apical endocytosis, but in the case of HA-Y543,R546, inhibition was incomplete. Jackman et al. (1994) have reported that inhibition of internalization of ricin in MDCK cells treated with cytochalasin is also incomplete. Since ricin is internalized by both clathrin and non clathrin pathways, it is possible that the drug completely inhibits one of the two pathways and not the other. Since VSV G protein lacks a cytoplasmic internalization signal for associating with coated pits (Thomas and Roth, 1994), the complete inhibition of internalization of G protein at the apical surface caused by cytochalasin D (Gottlieb et al., 1993) could occur through inhibition of a nonclathrin-mediated pathway. One interpretation of our observations is that polymerized actin at the apical membrane is not required for clathrin-mediated endocytosis, but that the polymerization state of subapical actin does influence the rate of this process. Gottlieb et al. (1993) observed that coated pits appear to be at the end of long, narrow invaginations at the apical surface of MDCK cells treated with cytochalasin D that did not appear to internalize cationized ferritin as measured by electron microscopy. They interpreted this as evidence that the pinching off of coated pits was inhibited. Alternatively, it is possible that membrane and soluble proteins enter the narrow tubes leading to coated pits more slowly, and that vesicle fission still occurs at some rate. Another explanation for our data is that HA-Y543,R546 with antibody bound to it can move into deeply invaginated coated pits and become sequestered from trypsin subsequently added to the culture medium. However, we observed continued internalization of both the membrane-bound HA-Y543,R546 and HRP in the fluid-phase in MDCK cells treated with cytochalasin D and it is difficult to understand how HRP might gain access to these deep invaginations but trypsin might not. Therefore, we favor an interpretation that actin is important for influencing the movement of proteins and solutes into coated pits but is not absolutely required for them to mature into coated vesicles.

We thank Dr. A. McDowall, G. Lawton, and K. Latham for help with electron microscopy.

This work was supported by grant GM41050 from the National Institutes of Health. During part of this work, M. G. Roth was an Established Investigator of the American Heart Association.

Received for publication 8 July 1994 and in revised form 15 March 1995.

References

Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1977. A mutation that impairs the ability of lipoprotein receptors to localize in coated pits on the cell surface of human fibroblasts. *Nature (Lond.)*. 270:695–699.

Bomsel, M., K. Prydz, R. G. Parton, J. Gruenberg, and K. Simons. 1989. Endocytosis in filter-grown Madin-Darby canine kidney cells. *J. Cell Biol.* 109: 3243–3258.

Brewer, C. B. 1994. Cytomegalovirus plasmid vectors for permanent lines of polarized epithelial cells. *Methods Cell Biol.* 43:233–246.

Brewer, C. B., and M. G. Roth. 1991. A single amino acid change in the cytoplasmic domain alters the polarized delivery of influenza virus hemagglutinin. *J. Cell Biol.* 114:413–421.

Damke, H., T. Baba, D. E. Warnock, and S. L. Schmid. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* 127:915–934.

Eker, P., P. K. Holm, B. van Deurs, and K. Sandvig. 1994. Selective regulation of apical endocytosis in polarized Madin-Darby canine kidney cells by mastoparan and cAMP. *J. Biol. Chem.* 269:18607–18615.

Fire, E., D. E. Zwart, M. G. Roth, and Y. I. Henis. 1991. Evidence from lateral mobility studies for dynamic interactions of a mutant influenza hemagglutinin with coated pits. *J. Cell Biol.* 115:1585–1594.

Gottlieb, T. A., I. E. Ivanov, M. Adesnik, and D. D. Sabatini. 1993. Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. *J. Cell Biol.* 120:695–710.

Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. *J. Cell Biol.* 109:2703–2720.

Hansen, S. H., K. Sandvig, and B. van Deurs. 1992. Internalization efficiency of the transferrin receptor. *Exp. Cell Res.* 199:19–28.

Hartfield, C., A. McDowall, B. Loveland, and K. Fischer-Lindahl. 1991. Cellular location of thymus-leukemia (TL) antigen as shown by immuno-cryoultramicrotomy. *J. Elect. Micros. Tech.* 18:148–156.

Heuser, J. E., and R. G. W. Anderson. 1989. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell Biol.* 108:389–400.

Jackman, M. R., W. Shurety, J. A. Ellis, and J. P. Luzio. 1994. Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D. *J. Cell Sci.* 107:2457–2556.

Kirchhausen, T. 1993. Coated pits and coated vesicles-sorting it all out. *Curr. Opin. Struct. Biol.* 3:182–188.

Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster, shibire*^{ts1}. *J. Cell Biol.* 97:499–507.

Ktistakis, N. T. , D. C. Thomas, and M. G. Roth. 1990. Characteristics of the tyrosine recognition signal for internalization of transmembrane surface glycoproteins. *J. Cell Biol.* 111:1393–1407.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488–492.

Lazarovits, J., and M. Roth. 1988. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. *Cell.* 53:743–752.

Le Bivic, A., Y. Sambuy, A. Patzak, N. Patil, M. Chao, and E. Rodriquez-Boulan. 1991. An internal deletion in the cytoplasmic tail reverses the apical localization of human NGF receptor in transfected MDCK cells. *J. Cell Biol.* 115:607–618.

Lee, T.-L., Y.-C. Lin, K. Mochitate, and F. Grinnell. 1993. Stress-relaxation of fibroblasts in collagen matrices triggers ectocytosis of plasma membrane vesicles containing actin, annexins II and VI, and β1 integrin receptors. *J. Cell Sci.* 105:167–177.

Matter, K., E. M. Yamamoto, and I. Mellman. 1994. Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. *J. Cell Biol.* 126:991–1004.

McGraw, T. E., B. Pytowski, J. Arzt, and C. Ferrone. 1991. Mutagenesis of the human transferrin receptor: two cytoplasmic phenylalanines are required for efficient internalization and a second-site mutation is capable of reverting an internalization-defective phenotype. *J. Cell Biol.* 112:853–861.

Melby, E. L., K. Prydz, S. Olsnes, and K. Sandvig. 1991. Effect of monensin on ricin and fluid phase transport in polarized MDCK cells. *J. Cell Biochem.* 47: 251–260.

Naim, H. Y., and M. G. Roth. 1994*a*. Characteristics of the internalization signal in the Y543 influenza virus hemagglutinin suggest a model for recognition of internalization signals containing tyrosine. *J. Biol. Chem.* 269:3928–3933.

Naim, H. Y., and M. G. Roth. 1994*b*. SV40 expression vectors. *Methods Cell Biol.* 43:233–246.

Nelson, W. J. 1992. Regulation of cell surface polarity from bacteria to mammals. *Science (Wash. DC).* 258:948–955.

Parton, R. G., K. Prydz, M. Bomsel, K. Simons, and G. Griffiths. 1989. Meeting of the apical and basolateral endocytic pathways of the Madin-Darby canine kidney cell in late endosomes. *J. Cell Biol.* 109:3259–3272.

Prydz, K., S. H. Hansen, K. Sandvig, and B. van Deurs. 1992. Effects of brefeldin A on endocytosis, transcytosis and transport to the Golgi complex in polarized MDCK cells. *J. Cell Biol.* 119:259–272.

Rodriquez-Boulan, E., and S. K. Powell. 1992. Polarity of epithelial and neuronal cells. *Annu. Rev. Cell Biol.* 8:395–427.

Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Schmid, S. L. 1993. Biochemical requirements for the formation of clathrin- and COP-coated transport vesicles. *Curr. Opin. Cell Biol.* 5:621–627.

Thomas, D. C., C. B. Brewer, and M. G. Roth. 1993. Vesicular stomatitis virus glycoprotein contains a dominant cytoplasmic basolateral sorting signal critically dependent upon a tyrosine. *J. Biol. Chem.* 268:3313–3320.

Thomas, D. C., and M. G. Roth. 1994. The basolateral targeting signal of the cytoplasmic domain of VSV G protein resembles a variety of intracellular targeting motifs related by primary sequence but having diverse targeting activities. *J. Biol. Chem.* 269:15732–15739.