

Membrane Protein Trafficking through the Common Apical Endosome Compartment of Polarized Caco-2 Cells

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By raising monoclonal antibodies to the apical surface of Caco-2 cells we have identified a membrane protein (p100) that internalizes and recycles constitutively between the apical plasma membrane and endosomes in the apical cytoplasm. By applying tracers bound to the transferrin receptor, which internalizes and recycles back to the basolateral border, we demonstrate that the apical endosomes containing p100 include a subset of multivesicular bodies (MVB), which are also accessible to proteins arriving from the basolateral endosome. Tracers bound to EGF receptors and α -2-macroglobulin, which internalize from the basolateral border and are degraded, probably in lysosomes, also pass through the p100-containing MVB. These studies therefore suggest that the apical cytoplasm of Caco-2 cells contains a population of MVB capable of receiving membrane proteins trafficking in from both apical and basolateral borders and then routing them to a variety of cell surface and intracellular destinations. The differential distribution of apical and basolateral tracers within the 50-nm-diameter tubules connected to these p100-positive apical MVB suggests that the destination of proteins trafficking from the MVB back to apical and basolateral surfaces is determined by the tubules to which they gain access.

INTRODUCTION

Membrane proteins internalized at the plasma membrane are delivered to the endosome. From this compartment they either recycle constitutively back to the surface from which they were internalized or they are diverted into alternative pathways that deliver them to new intracellular destinations such as lysosomes (Courtoy, 1991; Trowbridge *et al.*, 1993). In polarized epithelial cells, tracers internalizing from the apical and basolateral plasma membranes enter separate endosomal compartments (Parton *et al.*, 1989; Hughson and Hopkins, 1990). From these compartments internalized proteins either recycle back to the plasma membrane from which they were internalized, transfer toward lysosomes, or enter transcytotic pathways that carry them across the cell to new surface domains (Simons and Wandinger-Ness, 1990; Rodriguez-Bou-

lan and Powell, 1992). However, the detailed relationships between the various pathways of internalization in polarized epithelial cells remain to be clarified.

Our previous studies on Caco-2 cells (Hughson and Hopkins, 1990) suggested that apical and basolateral endocytic pathways connect in a compartment from which a recycling receptor (TFnR)¹ entering the cell from the basolateral border can return to the cell surface. However, studies of fluid phase tracers in MDCK cells suggested an alternative scheme in which the basolateral and apical endosomes were separate compartments that delivered their endocytosed contents to a common prelysosome compartment (Parton *et al.*,

¹ Abbreviations used: α -2 M, α -2 macroglobulin; DMEM, Dulbecco's modified minimum essential medium; EGF, epidermal growth factor; HRP, horseradish peroxidase; M6PR, mannose 6-phosphate receptor; MTOC, microtubule-organizing center; MVB, multivesicular bodies; PIgAR, polymeric IgA receptor; TfnR, transferrin receptor.

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1989). More recently (Apodaca *et al.*, 1994) it has been suggested that a membrane-bound tracer internalizing from the apical surface of MDCK cells can be found in apical endosomal elements which also contain TfnR. However, another recent study (Barroso and Sztul, 1994) was unable to find any co-localization between TfnR and apically internalized ricin. In addition, different labeling of apical endosomes was found by using a fluid-phase tracer as compared with ricin. These conflicting observations suggest the following: 1) the MDCK cell system may have much more in common with the Caco-2 system than previously realized; and 2) the use of fluid-phase and nonspecific membrane-bound tracers may lead to quite different views of endocytic traffic in polarized cells, especially when compared with specific membrane-bound tracers. Thus the exact connection(s) between apical and basolateral endocytic routes taken by recycling membrane proteins is unresolved. In this paper we investigate the relationship of specific apical and basolaterally internalized recycling membrane proteins for the first time in Caco-2 cells.

The route taken by basolaterally internalized membrane proteins targeted directly to the lysosome and its relationship to the other endocytic pathways in polarized cells has not yet been studied in detail. We have characterized the route from the basolateral surface to the lysosome, and show for the first time how it relates to the apical endocytic pathway.

We have identified an apical membrane protein (p100) that internalizes and recycles. Using tracers attached to this protein we show that it can enter endosomal compartments that are accessible to a variety of tracers bound to membrane proteins internalized from the basolateral border. These tracers are either in the process of recycling back to the basolateral border (TfnR) or transferring to lysosomes (EGFR and α -2 M). Together these observations suggest that membrane proteins internalized from both apical and basolateral surfaces and having a variety of subsequent destinations can all pass through a common, apical endosome compartment.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells were cultured and grown on filters as previously described (Hughson and Hopkins, 1990). HEp-2 cells (kindly provided by Dr. Dautry-Varsat, Pasteur Institute, Paris, France) were maintained in DMEM supplemented with 10% FCS.

Antibody Preparation

Monoclonal antibodies were raised against a confluent monolayer of 10-day-old Caco-2 cells. Cells were rinsed free of serum, fixed in 3% paraformaldehyde in PBS, scraped off the dish, and mixed with Freund's complete adjuvant. Two hundred microliters was administered intraperitoneally to BALB/c mice and subsequent boosts were given intraperitoneally every 2–3 wk. Test bleeds were

screened by indirect immunofluorescence. Fusion of spleen cells with PA1 cells was carried out as described (Isacke *et al.*, 1986).

To purify anti-p100, cloned hybridomas were grown in a "Cell Max Quad" hollow fiber cartridge system (Cellco Advanced Bioreactors, Germantown, MD). The medium was harvested and antibody purification carried out using a Fast Flow Sepharose S (Pharmacia, Uppsala, Sweden) ion exchange column.

Iodination

EGF (a gift from H. Gregory, ICI Pharmaceuticals), Extravidin (Sigma Chemical, St. Louis, MO), and monoclonal antibodies against p100, the transferrin receptor, (B3/25, a gift from I.S. Trowbridge, Salk Institute) and the EGF receptor (108, a gift from J. Schlessinger, New York University, New York, NY) were iodinated using the chloramine T method (Hunter & Greenwood, 1962), adapted as previously described (Hughson and Hopkins, 1990).

Quantitation of p100: Distribution and Trafficking

To determine the relative amounts of surface and intracellular p100 in Caco-2 and HEp-2 cells, cells fixed as for fluorescence (Hughson and Hopkins, 1990) were incubated with iodinated anti-p100 in the presence or absence of 0.4% saponin (antibody added apically to filter-grown Caco-2 cells). Unbound antibody was removed by washing and cells were solubilized in 2 M NaOH for counting.

To monitor the internalization of anti-p100, iodinated antibodies were bound to cells at 4°C. After rinsing, at which point 85% of the surface-bound label could be removed by acid stripping (with 200 mM acetic acid, 500 mM NaCl, pH 2.5), the cells were incubated at 37°C for up to 2 h. Media samples were precipitated with trichloroacetic acid (TCA), surface-bound antibodies were removed by acid stripping, and cell associated antibody was solubilized in 2 M NaOH. All samples were counted to track the anti-p100 through these fractions.

To investigate the constitutive internalization of anti-p100, cells were incubated in the presence or absence of excess anti-p100 for 4 h at 37°C, cooled to 4°C, and then both sets of filters were incubated for a further 1 h in excess anti-p100. After thorough rinsing, the binding of iodinated rabbit anti-mouse Ig was used to quantitate the amount of anti-p100 on the cell surface.

To investigate the recycling of p100, anti-p100 was labeled with NHS-SS-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions and applied apically to filter-grown Caco-2 cells at 37°C for 4 h. The cells were then treated with a mild reducing glutathione buffer (adapted from Bretscher and Lutter, 1988) at 4°C for 10 min. This treatment removed 87% of the surface label. The cells were then warmed to 37°C, and incubated for up to 2 h before being cooled again to 4°C and rinsed. Surface biotin was detected with iodinated Extravidin (Pierce) at 4°C.

Electron Microscopy

Anti-transferrin receptor antibody (B_{3/25}) and anti-p100 were conjugated to 8 nm colloidal gold following the method described by Slot and Geuze (1985). Anti-p100 and synthetic EGF (with the addition of four extra lysine residues on the carboxyl terminus) were conjugated to horseradish peroxidase using SPDP (Sigma Chemical) as described previously (Hopkins and Trowbridge, 1983; Miller *et al.*, 1986). α -2 M/HRP was prepared as described previously by Hopkins *et al.* (1994).

For the morphological analyses, filter-grown Caco-2 cells were incubated as described in RESULTS and in the figure legends. In one set of experiments cells were incubated with anti-TfnR/colloidal gold in the basal chamber for 30 or 45 min. The cells were then cooled to 4°C, anti-p100-HRP was added, and the incubation continued for a further 30 min. The cells were then washed apically and warmed to 37°C for intervals up to 120 min in 10% fetal calf serum/DMEM. The filters were then rinsed, fixed in 1% glutaral-

dehyde, 2% paraformaldehyde, 100 mM sodium cacodylate buffer, pH 7.4, for 15 min, and rinsed in 50 mM Tris, pH 7.6. After treatment with 0.15% wt/vol diaminobenzine and 0.03% H₂O₂ in 50 mM TRIS, pH 6.5, (Graham and Karnovsky, 1966) filters were embedded in Epon. Sections were cut and examined in a Philips CM12 microscope as previously described (Hughson and Hopkins, 1990).

In a second set of experiments, either Tfn/HRP, EGF/HRP, or α -2 M/HRP were allowed to internalize basolaterally for 30 min at 37°C, before washing at 37°C, followed by further incubations at 37°C for up to 4 h. Anti-p100-collodial gold was present in the apical chamber throughout these incubations. The cells were then washed in cold PBS, fixed, treated with diaminobenzine, and prepared for electron microscopy as above.

RESULTS

p100 Is a Membrane Protein Ag that Recycles Via the Apical Surface of Caco-2 Cells

To obtain antibodies that would allow us to follow an apical recycling pathway, we screened a panel of monoclonal antibodies that bound the apical surface of Caco-2 cells for their ability to internalize. We found one example which, based on the apparent M_r of its antigen, we named anti-p100 (Figure 1).

The approximate molecular weight of the antigen was determined by immunoblotting. Extracts of Caco-2 cells were run on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) gels and immunoblotted using anti-p100 antibody. This shows two clusters of bands, at approximately 100 and 200 kDa (the latter probably dimers, although this high molecular weight form could also arise from the addition of polyglycans) (Figure 2). The loss of signal after reduction suggests that the antibody recognizes a protein rather than a carbohydrate epitope. The multiplicity of bands most likely represents differential glycosylation because treatment of lysates to enzymatically remove *N*-linked oligosaccharides caused a reduction in molecular weight (our unpublished observations). The immunoreactivity partitioned into the detergent phase of TX-114 lysates, suggesting that p100 is an integral membrane protein (Bordier, 1981). An unfortunate inability to immunoprecipitate metabolically labeled antigen with this reagent has precluded further biochemical characterizations.

In Caco-2 cells, indirect immunofluorescence of permeabilized cells showed that p100 is present at steady state both on the apical surface and in internal vesicles within the apical cytoplasm. Intracellular vesicular staining with anti-p100 was also found in a variety of human epithelioid cell lines (HEp-2 and HeLa) as well as fibroblasts (AG1523). The molecular weights of the protein when extracted from these various cell types were similar (Figure 2).

The ratio of surface to intracellular p100 was quantified by comparing the binding of iodinated anti-p100 to nonpermeabilized and saponin-permeabilized cells. In polarized Caco-2 cells, more than 40% ($42 \pm 8\%$,

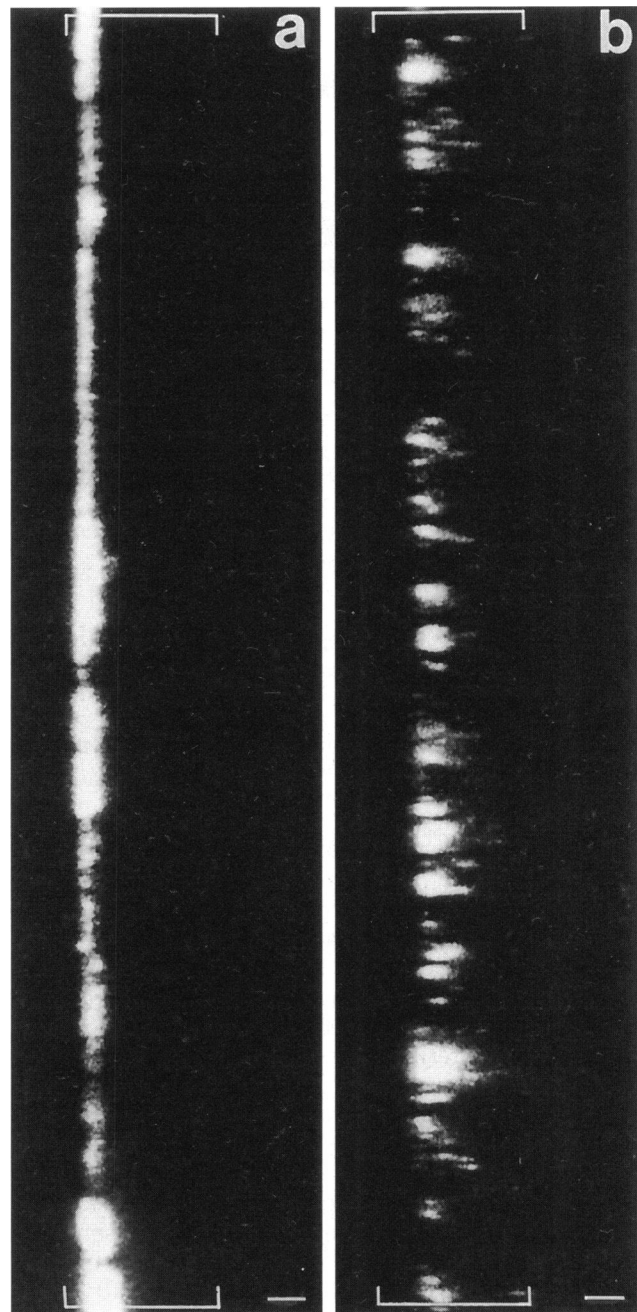


Figure 1. Internalization and distribution of p100 in Caco-2 cells shown by Z series section constructed from confocal optical sections. Polarized Caco-2 cells were incubated with anti-p100 at 4°C and then (a) fixed, permeabilized, and stained with a FITC-labeled anti-mouse antibody, or (b) warmed to 37°C for 30 min before cooling to 4°C, any remaining surface antibody was removed by stripping, fixation, and processing in the same manner. In panel a, fluorescence is confined to brush border; in panel b, brush border staining is lost and most of the p100 is within the apical cytoplasm. Brackets indicate the position of cell monolayer including brush border (on the left hand side). Bars, 5 μ m.

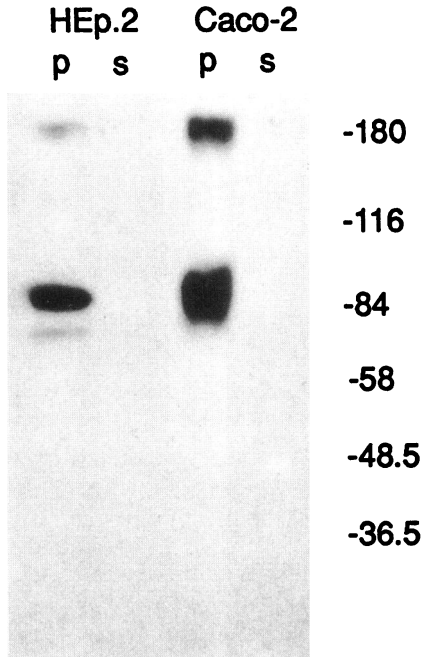


Figure 2. Immunoblotting of p100 in Caco-2 and HEp-2 cells. TX-114 lysates of solubilized Caco-2 cells and HEp-2 cells were partitioned, and both detergent pellets (p) and aqueous supernatants (s) were electrophoresed on nonreducing sodium dodecyl sulfate-PAGE followed by immuno-blot analysis. Relative molecular mass (kDa) is indicated.

$n = 3 \pm \text{SD}$) of the p100 was located on the apical cell surface.

The kinetics of internalization of p100 was determined by binding iodinated anti-p100 to cells at 4°C, warming to 37°C for up to 2 h, and then determining radioactivity present in the medium and within, as well as on the surface of the cells. As shown in Figure 3, Caco-2 cells internalize the apically bound anti-p100 at about 0.8%/min.

To ascertain if antibody binding was altering the distribution of p100, Caco-2 cells were incubated at 37°C for 4 h in the presence or absence of anti-p100. The amounts of p100 expressed at the surface of cells pre-incubated with antibody were essentially the same as those on control cells (our unpublished observations) demonstrating that the steady-state distribution of p100 is unaffected by antibody binding and suggesting that P100 internalization is probably constitutive.

The appearance of TCA soluble counts in the medium of Caco-2 cells after the uptake of anti-p100 (Figure 3) shows that less than 3% of the internalized antibody was degraded during the first 4 h of uptake. Longer incubations suggest that it takes almost 40 h for 40% of the internalized anti-p100 to be degraded by Caco-2 cells. Although this rate of degradation is faster than that of TFnR, a protein

internalizing from the basolateral border, it is significantly slower than the rate of degradation of EGFR when internalization and degradation is induced by ligand binding (our unpublished observations).

To determine whether internalized p100 recycled back to the cell surface, Caco-2 cells were loaded via the apical surface with biotinylated anti-p100 at 37°C for 4 h. They were then cooled to 4°C, and surface biotin was removed with glutathione treatment. The cells were then warmed to 37°C for intervals of up to 90 min and the biotin that returned to the surface during these intervals was quantified by binding of iodinated Extravidin. During the final 37°C incubation (Figure 4), a wave of internalized anti-p100 appears on the surface, which over the next 60–90 min then reduces. This latter reduction is probably not due to dissociation of the antibody because a wash at pH 2.5 is required to abrogate binding of the antibody to p100. This data suggests that internalized p100 is part of a mobile pool that repeatedly recycles.

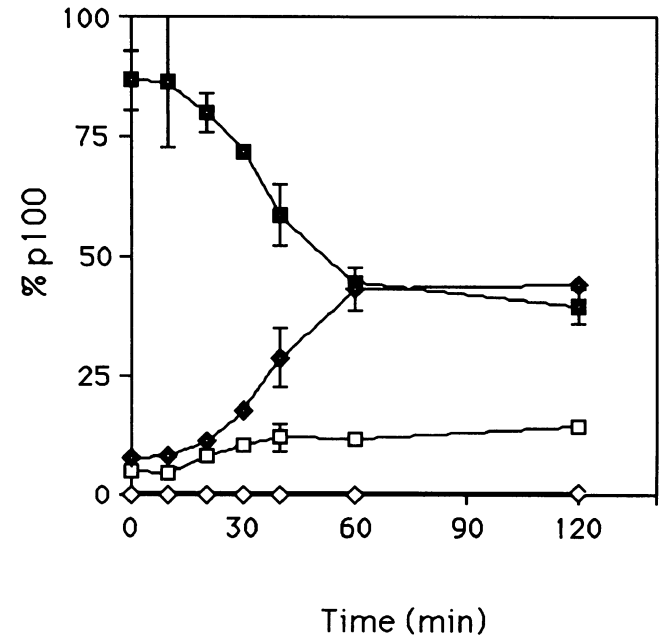


Figure 3. Internalization and processing of p100 by Caco-2 cells. Filter-grown Caco-2 cells were incubated with iodinated anti-p100 at 4°C, washed, and incubated at 37°C for various periods. At each time point, media were collected from triplicate sets of filters, which were then washed at pH 2.5 to release surface-bound counts. Acid-inaccessible counts were assumed to be intracellular. The media samples were precipitated with 10% TCA to give acid-soluble and acid-precipitable counts. Data is shown as the fraction of p100 in a particular pool at that time point (Surface, \blacksquare ; Internal, \blacklozenge ; Medium, TCA precipitable, \square ; Medium, TCA soluble, \diamond). $n=3\pm\text{SD}$.

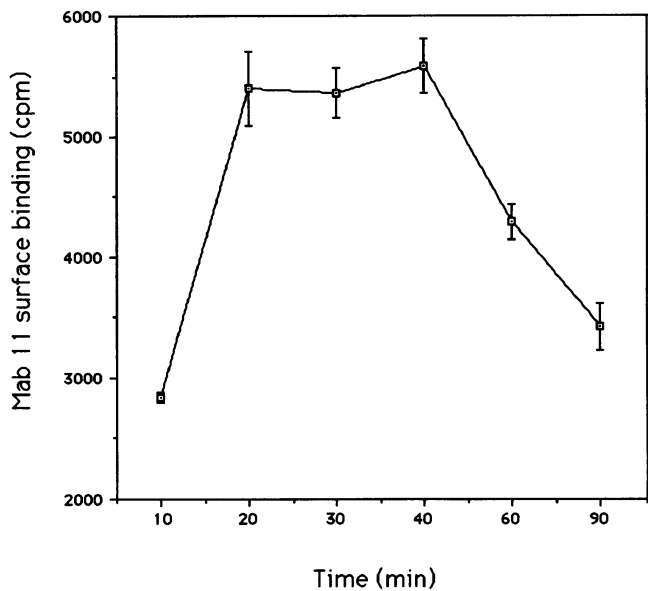


Figure 4. p100 recycles to the surface in Caco-2 cells. Caco-2 cells were incubated with SS-biotin-labeled anti-p100 in the apical chamber for 4 h at 37°C, cooled, and washed at 4°C. Surface accessible biotin label was then removed by reduction with glutathione. The cells were then warmed to 37°C, and after various time intervals, cooled and the presence of surface biotin assayed by the binding of iodinated Extravidin. $n = 3 \pm \text{SD}$.

The Apical Endocytic Pathway Taken by p100 Outlined by Electron Microscopy

To follow the route taken by internalized anti-p100, the antibody was coupled to HRP. This conjugate was allowed to bind to the apical surface at 4°C, the monolayer was rinsed, and then warmed to 37°C and incubated for up to 1 h. On warming, (Figures 5–8), the tracer internalized via coated pits at the base of the apical microvilli and over the next 60 min became distributed throughout endosomal elements within the apical cytoplasm. The HRP tracer did not penetrate the intercellular space between cells (Figure 8a) and was not seen, at any time, on the basolateral surface. Sections perpendicular to the apical surface showed a well defined terminal web of microfilaments separating the labeled endosomal elements from the plasma membrane (Figure 5, a and b).

The apical elements labeled by anti-p100 HRP included flattened saccules in addition to the 50-nm-diameter tubules and 0.2- μm - to 0.5- μm -diameter vacuoles that are characteristic of endosomes in many systems. These novel saccules were surrounded by a membrane that had the thickness and appearance typical of the plasma membrane (Figure 8b). These saccules often contained apically applied tracer which, unlike the evenly distributed reaction product seen on the apical surface, was concentrated in patches and

usually within their bulb-like ends (Figures 7a and 8b). The relationship of these saccules to the plasma membrane and to the other endosomal elements in the apical cytoplasm is unclear.

Thick (1 μM) sections (Figure 7a) best demonstrate the distribution of HRP reaction product within the elaborate network of 50-nm-diameter tubules. In thin sections (Figure 8, b and c) these tubules are easily discriminated from the flattened saccules because they have a consistent diameter, a thinner limiting membrane, they branch frequently, and their lumina are filled throughout with HRP tracer (Figure 8, b and c). Fifty-nanometer-diameter tubules are often seen in direct continuity with larger, vacuolar structures that also contain anti-p100/HRP reaction product (Figure 5a). Most of these vacuolar structures are typical multivesicular bodies (MVB) although they often contain only a few internal vesicles (Figure 5b).

The Relationship of the Apical Endosome to Basolateral Endocytic Pathways

To determine which of the endosomal elements labeled with internalized anti-p100 HRP tracer were accessible to tracers recycling from the basolateral surface, cells were loaded basolaterally with TfnR antibody ($B_{3/25}$) conjugated to colloidal gold. The ability of the $B_{3/25}$ gold conjugates to outline recycling pathways is discussed in detail below. The conjugate had essentially the same distribution as the TfnR/HRP tracer described previously (Hughson and Hopkins, 1990), being distributed in 50-nm-diameter tubules and MVB throughout both the basolateral (Figure 8d), and apical parts of the cell (see below). Anti-p100 HRP was introduced from the apical surface by binding at 4°C followed by rinses to remove unbound probe and then warming to 37°C. Anti-TfnR-gold complexes were present in the basolateral medium throughout the experiment. After these incubations, both tracers were co-localized within MVB throughout the apical cytoplasm (Figures 5, 6a, and 7a).

Although the two tracers (anti-TfnR-Gold and anti-p100 HRP) colocalize within the MVB, their distribution within the 50-nm-diameter tubules in the apical cytoplasm is strikingly different. Most tubules contain only p100 tracer (Figure 6a), although significant numbers are seen to contain anti-TfnR-gold. However, the 50-nm-diameter tubules in the apical cytoplasm that do contain significant amounts of basolateral gold tracer (Figure 7a), do not contain apically applied p100 tracer.

Within the basolateral cytoplasm, apical p100 tracer does not penetrate the MVB, and is only rarely found in 50-nm tubules/vesicles (see Figure 8d). Comparative quantitation of the distributions of the various tracers in these apical and basolateral endosomal tubules was attempted but we were unable to obtain

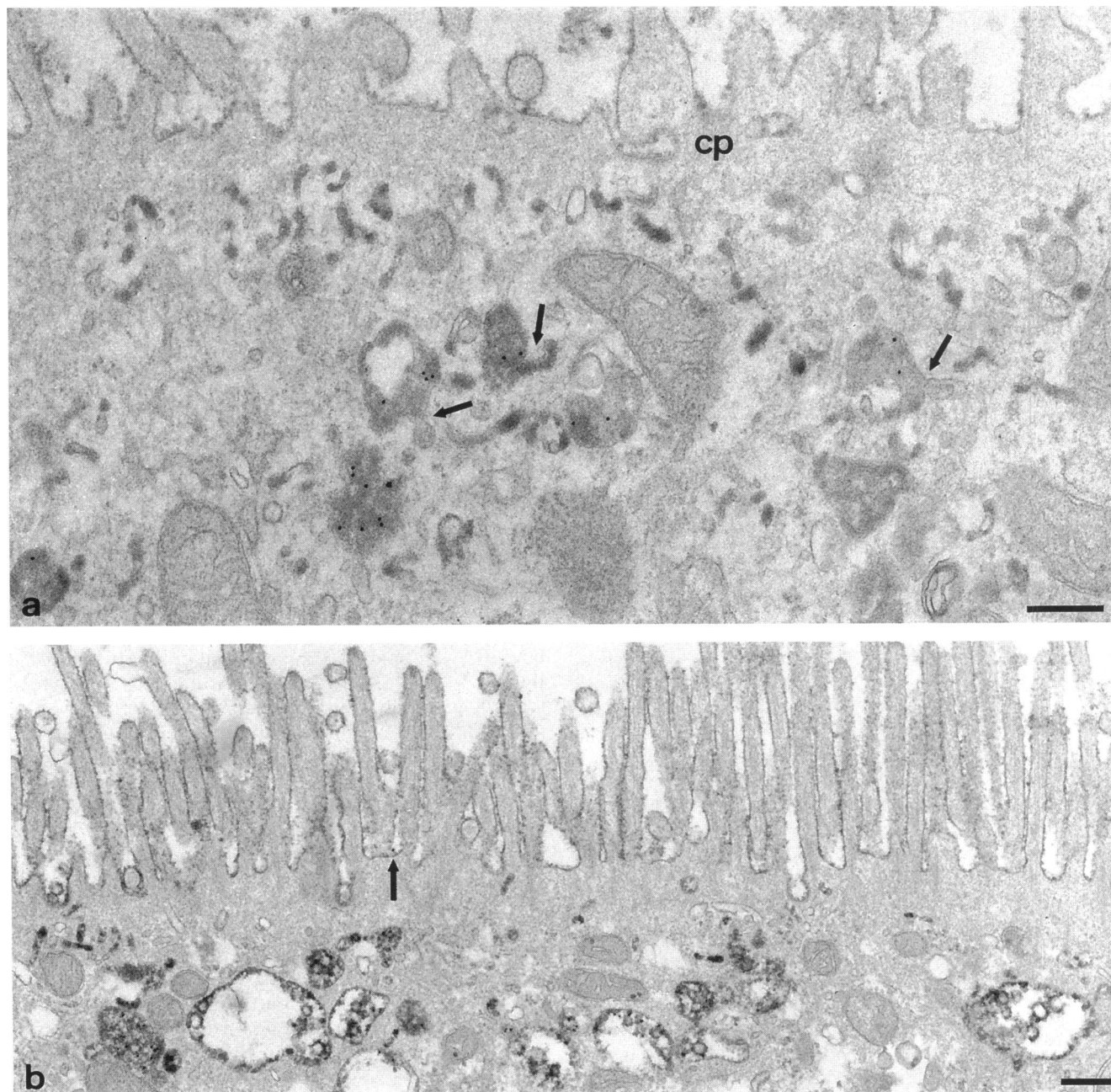


Figure 5. Distribution of p100 and TFnR in Caco-2 cells. (a) Anti-p100/HRP was introduced apically, and anti-TFnR/gold was introduced basolaterally. Section shows the apical cytoplasm of cells incubated with $B_{3/25}$ -gold in the basal medium for 45 min, cooled, anti-p100/HRP applied to the apical surface for 30 min, rinsed, and then warmed for 15 min. HRP reaction product is seen throughout the apical endocytic pathway from coated pits (cp) on the plasma membrane through 50-nm tubules and within small (~250 nm diameter) vacuoles containing basolateral tracer. Arrows indicate continuities between tubules and vacuoles. Bar, 0.2 μ m. (b) Anti-p100/HRP introduced apically, and anti-TFnR/gold was introduced basolaterally. Section shows the apical cytoplasm of cells incubated with $B_{3/25}$ -gold in basolateral medium for 30 min at 37°C, cooled, anti-p100/HRP applied to the apical surface for 30 min at 4°C, rinsed, and then warmed to 37°C for 30 min. HRP reaction product identifies the apical endocytic pathway from coated pits to MVB. With these tracers, MVB contain relatively few internal vesicles. Arrow indicates a single gold particle that has reached the apical surface. Bar, 0.2 μ m.

reproducible data because 50-nm tubules can only be identified with certainty when they are loaded with HRP reaction product.

To determine the relationship between the apical endocytic elements described above and the route from the basolateral surface to the lysosome, EGF

and α -2 M HRP were used as tracers. Thus EGF/HRP or α -2 M/HRP were introduced basolaterally while anti-p100/gold complexes were applied to the apical surface. Figures 6b and 7b show the results obtained with EGF/HRP and anti-p100/gold where the EGF/HRP conjugate was applied basolaterally for 45 min at 37°C. Anti-p100/colloidal gold was present in the apical medium throughout this incubation. Three kinds of labeling are seen in apical MVB; some contain only anti-p100 tracer, some only EGF/HRP, and some both tracers. Frequently, double-labeled MVB have an "hourglass" profile in which it appears the contents of two MVBs have become surrounded by a single perimeter membrane (Figures 6b and 7b).

Although the distribution of both basolaterally applied lysosomal tracers (EGF and α -2 M HRP) in apical MVB is essentially the same, EGF/HRP induces a distinctive change in the form of these structures. As shown in Figures 6b and 7b, MVB in the apical cytoplasm of EGF-treated cells are usually packed with internal vesicles and contain relatively little electron-lucent space (compare TfnR-loaded MVB in Figure 5b with EGF-loaded MVB in Figures 6b and 7b). After prolonged incubations (4 h plus) EGF/HRP and anti-p100/colloidal gold can be found together in dense, lysosome-like structures containing myelin figures and lipid droplets (our unpublished observations).

In preparations in which MVB in the apical cytoplasm were double labeled with anti-p100/gold and EGF/HRP, it was apparent that the 50-nm-diameter tubules in the apical cytoplasm contained only anti-p100 tracer (Figure 7b). This is despite the use of HRP tracers arriving from the basolateral borders, which being enzymic, are very sensitive probes capable of outlining the entire compartment occupied by the EGF. This failure to enter the tubules is in contrast to the distribution of the anti-TfnR-gold (Figure 7a) that is found in both MVB and tubules.

DISCUSSION

p100 Antibody Identifies an Apical Membrane Protein Which Internalizes and Recycles

By raising a monoclonal antibody against the Caco-2 apical cell surface, we have obtained a specific probe for a protein of approximately 100 kDa (p100), which internalizes. Its behavior in the detergent TX-114 suggests it is an integral membrane protein of the apical membrane.

Kinetic experiments using iodinated antibody showed that the p100 internalizes from the apical surface with a $t_{1/2}$ of approximately 40 min. This uptake appears to be constitutive and at steady state about 60% of the p100 is located within vesicles in the apical

cytoplasm. Experiments using biotinylated anti-P100 show that the protein can recycle to the apical surface rapidly ($t_{1/2}$ 5 min) and that its rate of degradation over the time scale used in most of our morphological experiments (< 2 h) is low (< 3%).

The vacuoles and tubules that contain p100 unequivocally outline the endocytic pathway from the apical border. However, although we know that p100 can recycle effectively, its distribution cannot be used to define the recycling compartment because we do not know what fraction may be en-route to the lysosome. Also, because p100 may be a lysosomal membrane protein and resistant to lysosomal hydrolases, we cannot use its long $t_{1/2}$ to argue that it is not delivered to lysosomes.

Lysosomal hydrolases are widely distributed along the apical endocytic pathway in Caco-2 cells. One lysosomal hydrolase in particular (α glucosidase) has been shown to be distributed on the apical boundary in Caco-2 cells (Klumperman *et al.*, 1991a) and its 110 kDa precursor is known to be transported to the surface as an integral membrane protein (Klumperman *et al.*, 1991b). However, p100 is not α glucosidase because considerably more p100 is found on the apical plasma membrane in double-label indirect immunofluorescence experiments (data not shown). There is also evidence to suggest that the mannose 6-phosphate receptor (M6PR) is apically distributed in these cells (Klumperman *et al.*, 1992). The MVB of Caco-2 cells have also been shown to contain significant amounts of the lysosomal hydrolase cathepsin C although this enzyme was not detected on the apical surface (Klumperman *et al.*, 1991a).

Some brush border hydrolases are also known to internalize constitutively and to recycle, and the rates of internalization and the extent of recycling and degradation described, for example, for the brush border peptidase DPPIV (Matter *et al.*, 1989, 1990a,b) are very similar to those we have described for p100. Previous work (Matter *et al.*, 1989) also showed that some DPPIV is delivered to lysosomes although, like p100, its main intracellular location was within apical endosomes from which it is able to recycle back to the apical surface.

It seems, therefore, that the apical endosomal elements in Caco-2 cells contain, at steady state, integral membrane proteins derived from the apical plasma membrane, and that a significant fraction of them can recycle back to the apical surface. Some of these membrane proteins may be lysosomal enzymes whereas others are probably brush border hydrolases. p100 is an integral membrane protein of the apical surface of unknown function, which displays a similar itinerary.

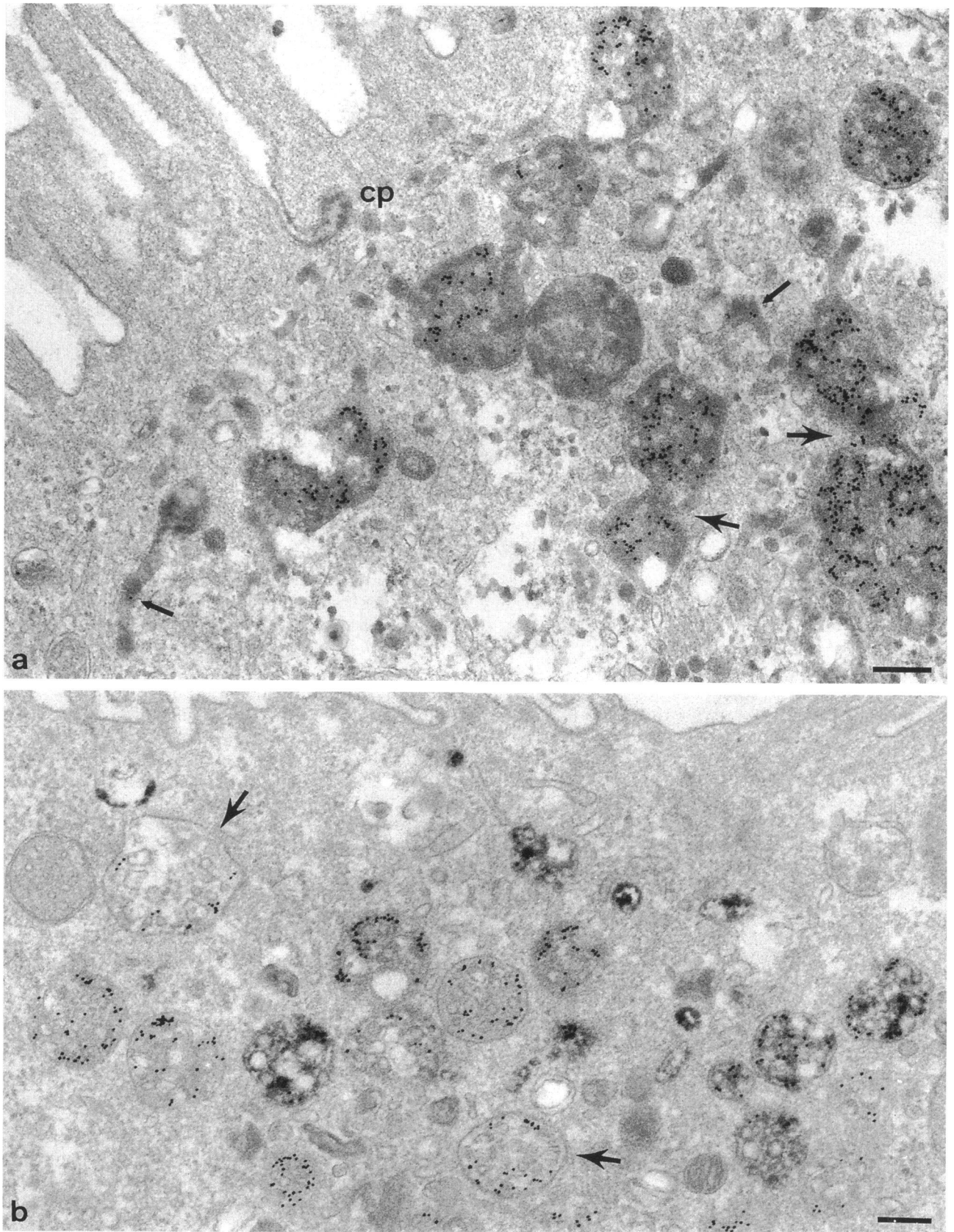


Figure 6.

The Apical Endosome Is an Important Crossroads for Membrane Traffic in Caco-2 Cells

In an earlier study (Hughson and Hopkins, 1990) where TFnR were shown to recycle back to the basolateral border with better than 95% efficiency, TFn-HRP was used to identify the compartments from which TFnR could recycle, and tracer was distributed throughout MVB in the apical cytoplasm. In the present study the TFnR of the MVB in the apical cytoplasm were labeled with B3/25 gold. We have also examined whether internalized B3/25 gold complexes have the same distribution within apical MVB as the distribution shown for TFnR in labeled cryosections. Our results (unpublished), indicate a similar distribution of receptors as analyzed by these two very different labeling approaches. Because TfnR in these cells have a prolonged $t_{1/2}$ and because TfnR are known to be very sensitive to proteolysis (Trowbridge and Omary, 1981) none of the compartments that contain recycling TfnR are likely to have significant proteolytic activity. The apically distributed TfnR is therefore either an insignificant fraction of the total traffic of this protein, or these apical endosomes represent an early, recycling stage of the endocytic pathway.

We have not directly measured the fraction of internalized TfnR that passes through the apical endosomes in this study. However, the kinetics of recycling shown in our earlier study (Hughson and Hopkins, 1990) suggests that as much as 50% of the receptor may recycle from this region after loading of the cell to steady state. Moreover, we estimate from labeled cryosections that about one-half of the gold particles coupled to anti-TfnR are over apical MVBs (our unpublished observations). These steady-state measurements suggest that a minimum of 50% of internalized TfnR must pass through the apical endosomes. It is of interest to note that Apodaca *et al.* (1994) have also estimated the fraction of TfnR in apical endosomes in MDCK cells using a biochemical assay. They show that approximately 50% of the receptor can be cross-linked by an apically internalized reagent. Together these data clearly show that the double-labeled apical MVBs represent an endosomal compartment from which recycling occurs.

The double label experiments in which anti-p100 is used to label apical MVB (discussed further below) also show that these p100-positive MVB receive EGFR and α -2 M as well as TFnR from the basolateral border. In other systems EGFR and α -2 M have been shown to label the endocytotic route to the lysosome (Trowbridge *et al.*, 1993) and it is clear, therefore, that the apical MVB containing TFnR are also on the endocytic pathway leading from the basolateral border to the lysosome.

In recent years there has been considerable debate concerning the compartments that make up the endocytic pathway (Griffiths and Gruenberg, 1991; Stoorvogel *et al.*, 1991; Dunn and Maxfield, 1992). There is, however, general agreement that one sub-compartment of the pathway can be clearly delineated by its content of recycling proteins, most typically TfnR (Mayor *et al.*, 1993; Trowbridge *et al.*, 1993). It has been suggested that ligands destined for the lysosome are transferred from this recycling endosomal compartment to an intermediate compartment (the prelysosome or "late endosome," which is rich in M6PR) (Griffiths *et al.*, 1988) and then to lysosomes that contain active hydrolases but lack M6PR (Kornfeld and Mellman, 1989).

Earlier studies on MDCK cells, which followed fluid phase tracers endocytosed either via the basolateral or the apical surface, concluded that apical and basolateral endosome compartments were not directly connected and that the two endocytotic pathways met only in the M6PR-rich pre-lysosome (Parton *et al.*, 1989). This conclusion was consistent with data from *in vitro* fusion studies, which showed that although apical and basolateral endosomes loaded with fluid phase tracers can fuse with themselves, they do not fuse with each other (Gruenberg and Howell, 1989). This picture of membrane traffic suggested that transcytosis requires a separate route, allowing transfer to apical cytoplasm while avoiding the prelysosome. Barroso and Sztul (1994) provided evidence for such a route, showing that the plgAR was sorted away from the TFnR before reaching an apical endosome that contained recycling plgAR and was accessible to apically internalized ricin. Subsequently, Apodaca *et al.* (1994) outlined an itinerary for basolaterally internalized

Figure 6 cont. Distribution of p100, TFnR, and EGF in Caco-2 cells. (a) Anti-p100/HRP was introduced apically, and anti-TFnR/gold was introduced basolaterally. Section shows the apical cytoplasm of cells with anti-p100/HRP applied apically for 30 min at 5°C, rinsed, and then warmed to 37°C. Anti-TFnR/gold was applied basolaterally during anti-p100 binding and for subsequent 45 min at 37°C. HRP reaction product outlines the apical endocytic pathway from the coated pit (cp) on the apical membrane, through the 50-nm-diameter tubules to the MVB. Small arrows indicate gold particles in tubules (highlighting the occasional double labeling), large arrows indicate glass profiles and tubular connections connecting MVB. (b) Anti-p100/gold was introduced apically, and EGF/HRP was introduced basolaterally. Section shows the apical cytoplasm of cells incubated with anti-p100-gold bound to the apical surface and EGF/HRP in the basal chamber for 45 min. Some MVB contain both tracers, some only gold tracer. MVB containing only gold tracer are usually larger and contain relatively few internal vesicles (large arrows). Bar, 0.2 μ m.

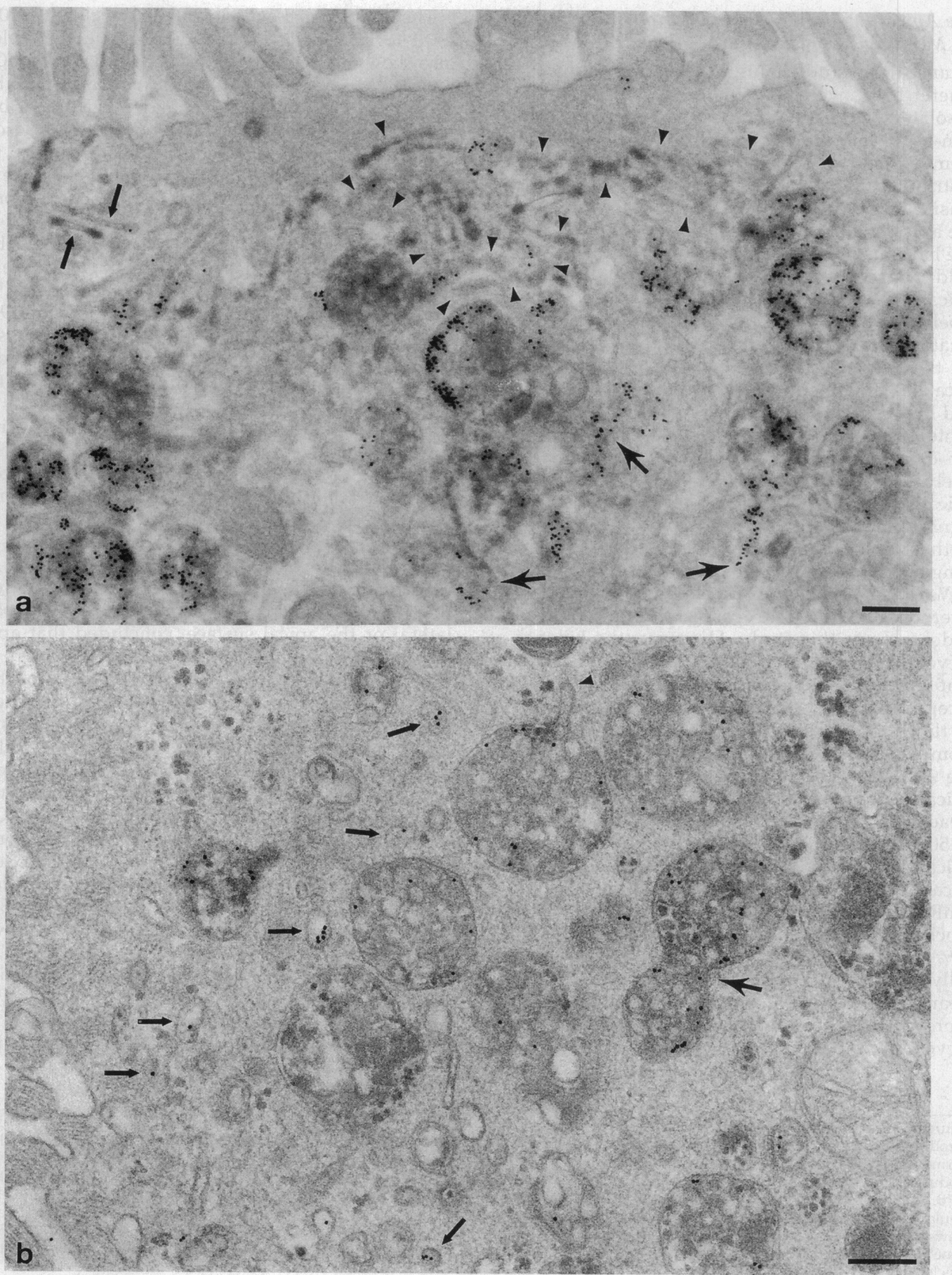


Figure 7.

proteins in MDCK cells that is similar to that which we find in Caco-2 cells. They show that both the pIgAR and the TfnR colocalize in an apical endosome. Our results here demonstrate that the apical compartment in Caco-2 cells reached by the TfnR also contains p100, thus showing that the common apical endosome contains apically recycling proteins other than those involved in transcytosis. Further work is required to establish how the apical vacuoles filled with IgA in MDCK relate to the common apical endosome described in the present study.

The Delivery of Tracers to Apical MVB

Because they can be labeled from the apical surface with anti-p100 tracers, the MVB in the apical cytoplasm are identifiable as a distinct subset of the various endosomal elements located in this area of the cell. It is clear that the form of these MVB is influenced by the kind of receptor they receive from the basolateral border. Thus recycling receptors such as TfnR and α -2 M receptors generate relatively few internal vesicles in these MVB compared with EGFR. This is in keeping with suggestions that vesiculation into MVB provides a means of removing membrane proteins like EGFR to the lumen of the endocytic pathway and that the number of internal vesicles increases with time (Haigler *et al.*, 1979; Futter *et al.*, 1993; van Deurs *et al.*, 1993). The presence of MVB in the apical cytoplasm packed with internal vesicles, and labeled with basolateral tracer yet unlabeled with apically derived anti-p100 clearly suggests that these are MVB that have formed in the basolateral cytoplasm and moved apically.

It is not clear if MVB containing basolaterally derived tracers can acquire apical tracers from p100-containing 50 nm tubules but the double-labeled "hourglass" profiles of MVB that are commonly observed in the apical cytoplasm suggest that these double-labeled elements can also arise by apically derived and basolaterally derived MVB fusing directly with each other. Our difficulty in finding double-labeled 50-nm-diameter tubules suggests that direct fusion

may be the primary means whereby double-labeled MVB are formed.

Electron microscopy of epithelioid A431 cells suggests that MVB arise in the peripheral cytoplasm, move centripetally, and concentrate in the pericentriolar area (Hopkins and Trowbridge, 1983). Our studies on living cells also show that gold-loaded MVB can be translocated through the cell by the microtubular cytoskeleton (de Brabander *et al.*, 1988; Hopkins *et al.*, 1990) and in both Caco-2 and MDCK cells there is good reason to believe that movement from the basolateral endosome to the apical surface also requires intact microtubules (Matter *et al.*, 1990b; Hunziker *et al.*, 1991; Barroso and Sztul, 1994). It is likely, therefore, that the movement of the basolateral MVB into the apical cytoplasm, which we suggest takes place in Caco-2 cells, will also depend upon the orientation of the microtubular cytoskeleton.

The Removal of Proteins from MVB: the Role of 50-nm Tubules in Routing to Apical and Basolateral Surfaces

The role of the 50-nm tubules seen in the apical cytoplasm connected to MVB is unclear; however, our previous observations strongly suggest that they may play a major role in recycling of internalized ligand/receptor complexes to the cell surface (Hopkins *et al.*, 1994). Our observations of the ability of recycling (TfnR and p100) tracers to penetrate the tubules, in contrast to the failure of EGF and α -2 M-HRP to appear in the 50-nm tubules in the apical cytoplasm, supports this contention. The virtual absence of 50-nm-diameter tubules containing both apical and basolateral tracer even though connected to MVB containing both tracers, suggests that some kind of mechanism regulates entry of tracer into these tubules.

The distribution of trafficking proteins that we have observed in 50-nm-diameter tubules in Caco-2 cells is more restricted than that in the 50-nm-diameter tubules of HEP-2 cells. There, both recycling tracers (TfnR) and tracers destined for the lysosome (α -2 M) are found to be co-localized in many of the 50-nm

Figure 7 cont. Distribution of p100, TfnR, and EGF in Caco-2 cells. (a) Anti-p100/HRP was introduced apically, and anti-TfnR/gold was introduced basolaterally. The figure shows a thick (1 μ m) section of cells treated with anti-p100/HRP bound to the apical surface and then incubated for 45 min with anti-TfnR/gold in the basal medium. The thick section demonstrates the complexity of the network of 50-nm-diameter tubules located below the terminal web and loaded with HRP tracer (arrowheads). Also in the apical cytoplasm are some flattened saccules containing HRP (small arrows). All of the MVB contain HRP and most contain gold. Linear arrays of gold particles extend from some MVB (large arrows); these identify 50-nm tubules containing the basolateral tracer. Within one of the flattened saccules immediately adjacent to the plasma membrane there is a single gold particle and adjacent to the apical membrane there is a group of four particles, indicating the efficient penetration of gold tracer from the basolateral border in this preparation. (b) Anti-p100/gold was introduced apically, and EGF/HRP was introduced basolaterally. The figure shows a conventional thin section of cells with EGF/HRP and anti-p100/gold applied for 45 min. The small arrows indicate tubules and vesicles containing gold but lacking HRP. The MVB are packed with internal vesicles and contain both HRP and gold tracers. The arrowhead indicates a 50-nm tubule connected to MVB lacking both tracers. Large arrow indicates hourglass profile suggesting MVBs fuse. The apical plasma membrane lies at the left hand margin of this micrograph. Bar, 0.2 μ m.

tubules connected to double-labeled MVB (Hopkins *et al.*, 1994). The greater separation observed in the 50-nm-diameter tubule system of Caco-2 cells may reflect the greater efficiency required in a more highly polarized organization.

In fibroblasts, evidence suggests that the TfnR in the pericentriolar recycling compartment comprising small tubules are returned preferentially to a special domain of the cell surface; the leading lamella (Hopkins *et al.*, 1994). It is conceivable, there-

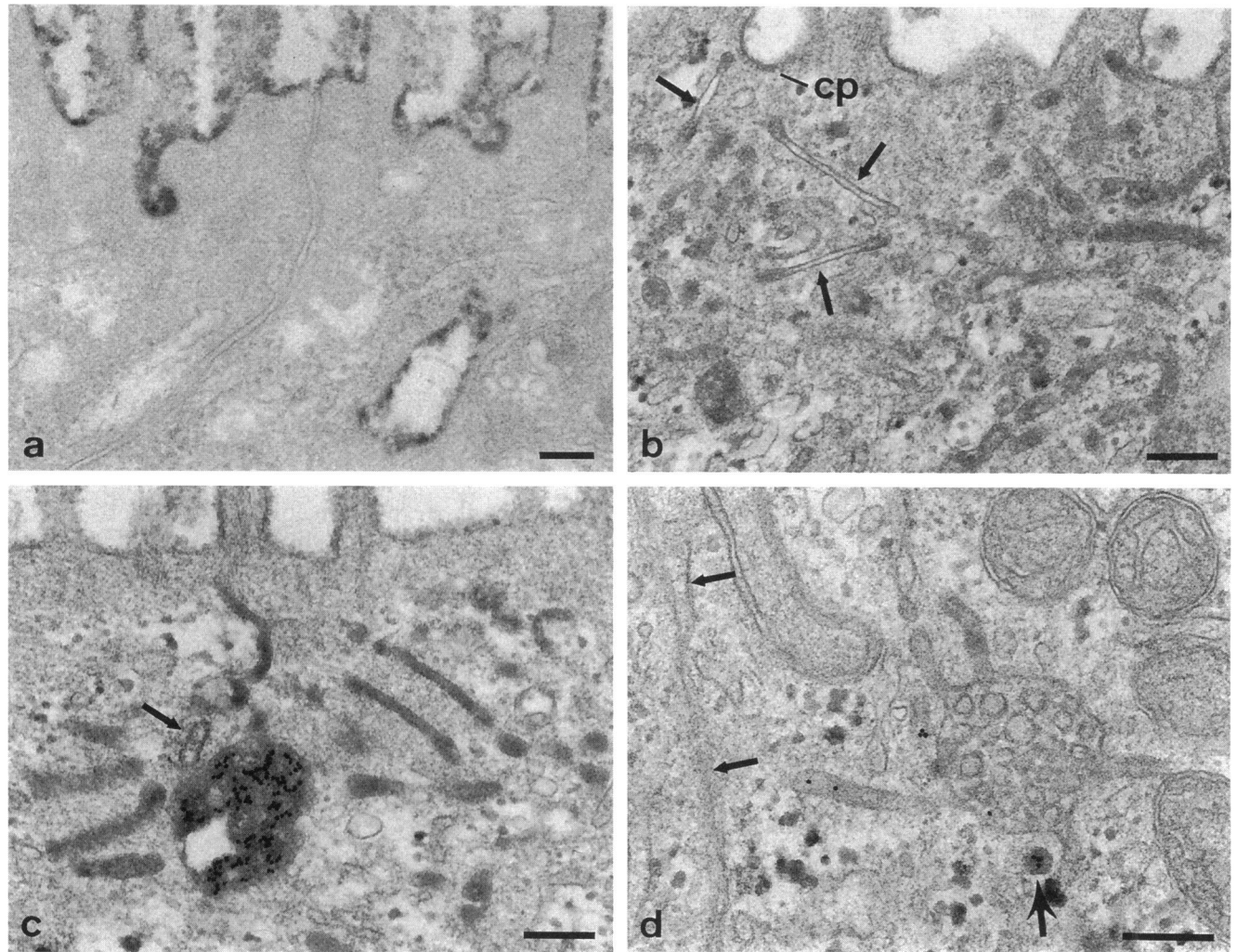


Figure 8. Distribution of p100 and TFnR in apical and basolateral regions of Caco-2 cells. (a) Anti-p100/HRP was introduced apically. The section shows the junction between two cells showing the distribution of anti-p100/HRP restricted to the apical plasma membrane. Cells were incubated with anti-p100/HRP for 60 min at 37°C. (b) Anti-p100/HRP was introduced apically. The figure shows endosomal elements adjacent to the apical membrane, which include flattened saccules (arrowheads) displaying membrane of similar thickness to the apical plasma membrane and containing HRP reaction product in slightly expanded ends. Also present are HRP-loaded 50-nm-diameter tubules: their limiting membranes are thinner and, because they are more sinuous and frequently branch, these tubules are less clearly displayed. Typically, reaction product fills their lumina. The cells were incubated with anti-p100/HRP at 4°C, rinsed, and chased for 15 min at 37°C. cp; coated pit. (c) Anti-p100/HRP was introduced apically, and anti-TFnR/gold was introduced basolaterally. The section shows 50-nm-diameter tubules containing anti-p100 tracer surrounding a vacuolar endosome labeled with HRP tracer and anti-TFnR/gold. A single profile of a saccule (arrow) serves to contrast the difference between the membranes of saccules and tubules. The cells were incubated with anti-TFnR/gold for 45 min, cooled, and then incubated with anti-p100/HRP at 4°C for 30 min, rinsed, and chased for 15 min at 37°C. (d) Anti-p100 was introduced apically, and anti-TFnR/gold was introduced basolaterally. The section shows endosomal elements adjacent to the basolateral border (small arrows) containing anti-TFnR/gold tracer. The gold tracer is present in the MVB and the 50-nm-diameter tubules with which it is connected. The large arrow indicates a vesicle/tubule containing both anti-p100/HRP and anti-TFnR/gold. Anti-p100/HRP was bound to the apical surface at 4°C, and then cells were warmed and incubated with anti-TFnR/gold in the basolateral medium for 45 min at 37°C. All bars, 0.2 μ m.

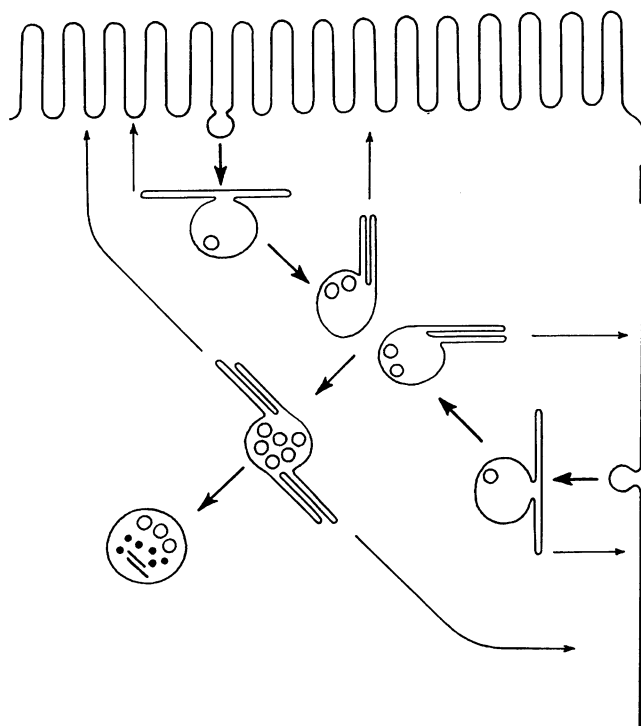


Figure 9. A proposal outlining endocytic membrane traffic in Caco-2 cells. Proteins entering Caco-2 cells from either surface first arrive in an endosome comprising vacuoles with connected tubules, from which recycling back to the plasma membrane occurs. With time, basolaterally derived endosomes move to the apical cytoplasm where they fuse with apically derived MVBs. During and after these stages, recycling of membrane to the appropriate surfaces, probably via the tubules, continues. After fusion, the MVB containing material internalized from both surfaces continues to accumulate internal vesicles and to recycle membrane to both surfaces. Eventually recycling and inward vesiculation is completed, and the maturing organelle will eventually become part of a lysosome.

fore, that there are subsets of 50-nm-diameter tubules in the recycling endosome compartment of most cell types, which can selectively target the membrane proteins fluxing through them. It now becomes of special interest to identify the mechanisms that determine entry of trafficking membrane proteins into these tubules and the factors that determine the orientation of these tubules with regard to cell surface domains.

Conclusions

On the basis of the observations we have made in Caco-2 cells, we suggest that the endocytic pathway that enters the cell from the basolateral border consists of an endosome compartment that includes MVB where sorting takes place and 50-nm-diameter tubules that recycle proteins back to the cell surface. Our observations further suggest that this endosome compartment is able to fuse with the MVB of

the apical endocytic pathway, which are able to recycle proteins back to the apical surface. The common endosome formed by the apical and basolateral MVB fusing is located in the apical cytoplasm and is able to continue recycling proteins back to their appropriate cell surface domain. We suggest that when all of the proteins that need to be retrieved (including M6PR) have been removed, the MVB can no longer be regarded as endosomes and should then be considered lysosomes. A scheme outlining these proposals is shown in Figure 9.

The sorting mechanisms that regulate selective routing through these endosomal compartments must play a pivotal role in maintaining the polarized phenotype of the Caco-2 cell. They also present an attractive target for pharmaceutical studies intent upon achieving the transcellular delivery of hydrophilic, macromolecular drugs.

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