Endocytic Pathways in Polarized Caco-2 Cells: Identification of An Endosomal Compartment Accessible from Both Apical and Basolateral Surfaces

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Abstract. The enterocyte-like cell line Caco-2 forms a polarized epithelium when grown on filters. We have investigated the interaction of endocytic pathways from the apical and basolateral surfaces. The transferrin receptor was an appropriate marker for the basolateral route; uptake of radiolabeled transferrin was highly polarized, and recycling of this ligand back to the basolateral surface occurred with an efficiency of 95%, even after prolonged incubations with transferrin. Using a transferrin-peroxidase conjugate to delineate the morphological pathway, we have identified an early endocytic compartment in the basolateral cytoplasm of the cells. Longer incubations revealed a deeper endocytic compartment in the apical cytoplasm. Concanavalin A complexed to gold was used to simultaneously label the apical endocytic route. After 60 min, extensive mixing of the two labels was seen in endocytic elements throughout the apical cytoplasm, including in the Golgi area, but never in the basal cytoplasm. Using a second double labeling procedure in which antitransferrin receptor antibody complexed to gold was applied to the basolateral surface for up to 2 h and free peroxidase applied to the apical surface for shorter periods, we demonstrated that this apical marker rapidly (within 5 min) reached endosomes containing antibody-gold. Our results indicate that, in Caco-2 cells, the endocytic pathways from the apical and basolateral surfaces meet in an endosomal compartment from which transferrin can still be recycled.

SIMPLE epithelia consist of sheets of polarized cells. The surface membrane of each cell is divided into two domains, the apical surface facing the lumen or exterior milieu and the basolateral surface facing the serosal environment. These two domains are separated by tight junctions, which also help to seal the epithelium and thus form a relatively impermeable barrier between the two environments. Each surface has its own characteristic set of proteins and lipids (Simons and Fuller, 1985). For example, small intestinal enterocytes have a specialized apical surface, the brush border, which carries peptidases, disaccharidases and amino acid and glucose transporters (Hauri et al., 1985; Hediger et al., 1987). Conversely, Na^+K^+ATPase is confined to the basolateral surface of the Madin-Darby canine kidney (MDCK) cell line (Caplan et al., 1986).

These integral membrane proteins are all thought to be resident cell surface proteins. Another population of proteins found on the plasma membrane surface internalize into the cell by endocytosis (Helenius et al., 1983; Hopkins, 1986). Many of these have been characterized in nonpolarized systems. Some, such as the transferrin receptor (and its ligand) and the low density lipoprotein receptor, recycle back to the cell surface (Dautry-Varsat et al., 1983; Klausner et al., 1983; Brown et al., 1983), whereas others such as the epidermal growth factor (EGF)[1] receptor are routed to the lysosomes with their ligand (Stoscheck and Carpenter, 1984; Miller et al., 1986). It has been shown in some epithelial cells that certain receptors are characteristic of a particular domain. In hepatocytes, the EGF receptor is internalized only from the sinosoidal and lateral surfaces (Dunn et al., 1986). The polymeric IgA receptor follows a more complicated itinerary, for it internalizes from one domain (the basolateral) in several epithelial cell types (Underdown, 1986) and is transported to the opposite (apical) domain. There is also evidence for receptor-mediated endocytosis from the apical surface of neonatal and adult enterocytes (Abrahamson and Rodewald, 1981; Levine et al., 1984).

Little is known about the way in which the endocytic pathways of polarized epithelial cells interact. A study on exocrine acinar cells using nonspecific endocytic markers (Oliver, 1982) showed that the endocytic routes from the apical and basolateral routes joined, after prolonged incubations with both markers. The point at which the two pathways intersect was not investigated.

1. *Abbreviations used in this paper*: ATR, monoclonal antibody against the human transferrin receptor; Con A, concanavalin A; DAB, diaminobenzidine; EGF, epidermal growth factor; HRP, horse radish peroxidase; Tr-HRP, transferrin-peroxidase.

We have begun to investigate the endocytic routes in the Caco-2 cell line. These cells, which are derived from a human colon carcinoma (Pinto et al., 1983), form a tight monolayer when grown on a permeable support. They have an enterocyte-like morphology, express brush border enzymes (Hauri et al., 1985) and exhibit other characteristics of the ileum (Dix et al., 1987; Hughes et al., 1987; Faust and Albers, 1988). By growing the cells on filters, we have a highly polarized system that gives relatively free access to both surfaces. This system has far more potential for experimental manipulation than the in vivo and explant models previously employed (Blok et al., 1981; Gonella and Neutra, 1984). We show that transferrin and its receptor are a good marker for the basolateral endocytic system in Caco-2 cells and demonstrate that at some points on this pathway they are found in the same compartment as markers introduced from the apical surface.

Materials and Methods

Cell Culture

Wild-type Caco-2 cells were cultured, and plated for experiments on to Transwell filters (Costar, Cambridge, MA), as described elsewhere (Hughson et al., 1989). They were used for experiments between 12 and 19 d after plating. The transepithelial resistance, measured using a device similar to that described (Fuller et al., 1984), was as least 200 ohms-cm^2.

Transferrin-Peroxidase (Tr-HRP) Conjugation

4 mg transferrin (iron-free; Sigma Chemical Co., St. Louis, MO) was conjugated to 6 mg horse radish peroxidase (HRP) (type 2; Sigma Chemical Co.) using *N*-succinimidyl-3-(2-pyridyldithio)-propionate as previously described (Hopkins, 1983). The conjugate was purified from free HRP on a Biogel P60 column (Bio-Rad Laboratories, Richmond, CA). On SDS-PAGE, the conjugate ran as a single band of ~120 kD.

Iodinations

EGF, transferrin, and the B3/25 monoclonal antibody (ATR) against the transferrin receptor (a gift from Dr. Ian Trowbridge, Salk Institute) were iodinated by the chloramine T (Sigma Chemical Co.) method (Hunter and Greenwood, 1962) using 0.5 mCi ^{125}I (Amersham Corp., Arlington Heights, IL) and 4 μg EGF, 150 μg transferrin, or 22 μg ATR antibody. Transferrin and ATR antibody were incubated with chloromine T for 2 min, and EGF for 45 sec, at room temperature, before addition of sodium metabisulphite (BDH Chemicals Ltd., Dageham, Essex, UK) to stop the reaction. Tr-HRP was iodinated by the "Iodogen" method (Fraker and Speck, 1978). Briefly, 60 μg protein in 100 μl PBS, 25 μl 0.4 M sodium phosphate buffer and 0.5 mCi ^{125}I were reacted together with 10 μg 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Sigma Chemical Co.) for 5 min at room temperature. 200 μl potassium iodide was then added to give a final concentration of 0.25 M. The conjugates were separated from free iodide using either a Biogel P60 (transferrin, Tr-HRP, and ATR-antibody) or P10 (EGF) column.

Gold Conjugates

8 and 10 nm colloidal gold were made according to the method of Slot and Geuze (1985). ATR antibody was conjugated to gold as described (Hopkins and Trowbridge, 1983). The concanavalin A (Con A)-gold complex was made according to Benhamou and Ouellette (1986). Con A (Sigma Chemical Co.) was dialyzed against distilled water overnight. The amount of protein needed to stabilize the gold was determined by adding different aliquots of protein to the gold colloid. NaCl was added to 1% (final concentration), and the tubes were spun in a microfuge. The gold was stable when the resulting pellet could be resuspended. 20–40 μg Con A/ml were needed. The gold complex was spun for 40 min, at 25,000 rpm in a rotor (Ti60; Beckman Instruments, Inc., Palo Alto, CA) in an ultracentrifuge, (L8-55, Beckman Instruments, Inc.) and the pellet was resuspended in PBS with 0.05% Carbowax (BDH Chemicals Ltd.).

Transcytosis, Internalization, and Recycling Studies

Filters were incubated in serum-free medium (DME) plus 0.1% BSA (Sigma Chemical Co.) for 1 h before all tracer experiments. For iodinated ligand uptake studies, the filters were incubated in serum-free medium and BSA, with 1 ml in the apical part of the chamber and 1.5 ml basolaterally. Whenever possible, solutions were only put in the bottom chamber when fluid was present in the upper chamber to avoid hydrostatic pressures lifting the cells off the filter. Radiolabeled ligand (EGF, transferrin, 1×10^5 cpm/ml; Tr-HRP, 1×10^6 cpm/ml) was applied apically or basolaterally as appropriate, with or without cold ligand (transferrin, 0.5 mg/ml; EGF, 50 ng/ml), and the cells were incubated at 37°C, 5% CO_2, for the desired period. The cells were then placed on ice, washed several times in cold PBS containing 0.1 mM calcium and 0.05 mM magnesium, followed by two acid washes (0.15 M NaCl, 0.5 M acetic acid, pH 2.7), and then the filters were cut out of their holder with a scalpel and counted in gamma counter. As a control for nonspecific adsorption of radiolabeled ligand to the filter, filters without cells were incubated overnight with medium containing serum, and then radiolabeled ligand was applied as in a standard experiment. The number of counts which bound to the empty filter was negligible.

The passage of tracers across the monolayer was followed using radiolabeled ligand (transferrin, 5×10^5 cpm/ml; EGF, 2×10^5 cpm/ml) applied either apically or basolaterally. The filters were incubated at 37°C for up to 4 h. Counts appearing in the opposite chamber as a result of crossing the monolayer were expressed as a percentage of the counts originally applied.

Recycling experiments were performed as follows: filters were set up as described above, but with ~2×10^6 cpm/ml [^{125}I]transferrin ([^{125}I]Tr-HRP, 4×10^6 cpm/ml; [^{125}I]ATR antibody, 5×10^5 cpm) in the basal chamber. After incubation at 37°C for the given length of time, the filters were placed on ice and washed carefully but extensively with cold PBS (containing calcium and magnesium) until the washings showed no counts above background. This was to remove all nonspecific ligand bound to the cells and filter. The filters were then either cut out and counted, or given fresh, prewarmed medium (in experiments with [^{125}I]transferrin and [^{125}I]Tr-HRP, the medium contained excess cold transferrin to prevent reinternalization of released radiolabeled transferrin) and incubated at 37°C. The medium was replaced halfway through the longer chases, apical, and basolateral media always being collected separately. After the "chase," the cells were washed until the counts were negligible and the filters, media, and washings counted.

To analyze medium for the presence of degraded ligand, samples were passed over a Biogel P6 (EGF), P60 (transferrin), or P100 (ATR antibody) column. The profile of counts appearing in the fractions was compared to that of control iodinated ligand, and corrections for free iodide made accordingly, before estimating the amount of degraded ligand.

Fluorescence Microscopy Studies

Indirect immunofluorescence was performed as described (Hughson et al., 1989) on filter-grown Caco-2 cells permeabilized with 0.05% saponin (Sigma Chemical Co.). The ATR antibody was used as the first antibody (1:1,000), and a sheep anti-mouse IgG antibody conjugated to FITC (Seralab) was used in the second step. Photographs were taken on a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY; or Polyvar microscope, Reichert Scientific Instruments, Buffalo, NY).

To make FITC-transferrin, 0.5 mg FITC (Sigma Chemical Co.), dissolved in 100 μl ethanol, was added to 5 mg transferrin and 23 mg $NaHCO_3$. This was made up to 1 ml with water and then dialyzed overnight against PBS. The conjugate was applied (1:10) to the filters apically or basolaterally in DME plus 0.1% BSA, and the cells were incubated at 37°C, in 5% CO_2, for 60 min. The cells were fixed in 3% paraformaldehyde in PBS, quenched in 50 mM ammonium chloride and examined on a fluorescence microscope (Carl Zeiss, Inc.) or on a laser scanning confocal apparatus (500; Lasersharp MRC, Bio-Rad Microscience Ltd., Hemel Hempstead, UK) in conjunction with an Optophot microscope (Nikon Inc., Garden City, NY).

Semi-thin Frozen Sections

Filter-grown Caco-2 cells were fixed with 3% paraformaldehyde in PBS for 30 min. The filter was excised and pieces were briefly immersed in 10% gelatin at 37°C, and then placed on ice for 10 min. They were then fixed for a further 30 min in paraformaldehyde. Small triangles were cut out of the gelatin "sandwich," infiltrated with 2.3 M sucrose, mounted on copper stubs, and frozen in liquid nitrogen. 0.5 μm frozen sections were cut on a recryo-ultramicrotome (MT2-B; Sorvall Instruments, Du Pont Company,

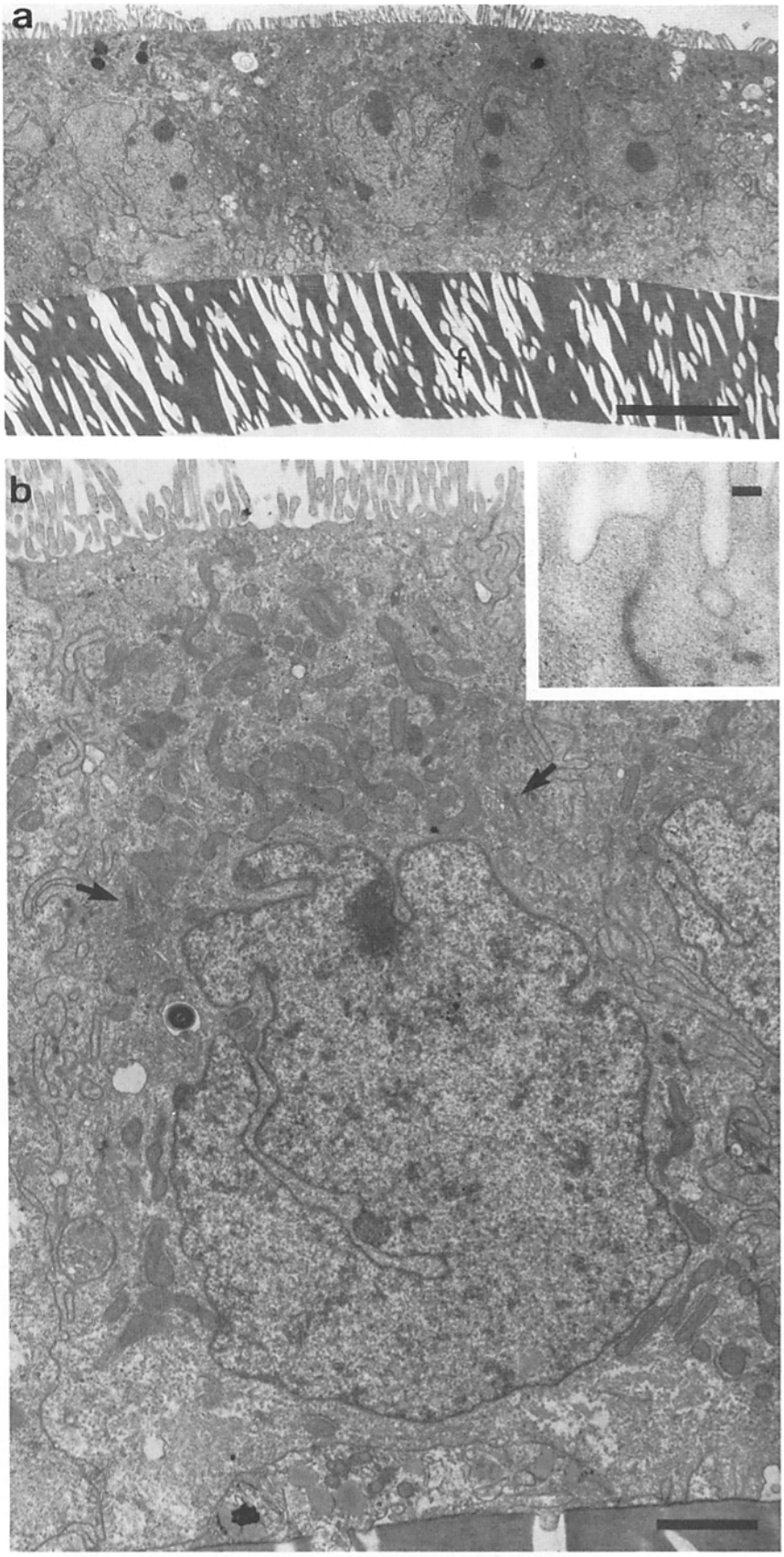

Figure 1. Electron micrographs of a monolayer of Caco-2 cells. Cells were seeded onto Transwell filters and grown for 14 d. After fixing with Karnovsky fluid, cells were postfixed in 1% reduced osmium tetroxide and processed for electron microscopy. Sections were stained with uranyl acetate and lead citrate. (*a*) Low power magnification of the monolayer. (*b*) Higher magnification of a Caco-2 cell, showing position of the Golgi apparatus (*arrows*). Inset shows a tight junctional complex from a monolayer that was incubated with HRP basolaterally before fixation. DAB reaction product is seen in the lateral space up to the tight junction. *f*, filter. Bars, (*a*) 10 μm and (*b*) 2 μm; *inset*, 0.1 μm.

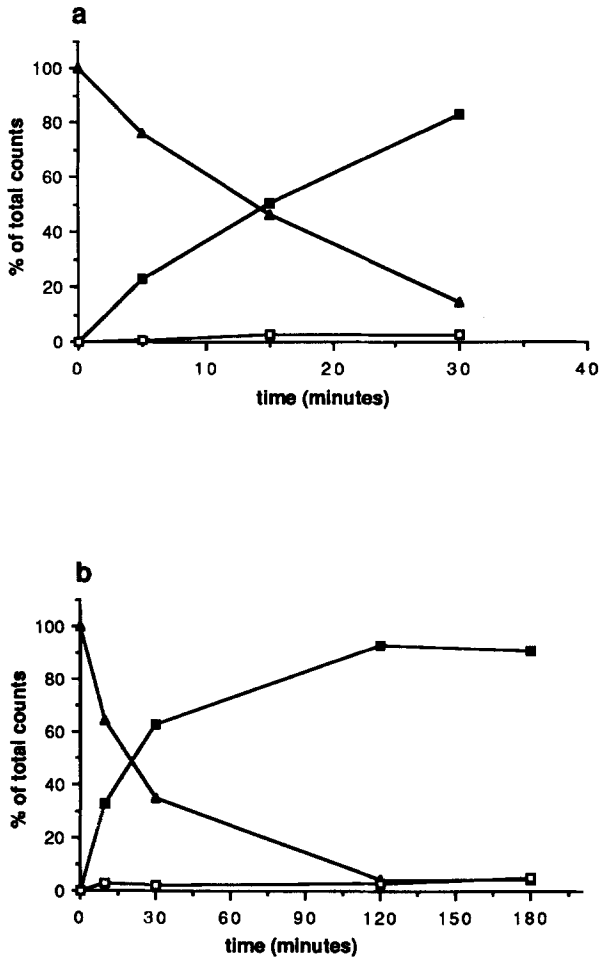

Figure 2. Transferrin recycles basolaterally in Caco-2 cells. Triplicate sets of filter-grown cells were incubated in serum-free medium for 1 h. [^{125}I]Transferrin was added basolaterally, and the cells were incubated at 37°C for (*a*) 10 min or (*b*) 120 min. The cells were placed on ice and rinsed with PBS until no counts remained in the washes. The filters were then either counted or given prewarmed chase medium containing 0.5 mg/ml cold transferrin and incubated at 37°C. At each time point, cells (△), apical (□), and basolateral (■) media were collected separately and counted. Standard errors were <10%, except where counts were <1 × 10^3 cpm, when standard errors were <20%.

Wilmington, DE). The thawed sections were labeled with the ATR antibody essentially as described for whole cell fluorescence.

Electron Microscopy Tracer Experiments

HRP (1–10 mg/ml) was applied to either side of the filter in DME with 0.1% BSA. Incubations were done at 37°C, with 5% CO_2, for 5–60 min. Tr-HRP (~30 μg transferrin/ml) was applied for 1 h at 4°C on a shaker in

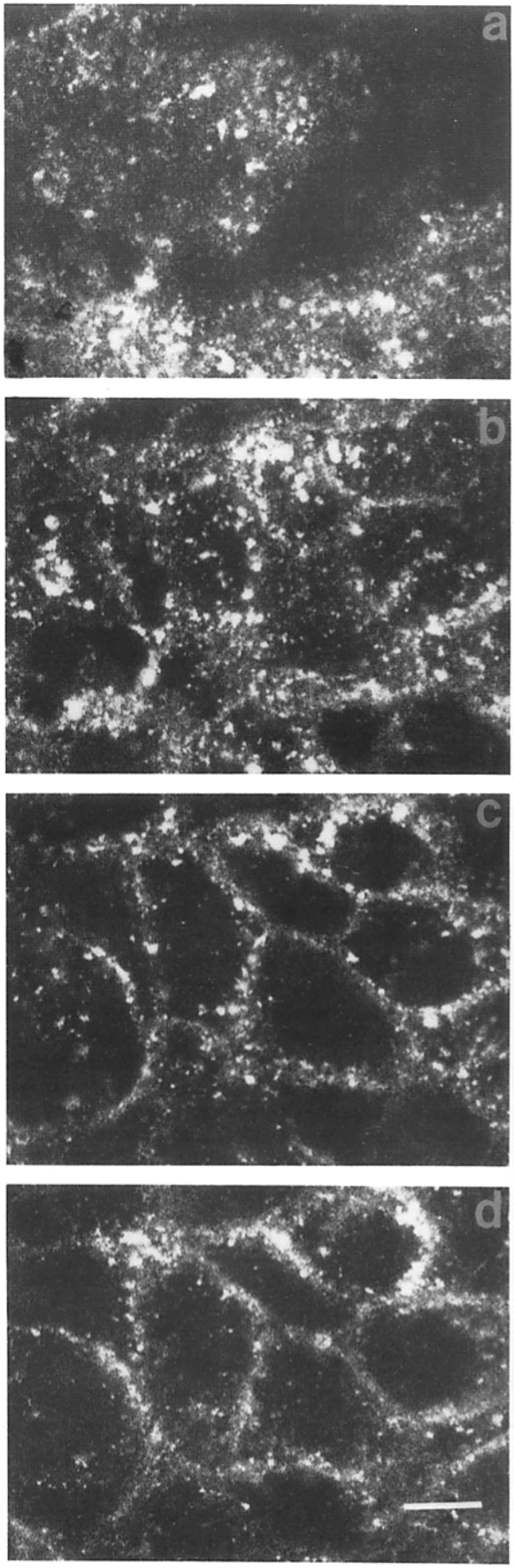

Figure 3. Uptake of transferrin-FITC by Caco-2 cells. Filter-grown cells were incubated with transferrin-FITC present in the basolateral chamber for 60 min. Cells were placed on ice, washed in PBS and fixed with 3% paraformaldehyde. A series of optical sections, 2-μm apart, were taken through the monolayer using the confocal microscope, starting at the apical side of the monolayer. There is strong labeling in the apical cytoplasm (*a* and *b*) and lateral to the nucleus (*c* and *d*) but only small punctate dots in the basal cytoplasm (*d*). Bar, 10 μm.

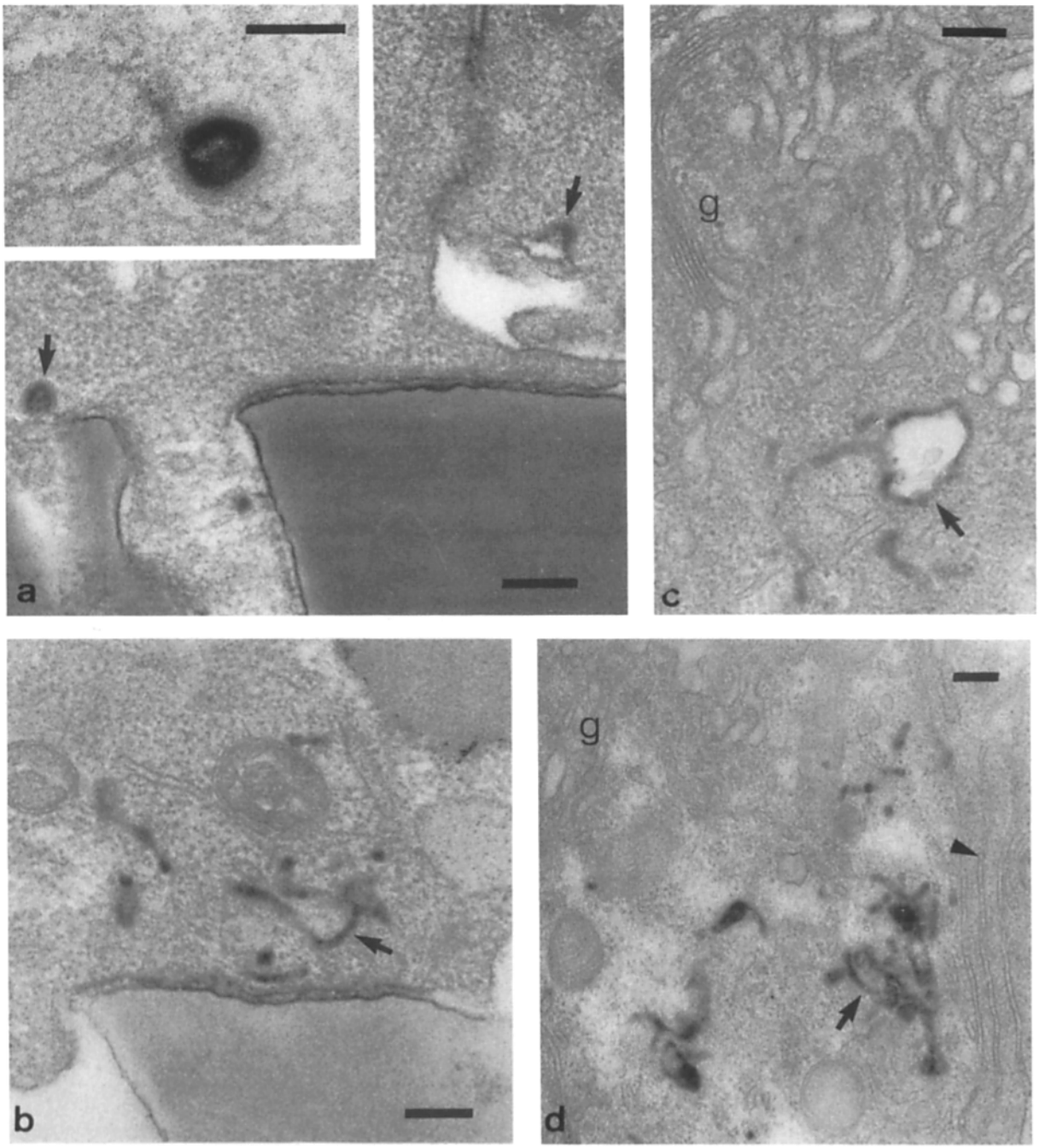

Figure 4. The basolateral endocytic route identified by Tr-HRP. Caco-2 cells were incubated at 4°C with medium containing Tr-HRP in the basal part of the filter chamber for 1 h. The medium was then replaced with prewarmed medium containing Tr-HRP basolaterally, and the incubation was continued at 37°C for (*a*) 5 min, (*b* and *c*) 15 min, or (*d*) 60 min. The cells were then fixed with 1% glutaraldehyde, processed with DAB to reveal the presence of HRP, and prepared for electron microscopy. The sections were stained with lead citrate only, which does not stain glycogen; hence some areas of the cytoplasm have no contrast. Tr-HRP is first observed in coated pits on the basal and lateral surfaces (*a*). Inset shows the detail of a coated pit budding off the lateral membrane. Label then appears in smooth tubular elements in the basal cytoplasm (*b*). Reaction product is first seen in endocytic structures in the Golgi area, apical and lateral to the nucleus after 15 min (*c*). These elements become more elaborate with prolonged incubations (*d*). Endosomes labeled under the same conditions are also shown on Figs. 8 and 9. Arrows indicate labeled pits (*a*) or endosomal elements (*c–d*). Arrowhead shows lateral membrane; *g*, Golgi apparatus. Bars, (*a–d*), 0.2 μm; *inset*, 0.1 μm.

buffered medium (DME with only 37 mg/100 ml $NaHCO_3$, 10 mM Hepes, and 0.1% BSA). This was then replaced with prewarmed serum-free medium (plus BSA), still containing Tr-HRP and the filter was incubated at 37°C, 5% CO_2, for 5–60 min. This protocol was found to be necessary, especially for short time periods, to allow for the time lag in conjugate diffusing across the filter. After all peroxidase experiments, cells were placed on ice and fixed with 1% glutaraldehyde (Polysciences Inc., Warrington, PA) in 0.2 M sodium cacodylate buffer, pH 7.4, for 15 min and then the cells were washed several times in buffer. The presence of HRP was revealed using the diaminobenzidine (DAB) technique (Graham and Karnovsky, 1966). As a control for fluid-phase uptake of Tr-HRP, cells were incubated with equivalent concentrations of free HRP. Negligible staining of endosomes was seen.

Gold conjugates (300 μl/filter) were spun in a microfuge for 20 min be-

*Table I. Tr-HRP Recycles Basolaterally in Caco-2 Cells**

Time (min)	Percentage of total counts		
	Cell-associated	Apical	Basolateral
0	100	0	0
20	17	8	75

* The experiment was performed as described for Fig. 2, but using [^{125}I]Tr-HRP instead of [^{125}I]transferrin.

fore use. ATR-gold was resuspended in DME plus BSA and then applied basolaterally to the filters. Incubations were carried out at 37°C for 1–2 h. Con A-gold was resuspended in buffered medium with 0.1 mM magnesium chloride and no BSA. This was applied apically (1 ml/filter), and the cells were incubated at 4°C for 1 h on a shaker. After 1 h, the medium was removed and replaced with prewarmed DME with BSA and the cells were placed at 37°C for the desired time period.

After labeling with gold complexes, the cells were fixed with glutaraldehyde. All experimental samples were postfixed with 1% osmium tetroxide for 1 h and processed further for electron microscopy by standard techniques. For routine electron microscopy, cells were fixed with Karnovsky fluid (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.5), 1:1 with PBS for 2 h at room temperature, postfixed in reduced osmium tetroxide and processed further as before. Sections were examined on an electron microscope (CM12; Philips Electronic Instruments, Inc., Mahwah, NJ). Reynold's lead citrate stain alone was used for peroxidase samples because when uranyl acetate was used, the glycogen in the cells stained strongly and it was difficult to distinguish the DAB reaction product. Hence, the contrast of the sections is sometimes weak.

Results

Characterization of the Polarized Monolayer

The Caco-2 cells grown in our laboratory on Transwell filters are highly polarized with respect to the expression of surface membrane proteins and secretion (Hughson et al., 1989), and they form tight monolayers with a transepithelial resistance of >200 ohms-cm^2. The morphology of the cells is also polarized (Fig. 1). Seen in section, the cells form a typical lining epithelium and, although they are not as tall as normal human enterocytes, they have a well-developed brush border and their nuclei are usually basally placed. Electron microscopy shows their lateral membranes to be closely opposed (unlike cells grown on plastic; Pinto et al., 1983), highly interdigitated and connected by junctional complexes. Their Golgi stacks are usually found in the apical cytoplasm above the nuclei but may lie more laterally between the nucleus and basolateral border. The cells often contain substantial amounts of glycogen.

The Polarized Expression of Transferrin and EGF Receptors

Preliminary studies using antibodies specific for the transferrin and EGF receptors or their ligands conjugated to FITC demonstrated that these receptor populations were located predominantly on the basolateral surface of the polarized monolayers. Analyses using radiolabeled ligands confirmed these observations (as reported elsewhere for EGF; Hughson et al., 1989). Thus, for example, when monolayers were incubated with [^{125}I]transferrin in the basal chamber, there was a significant uptake of label that was inhibited by cold transferrin. Only 0.5% of these counts appeared in the apical chamber after 4 h, indicating a very low level of leakage (or transcytosis). No degraded counts could be detected. This was in contrast to the results obtained with EGF. After 4 h of incubation with basolaterally applied [^{125}I]EGF, 3% of

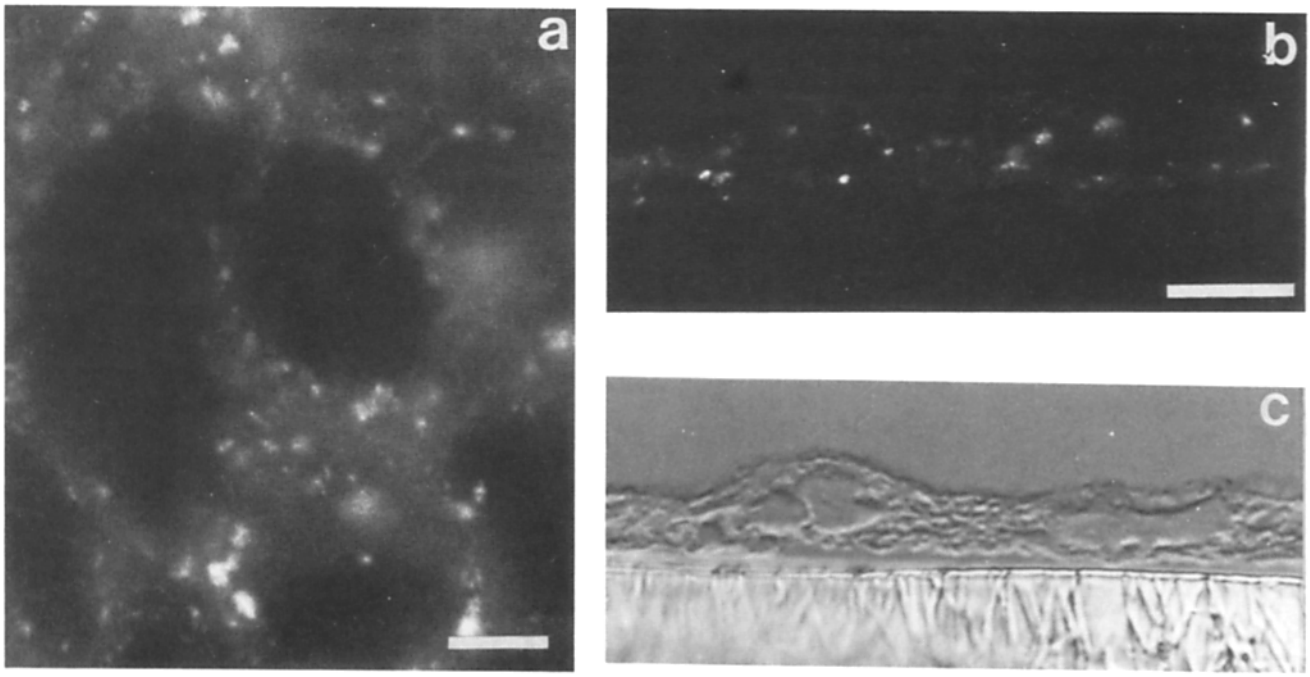

Figure 5. Localization of the transferrin receptor in Caco-2 cells. Filter-grown cells were fixed with 3% paraformaldehyde and (*a*) labeled for the transferrin receptor by indirect immunofluorescence. In *b*, fixed cells were embedded in gelatin (see Materials and Methods), prepared for cryomicrotomy and semi-thin frozen sections were cut. The sections were labeled by indirect immunofluorescence. Concentrations of fluorescent label are seen mainly lateral and apical to the nucleus. (*c*) Nomarski image of monolayer shown in *b*. Bars, (*a*) 5 μm; (*b*) 10 μm.

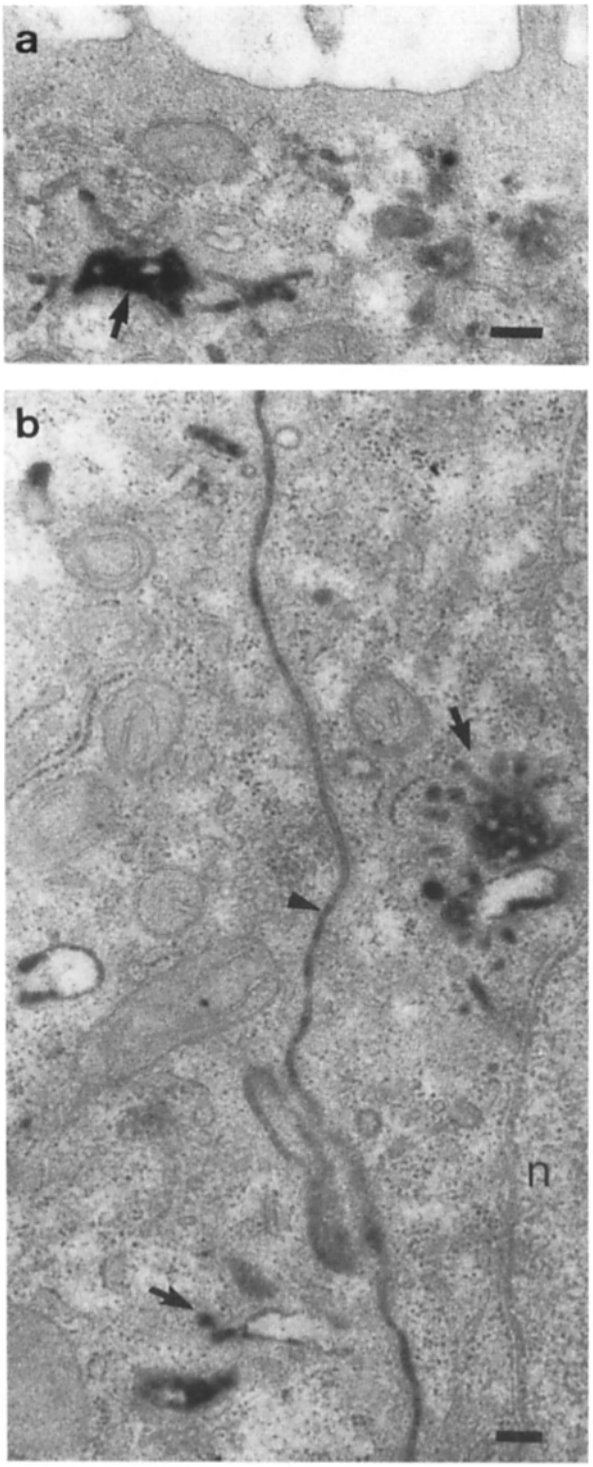

Figure 6. Endosomes labeled by a fluid-phase marker applied at the basolateral surface. Filter-grown cells were incubated with free HRP in the basal chamber for 1 h at 37°C, fixed, and processed to develop the HRP reaction. HRP is present in the lateral space (*b*, *arrowhead*) and in endosomal elements (*arrows*) in the lateral cytoplasm (*b*) and underneath the brush border (*a*). *n*, nucleus. Bars, 0.2 μm.

the total counts appeared in the apical chamber. As only 40% of this label migrated with intact EGF on a Biogel column, it was concluded that much of the label resulted from degradation of internalized peptide.

When monolayers were incubated with [^{125}I]transferrin in the apical chamber uptake is low but significant. Morphological studies with transferrin-FITC indicated that this uptake was because of internalization into isolated groups of cells that can be found occasionally on the surface of the monolayer (data not shown).

Internalization from the Basolateral Surface

Uptake and Recycling of Transferrin. The presence of the filter at the base of the monolayer reduces the efficiency with which conventional pulse-chase procedures can be carried out. Nevertheless, by preincubating at 37°C to preload the cells with [^{125}I]transferrin, rinsing thoroughly at 5°C and then chasing with excess cold transferrin (to reduce internalization of any recycled [^{125}I]transferrin) the release of internalized transferrin provides a direct indication of recycling. As shown in Fig. 2 *a* and Table I, after being preloaded for 10 and 20 min with radiolabel, the cells rapidly recycle >80% of their transferrin to the basal chamber. If, however, the monolayer is preloaded for 120 min, while release remains predominantly (>95%) basal, the kinetics of release are different. The initial rapid phase of 30 min is followed by a slower, more prolonged phase. Eventually after 2 h, 95% of the label can be chased out into the basolateral medium (Fig. 2 *b*). The release observed after the 10–20-min load, and the initial rapid phase of release following the 120-min load, both indicate that transferrin initially enters a compartment from which recycling is rapid and almost 100% efficient. More prolonged loading, however, involves a larger capacity compartment from which recycling is much slower. Passage over a P60 Biogel column showed that the released counts were because of recycled intact transferrin, as negligible amounts of label migrated with the free iodide peak.

The absence of radiolabel in the apical chamber and the data suggesting that the internalized [^{125}I]transferrin remains undegraded (even 4 h) indicates that the pathways involved in this intracellular processing do not include lysosomal elements.

Morphology of the Transferrin Processing Pathways

Light microscopy using transferrin-FITC as a tracer applied basolaterally showed that after incubation at 37°C for 60 min, the internalized transferrin penetrates compartments throughout the cell. Optical sectioning in the confocal microscope revealed that small punctate label is seen in the basal cytoplasm (Fig. 3 *d*). Larger concentrations are observed lateral to the nucleus (Fig. 3 *c*) and substantial labeling is present within the apical cytoplasm (Figs. 3 *a* and *b*). For electron microscopy, Tr-HRP was used as a tracer. By radiolabeling the conjugate it could be shown that the uptake was inhibited by excess cold transferrin and that it had the same short term kinetics for recycling as [^{125}I]transferrin (Table I).

When monolayers were incubated with Tr-HRP in the basal chamber for 1 h at 4°C, DAB reaction product was diffusely distributed within the intercellular spaces and concentrated in coated pits on the lower part of the lateral membrane and occasionally on the basal membrane (Fig. 4 *a*). When the temperature was raised to 37°C after this labeling up period and the incubation continued with Tr-HRP in the medium, peripheral endosomal structures in the form of smooth, sometimes branching tubules in the basolateral

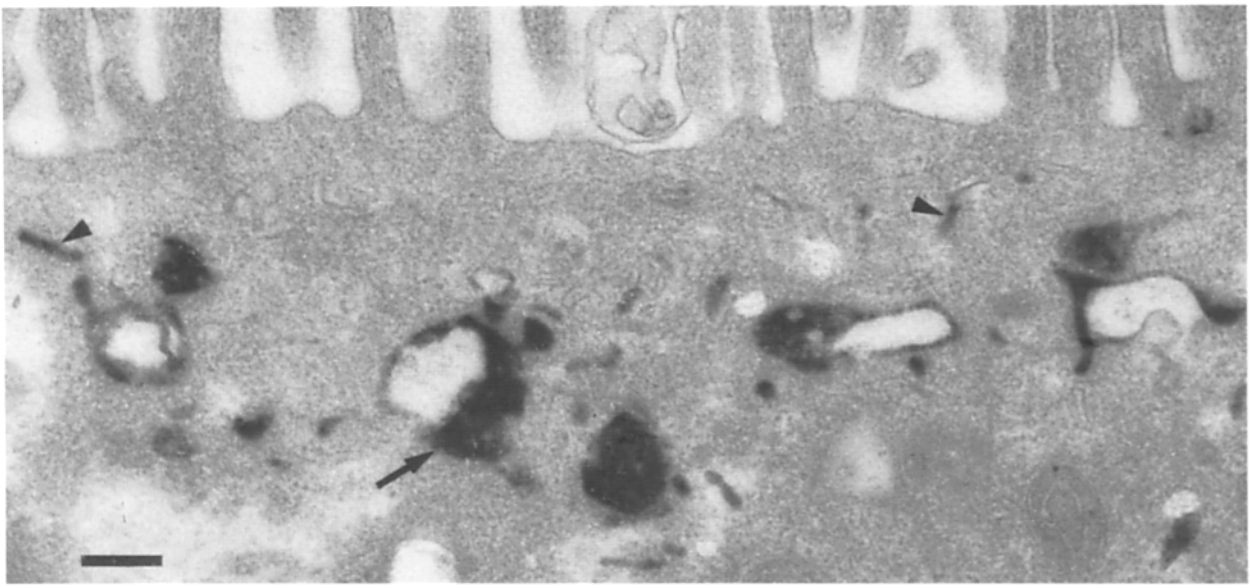

Figure 7. Endosomes accessible by markers from the apical surface. Caco-2 cells were incubated with HRP (3 mg/ml) in the apical chamber for 60 min at 37°C before fixing, and development of the DAB reaction. Label is present in endosomal elements under the brush border, including tubules (*arrowheads*) and vacuolar elements (*arrow*). Bar, 0.2 μm.

cytoplasm became labeled within 5 min (Fig. 4 *b*). After 10 min of incubation at 37°C, small vacuolar elements adjacent to the nucleus also labeled. These structures were in the basal cytoplasm; no label was evident in the apical region of the cell at this time. After 15 min chase at 37°C, conjugate reached elements in the Golgi area (Fig. 4 *c*). These were predominantly vacuolar elements with extensive branching tubules. At this time, a few small endosomal tubular elements could also be found in the apical cytoplasm just below the brush border. After a 60-min incubation at 37°C, the most prominently labeled endosomal elements were in the Golgi area above and lateral to the nucleus (Fig. 4 *d*) and in the apical cytoplasm below the brush border. The elements in the Golgi area now included tubular elements and prominent multivesicular bodies.

Incubating the basolateral surface of the monolayer with gold particles conjugated to ATR antibody, for 60 min at 37°C, labeled intracellular elements with the same form and distribution as those seen with Tr-HRP. Experiments similar to those described above for [^{125}I]transferrin, but using [^{125}I]ATR antibody, demonstrated that this label also recycled in a polarized fashion to the basolateral surface (data not shown).

The Distribution of Transferrin Receptors

Receptors were localized by light microscopy using the ATR antibody on permeabilized cells and on cryosections. As shown in Fig. 5, the transferrin receptor in these cells is localized in a variety of elements throughout the cell cytoplasm. The largest and most strongly labeled structures are in the perinuclear area although positive structures are also present in both basal and apical regions of the cell. The overall pattern of fluorescence thus closely resembles that seen in cells labeled with transferrin-FITC (Fig. 3).

Uptake of a Fluid-Phase Marker

Free HRP was used as a fluid-phase endocytic marker from the basolateral surface to see if this pathway differed from the basolateral recycling route delineated by Tr-HRP. After 60 min of incubation, essentially similar endocytic elements to those seen with Tr-HRP contained peroxidase (Fig. 6). However, labeling of coated pits at the basolateral membrane and in the tubular endosome elements of the Golgi area was weaker.

Uptake from the Apical Surface

Coated pits are frequently observed on the apical membrane at the base of the microvilli in polarized monolayers, and it has been demonstrated with cobalamin-intrinsic factor complexes (Dix et al., 1987) that receptor mediated endocytosis can occur from this boundary in Caco-2 cells. However, this receptor is not expressed in sufficient amounts for morphological studies and no other mobile receptor populations in high copy number have yet been identified on the apical membrane of Caco-2 cells. Morphological studies of endocytosis must therefore use either fluid-phase tracers (HRP) or ligands of broad specificity such as plant lectins.

When Caco-2 monolayers were incubated with HRP in the apical chamber, within 5 min DAB reaction product was present in coated pits and also in very fine tubules and small tubular endosomes immediately below the brush border. After 15 min, larger endosomes became labeled, and, by 30 min, vacuolar endosomes including multivesicular bodies were present deeper in the cell. After 60 min, labeled elements were widespread in the apical cytoplasm (Fig. 7) but were infrequently seen in the Golgi area. No labeled elements occurred in the basal regions below the nucleus.

Incubation with Con A–gold at 4°C (60 min) followed by rinsing and then incubation at 37°C for 60 min introduced the gold conjugate into the endocytic pathway. As expected for a label with such broad specificity (terminal D-mannose and D-glucose on glycoproteins and glycolipids), large amounts remained on the surface even after 60 min at 37°C. There was, however, significant uptake into apical endo-

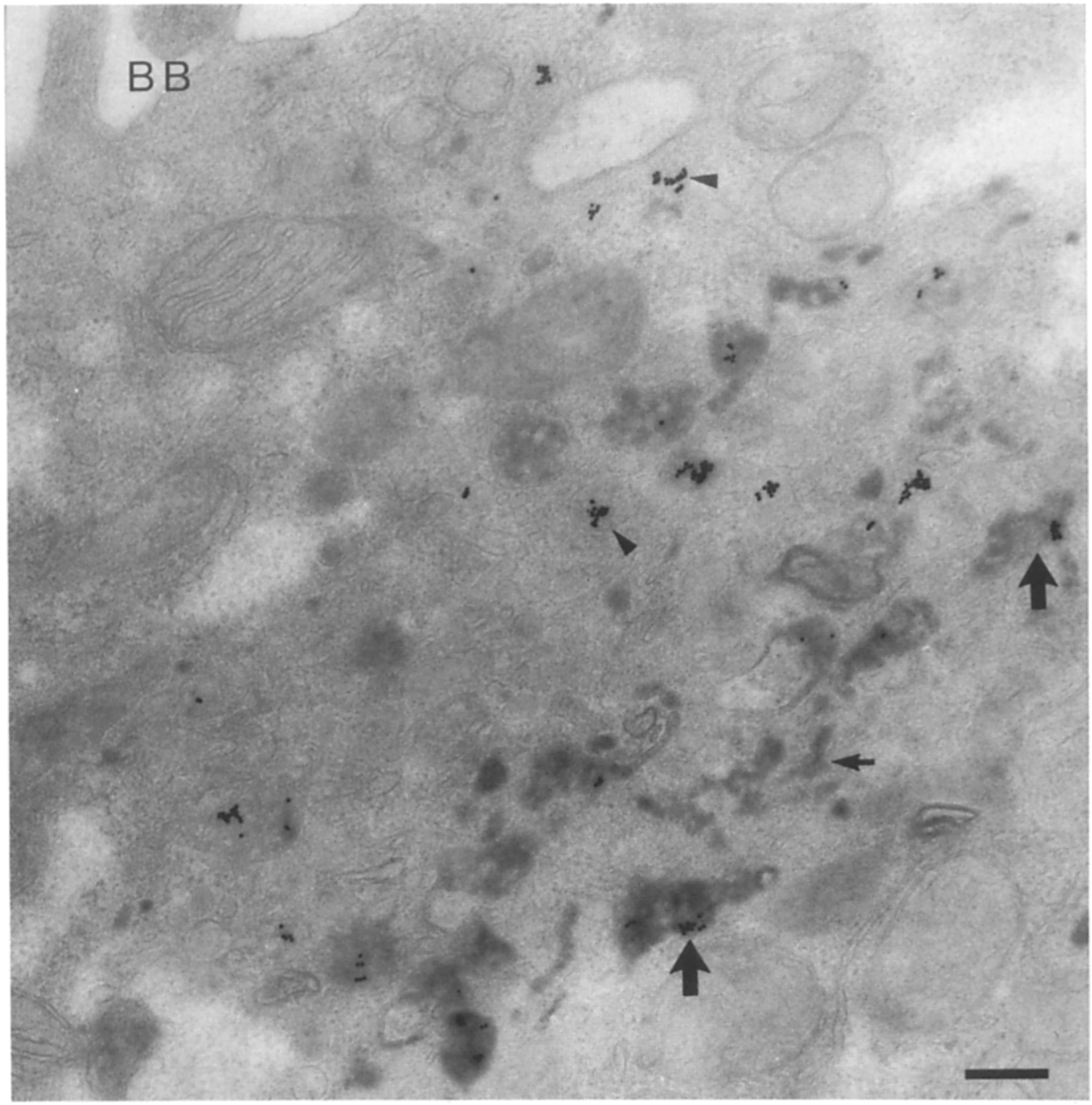

Figure 8. Apical and basolateral endocytic pathways meet in the apical part of the cell. Monolayers of cells were labeled for 60 min with Tr-HRP in the basal chamber of the filter and Con A–gold in the apical chamber, as described in Materials and Methods. Although some endosomal elements contain only peroxidase (*small arrow*) or gold particles (*arrowheads*), a substantial number of these deep apical endosomes are labeled with both peroxidase and gold particles (*large arrows*). All the gold particles are within membrane-bound structures. The micrograph has been printed at a light density so that the double label can be clearly seen. *BB*, brush border. Bar, 0.2 μm.

somes, and, in general, the elements labeled with gold were similar to those identified with free HRP (Fig. 8) although larger numbers were seen in the Golgi region (Fig. 9). Only rarely were particles found in endosomal elements in the basal cytoplasm.

Accessibility of Common Endosome Elements from Apical and Basolateral Surfaces

Our data showing that transferrin/transferrin receptors internalized at the basolateral border remain undergraded and become distributed within endosomal elements in the apical cytoplasm suggested that these ligand-receptor complexes may be able to penetrate compartments that are also accessible to ligands endocytosed from the apical border. This possibility was explored with two kinds of double label procedures.

In the first procedure, monolayers were incubated with their basolateral border exposed to Tr-HRP and the apical surface exposed to Con A–gold. At all time points examined, the majority of the labeled endosomes contained only one tracer. However, mixing of the two elements was observed after 15 min. After 60 min at 37°C, 25% of the gold-labeled elements also contained Tr-HRP. All of these double labeled elements were in the apical cytoplasm; some were distrib-

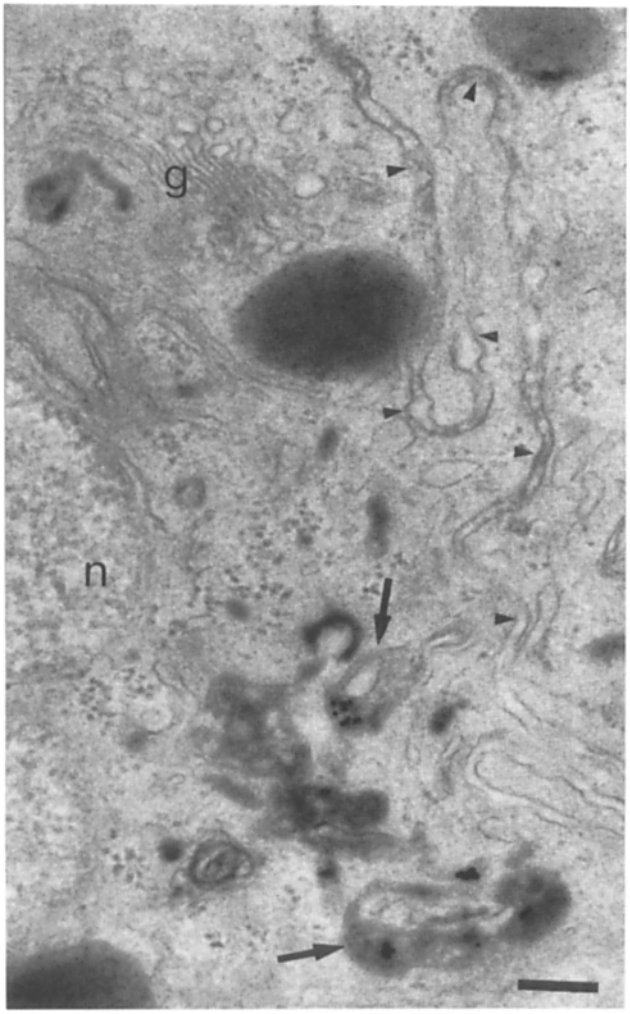

Figure 9. Endosomes in the Golgi area are also accessible to label from both surfaces. Labeling was performed as for Fig. 8. The lateral membrane, which is convoluted and has been sectioned tangentially, is outlined with arrowheads. Arrows indicate double-labeled elements. *n*, nucleus, *g*, Golgi apparatus. Bar, 0.2 μm.

uted just below the brush border (Fig. 8) whilst others were in the Golgi area (Fig. 9). The tight junctions remained firmly closed throughout these experiments.

The observations described here and those on the single labeling experiments suggested that the labeled endosomes in the apical cytoplasm were penetrated from the basolateral border only after prolonged incubations with basolateral tracer. They thus probably represent the late endosome identified in the [^{125}I]transferrin recycling studies (Fig. 2). To investigate further the relationship of this compartment to the apical endocytic pathways, the endocytic routes from the basolateral border was loaded with ATR-gold (by incubating for 1 to 2 h at 37°C), and then the accessibility of this compartment was probed from the apical surface by short incubations with free HRP. As shown in Fig. 10 (*inset*), after only 5 min with HRP, double labeled elements are detectable. Approximately 7% of the HRP-labeled endosomes contained gold-particles. After 30 min, ~20% of the internalized HRP had reached gold-containing endosomes. With longer HRP incubations, the penetration of gold-loaded elements became very extensive (Fig. 10).

Discussion

The uptake and intracellular processing of transferrin and EGF from the basolateral border of Caco-2 cells are, in most respects, similar to that observed in unpolarized epithelioid and fibroblastic cell lines. For transferrin there is a rapid short term recycling pathway (which returns the internalized ligand back to the surface where it was endocytosed within minutes) (Dautry-Varsat et al., 1983; Klausner et al., 1983; Hopkins and Trowbridge, 1983) and a longer larger capacity pathway from which recycling is much less rapid (Hopkins, 1983). The transferrin is efficiently recycled back to the basolateral surface, as demonstrated indirectly in MDCK cells (Fuller and Simons, 1986). There is no evidence (from this and other studies: Stoorvogel et al., 1988; Futter and Hopkins, 1989) that the transferrin-receptor complexes are degraded during the intracellular processing even when they are conjugated to HRP or gold-antibody complexes. Significant amounts of EGF, on the other hand, appear to be delivered to a degradative compartment (presumably the lysosome). No evidence was obtained in our experiments for an uptake pathway arising at the basolateral border that specifically delivered either of these ligands to the apical surface. This is in contrast to the results of a previous study using EGF in the MDCK cell line (Maratos-Flier et al., 1987).

Morphological analyses identify the intracellular pathways that process transferrin as consisting of peripheral or "early" elements below the basolateral border and deeper, later elements in the Golgi area. This arrangement is also similar to that which has been observed in "unpolarized" tissue culture cells (Hopkins and Trowbridge, 1983). The time taken for the tracer to reach this compartment is not significantly different from that recorded for unpolarized cells (10–30 min) (Hopkins, 1983; Yamashiro et al., 1984; Willingham et al., 1984; Stoorvogel et al., 1988). In Caco-2 cells, there is also a late, more apical compartment underneath the brush border where Tr-HRP is seen from 15 min onwards. Together our data suggest that the deeper, late endosome identified by the [^{125}I]transferrin studies lies in the endosomal elements of the apical cytoplasm and Golgi area. The absence of degradation of [^{125}I]transferrin demonstrates that these elements are endosomal rather than lysosomal. It is of interest that HRP introduced at the basolateral border also shows a distribution within apical elements similar to transferrin. A similar distribution has been reported in both intact epithelial tissue (Hugon and Borgers, 1967) and other polarized epithelial cell systems (von Bonsdorf et al., 1985). Presumably, as with EGF, some fluid-phase HRP is also delivered to lysosomes.

Uptake at the apical membrane in enterocytes has been studied extensively. In the neonate, the apical endocytic system is elaborately developed and is capable of delivering endocytosed ligands either to an extensive lysosomal compartment in the apical cytoplasm or via the basolateral border, to the intercellular space (Gonnella and Neutra, 1984; Abraham and Rodewald, 1981). Both routes include endosomal elements in the apical cytoplasm. In the enterocyte of the adult mucosa, no evidence has been found for a receptor-mediated transcytotic pathway from the apical surface to the basolateral border. Similarly, with the probes that we have at present, no apical to basolateral transcytotic pathway could be identified in the Caco-2 system (Hughson and Hopkins,

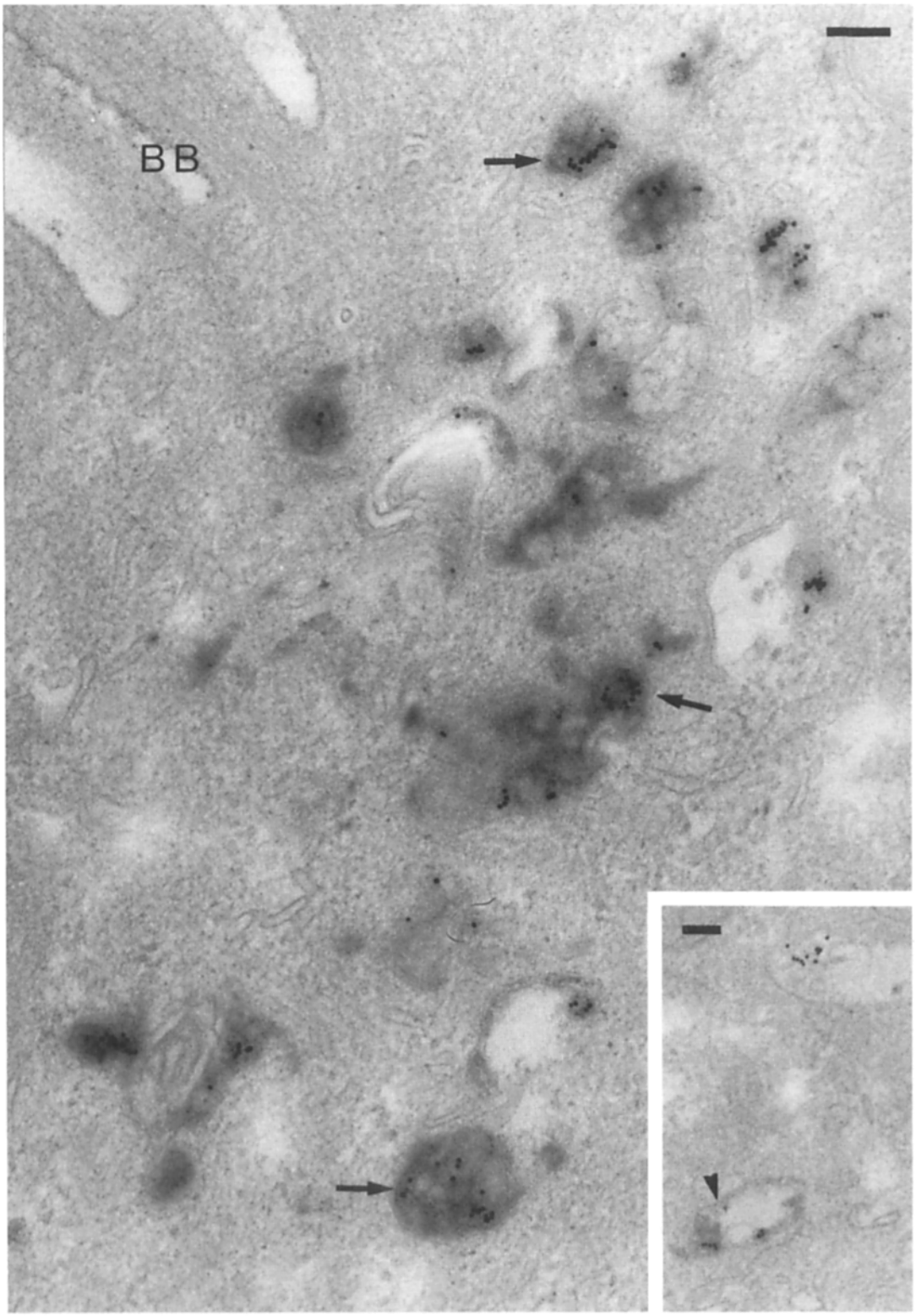

Figure 10. Double labeling of apical endosomes with ATR-gold applied from the basolateral surface for 120 min and HRP applied for the final 60 min from the apical surface. The distribution of ATR-gold is the same in both single and double labeling experiments. Extensive mixing (*arrows*) is seen in elements close to the brush border (*BB*) and deeper in the cell. Inset shows endosomes from a cell incubated with ATR-gold for 60 min and apically applied HRP for the last 5 min. One endosome contains both labels (*arrowhead*). Bars, 0.1 μm.

unpublished observations). Uptake studies in several mammalian systems have shown fluid-phase markers penetrating endosomal elements throughout the apical cytoplasm (Cornell et al., 1971; Blok et al., 1981). Our observations with HRP on polarized Caco-2 cells identify a similar system. They also indicate that the peripheral endosome system identified in the basolateral cytoplasm with transferrin tracers is inaccessible to apically applied fluid-phase tracer.

However, the double label experiments in which tracers are applied from apical and basolateral borders demonstrate that endocytic routes from these two boundaries can reach common endosomal elements. These elements, which lie in the apical cytoplasm and Golgi area, probably correspond to the late endosome identified after prolonged incubations with basolaterally applied radiolabeled transferrin. The more apical elements of this compartment are accessible within minutes to tracers applied at the apical boundary.

A previous study has shown that tracers applied to apical and basolateral surfaces of polarized epithelial cells can reach a common compartment. This, however, represents rather special circumstances since it described the recycling of apical membrane (poststimulation) in secretory cells (Oliver, 1982). In this system, the common compartment was identified as a lysosome or prelysosomal compartment. It is therefore different from the endosome identified in the present study, as our data suggests that transferrin recycles from this compartment to the basolateral surface.

The observations made on Caco-2 cells in the present study raise many questions on membrane trafficking in polarized epithelial cells. For example, what signals and mechanisms operate in the late endosome to allow efficient sorting of the transferrin receptor back to the basolateral membrane? Our results are also of significance for research into drug delivery across the intestinal epithelium. Even though a direct transcytotic pathway (apical to basolateral) may not exist in adult enterocytes, it may be possible to exploit the finding that apically applied ligands reach the same compartment as membrane proteins recycling to the basolateral membrane.

We would like to thank Mark Shipman and Adele Gibson for their excellent technical assistance.

The work was supported by grants provided by the Cancer Research Campaign and the Medical Research Council to Colin Hopkins.

Received for publication 7 July 1989 and in revised form 11 October 1989.

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