Meeting of the Apical and Basolateral Endocytic Pathways of the Madin–Darby Canine Kidney Cell in Late Endosomes

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Abstract. Electron microscopic approaches have been used to study the endocytic pathways from the apical and basolateral surface domains of the polarized epithelial cell, MDCK strain I, grown on polycarbonate filters. The cells were incubated at 37°C in the presence of two distinguishable markers administered separately to the apical or the basolateral domain. Initially each marker was visualized within distinct apical or basolateral peripheral endosomes. However, after 15 min at 37°C, both markers were observed within common perinuclear structures. The compartment in which meeting first occurred was shown to be a late endosome (prelysosome) that labeled extensively with antibodies against the cation-independent mannose-6-phosphate receptor (MPR) on cryosections. With increasing incubation times, markers passed from these MPR-positive structures into a common set of MPR-negative lysosomes that were mainly located in the apical half of the cell.

A detailed quantitative analysis of the endocytic pathways was carried out using stereological techniques in conjunction with horseradish peroxidase and acid phosphatase cytochemistry. This enabled us to estimate the absolute volumes and membrane surface areas of the endocytic organelles involved in apical and basolateral endocytosis.

Selected plasma membrane components of mammalian cells are continuously taken into the cell by endocytosis (Steinman et al., 1983; Goldstein et al., 1985). After 5 min at 37°C, internalized markers enter tubulovesicular elements, termed early or peripheral endosomes, before being routed to lysosomes or recycled back to the plasma membrane (Storrie, 1988; Courtoy, 1989; Gruenberg and Howell, 1990). Ligands destined for lysosomes pass from early endosomes to juxtanuclear late endosomes (Helenius et al., 1983; Hopkins, 1986), also referred to as prelysosomes (Kornfeld and Mellman, 1989), with kinetics that appear to depend on both the cell type and the ligand used (Hubbard, 1989). Late endosomes are enriched in both the cation-independent mannose-6-phosphate receptor (MPR) and lysosomal proteins as compared with early endosomes (Geuze et al., 1988; Griffiths et al., 1988) and have been proposed to be the target of trans-Golgi network (TGN)-derived vesicles containing newly synthesized lysosomal enzymes on their way to lysosomes (Kornfeld and Mellman, 1989). Late endosomes are more acidic than both early endosomes (Schmid et al., 1989; Fuchs et al., 1989) and the TGN (Griffiths et al., 1988). The low pH may facilitate dissociation of lysosomal enzymes from MPRs, allowing the latter to recycle back to the TGN for further rounds of transport, while the lysosomal enzymes pass to lysosomes (Goda and Pfeffer, 1988). In agreement with this model is the finding that at later times endocytic markers reach MPR-negative structures containing lysosomal proteins; the latter are most likely functional lysosomes (Brown et al., 1986; Geuze et al., 1988; Griffiths et al., 1988).

In the polarized epithelial cell, where the surface membrane is divided into two domains of distinct composition, continual endocytosis and recycling also occur (Simons and Fuller, 1985). Moreover, there is extensive membrane traffic between the two domains (transcytosis) (Abrahamson and Rodewald, 1981; Limet et al., 1985; Hoppe et al., 1985; Mostov and Simister, 1985; von Bonsdorff et al., 1985). Despite this process, the cell maintains its polarity with high efficiency (Fuller and Simons, 1986).

We have studied endocytosis in polarized monolayers of MDCK cells grown on polycarbonate filters. In the preceding paper (Bomsel et al., 1989), it was shown that markers internalized from the two surface domains of the MDCK cell were initially located in distinct early endocytic structures but that meeting of the markers occurred within 15 min after the onset of internalization. In this study, we have characterized and quantitated the endocytic pathways from the apical and basolateral domains of the MDCK cell by an ultrastructural approach and have demonstrated that the two pathways meet in late endosomes (or prelysosomes) enriched in the MPR.

Materials and Methods

Cells

MDCK strain I cells were grown on 0.4-μm-pore polycarbonate filters

1. Abbreviations used in this paper: HRP, horseradish peroxidase; MPR, mannose-6-phosphate receptor; Sv, surface density; TGN, trans-Golgi network.
The Journal of Cell Biology, Volume 109, 1989

sylvania (Costar Corp., Cambridge, MA) for 3–4 d (Bomsel et al., 1989). The electrical resistance across the bilayer was routinely assayed as described previously (Fuller et al., 1984) and was always >2,000 Ω·cm².

Markers of Endocytosis

Cells were washed twice with the incubation medium (MEM, containing 0.2% BSA, 350 mg/liter Na HCO₃, and 10 mM Hepes). They were incubated for various times at 37°C or 20°C, in the incubation buffer with horseradish peroxidase (HRP, 10 mg/ml), added to the apical medium (Sigma Chemical Co., St. Louis, MO) and BSA-coated gold particles (OD₀/₅₀ = 20) in the basolateral medium. 13–14 nm gold was prepared by the tannic acid method (Slot and Geuze, 1985) and bound to 200 μg/ml BSA at pH 7.0.

Due to the relatively low endocytic rate from the apical surface of the MDCK cell (von Bonsdorff et al., 1985), colloidal gold-BSA did not give a sufficiently high signal when administered apically. For this reason, in those experiments in which HRP was applied basolaterally, a membrane marker was used to follow endocytosis from the apical surface. An anti-MDCK rabbit polyclonal antibody against MDCK strain II cells (a gift from J. Gruenberg, European Molecular Biology Laboratory) was bound to the apical cell surface (30 min on ice). After washing three times over 30 min, the cells were incubated with protein A-gold for 30 min on ice and then warmed to 37°C for various times while 10 mg/ml HRP was administered basolaterally. Alternatively, cationized ferritin (0.1 mg/ml; Sigma Chemical Co.) was bound to the apical surface (5 min on ice) and then warmed to 37°C for various times with HRP in the basolateral medium.

To terminate the incubations, cells were either fixed without a washing step or washed twice with ice-cold incubation buffer before fixation, as described below.

Electron Microscopy

Cells on polycarbonate filters were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (cacodylate buffer) for 30 min. To visualize HRP, the fixed cells were rinsed with cacodylate buffer, incubated in cacodylate buffer containing 0.1% diaminobenzidine (DAB; Sigma Chemical Co.) for 1 min and then H₂O₂ was added to a final concentration of 0.01% to initiate the reaction. After a 2-min incubation in the dark, the DAB solution was removed and the cells were washed with cacodylate buffer (Marsh et al., 1986). The cells were postfixed for 60 min in 1% OsO₄ containing 1.5% potassium ferricyanide, or with 2% aqueous OsO₄ and block-stained for 1 h with 1% uranyl acetate in 50 mM maleate buffer, pH 5.2. Cells were dehydrated in an alcohol series, and embedded in Epon. Sectioning was performed perpendicular to the plane of the filter to obtain vertical sections as required for the stereological analysis. The cells showed no endogenous peroxidase activity.

Acid phosphatase was visualized by the lead capture method as described previously (Griffiths et al., 1983b) with a 75-min incubation with the reaction mixture. When the same sample was to be stained for acid phosphatase and HRP, the staining for acid phosphatase was performed before the staining with DAB.

Morphometric Analysis

Mean Cell Volume. The absolute volume of the MDCK I cell was estimated by measuring the mean area of filter covered by one cell (24 μm²) and the mean cell height (9.38 μm) across the monolayer (Griffiths et al., 1984, 1989b). The former was estimated by counting the number of cells per area of filter on light micrographs after staining the nuclei with Hoechst dye, and the latter was measured as outlined below.

Stereology. For all the stereological analysis Epon blocks were sectioned perpendicular to the plane of the filter to obtain the necessary vertical sections (Baddeley et al., 1986). Photographs were taken at three different magnification levels as described below and all the measurements were made on negatives enlarged 4.1 times on an EM♭-designed projector system.

The three magnification levels used were as follows: Level 1: a primary magnification of 2,800× was used to gain estimates for several morphological features of the MDCK I cell. Sections were cut from a total of six Epon blocks after embedding two filters of MDCK I cells. 35 random micrographs were taken along the monolayer by using the translation controls of the microscope.

The surface to volume ratio (SV) of the MDCK I cell was estimated using the method of Baddeley et al. (1986). A cycloid lattice grid (Cruz-Orive and Hunziker, 1986) was laid over the projected negatives. Intersections of the cyclod with the apical, basal and lateral surface domains and the points over the cell were counted and SV was calculated as described previously (Griffiths et al., 1989b).

The volume density of the cytoplasm (the ratio of the cytoplasmic volume to the cell volume) was estimated using the same negatives and a square double-lattice grid to relate the number of points over the nucleus to the number of points over the cell. The mean cell height across the monolayer was directly measured on the same negatives at three different fixed positions across the negative.

Magnification level 2: a primary magnification of 10,500× was used to obtain estimates for the volume density of HRP or acid phosphatase-labeled compartments. 25–35 random micrographs were analyzed using a double-square lattice grid. Points over HRP or acid phosphatase-labeled structures were related to the points over the cytoplasm of the cell.

Magnification level 3: 20–30 micrographs were taken at a primary magnification of 33,000× after systematically searching for HRP or acid phosphatase-labeled structures. The SV of the labeled structures was estimated by using square lattice grids of various sizes and relating the number of points over the structures of interest to the intersections the grid lines made with the limiting membrane. The SV of these structures was calculated as described by Weibel (1979).

Cryomicrotomy and Immunolabeling

Filter-grown MDCK cells were processed for EM after fixation either as described above for plastic sections or alternatively in 1% glutaraldehyde in 250 mM Hepes, pH 7.4. After washing, the filters were cut into segments and incubated in 10% gelatin in PBS for 10 min at 37°C. Two pieces of filters were then laid on top of each other and the gelatin was allowed to set at 4°C. The gelatin was cross-linked with 8% paraformaldehyde in 250 mM Hepes, pH 7.4, for 1 h at room temperature. The samples were then mounted on copper stubs with the filter perpendicular to the plane of sectioning and sectioned on a ultramicrotome (model OMU4; Reichert, Vienna, Austria) with cryoattachment. Labeling was performed as described previously (Griffiths et al., 1983a, 1984). The affinity-purified anti-HRP was a gift from J. Gruenberg (European Molecular Biology Laboratory). The antisera against chicken 215K cation-independent mannose-6-phosphate receptor was prepared by K. Römsch and B. Hoffack (European Molecular Biology Laboratory). This antisera shows specificity only for the cation-independent MPR by immunoblotting of MDCK cell homogenates.

Results

Meeting of the Endocytic Pathways from the Two Surface Domains of MDCK Cells

HRP was administered independently to the two domains of MDCK I cells grown on polycarbonate filters. At early times of internalization (5–10 min at 37°C), apically administered HRP was visualized close to the apical surface of the cell, whereas basolateral HRP was observed in profiles along the lateral and basal surfaces. A similar distribution of HRP was shown by von Bonsdorff et al. (1985) and is in good agreement with the confocal microscopic observations of Bomsel et al. (1989). These peripheral structures correspond to the early endosomes described in the companion paper.

By EM the apical and basolateral early endosomes appeared morphologically similar (Fig. 1, a, b, and d). Both had tubular and vesicular portions as observed in other cells (Geuze et al., 1983; Marsh et al., 1986). HRP reaction product often appeared to be limited to the periphery of such structures and to surround an electron-lucent lumen (Fig. 1). Such images have been observed by other workers and assumed to represent spherical vesicles (Steinman et al., 1976; de Chastellier et al., 1987). However, in some of our images an inner membrane is visible (Fig. 1, a and b), suggesting that a tubule, or more likely, cisterna, containing HRP may surround an electron-lucent area. In addition to these structures, a small number of noncoated vesicles directly underlying...
eral marker prevented any possible problems with access of the basolateral surfaces of the cell. After continuous internalization of these markers for a period of 15 min or longer at 37°C, colocalization of HRP and gold particles was observed within perinuclear structures lying close to the apical surface of the cell (Fig. 5b). Labeling for MPR was visualized within structures in close proximity to the lateral and basal surfaces, respectively (Fig. 3). However, after 15 min of internalization, colocalization of HRP and gold particles was apparent within structures predominantly in the supranuclear portion of the cell close to the Golgi stacks (Fig. 4). Identical results were obtained with cationized ferritin as a marker of the apical surface and HRP as a basolateral marker (see Fig. 5d). These results are therefore in good agreement with the biochemical and confocal microscopic results presented in the companion paper (Bomsel et al., 1989). The organelles containing the two markers had numerous vesicular inclusions and resembled the multivesicular bodies previously described in these cells (von Bonsdorff et al., 1985) and in other cell types (McKanna et al., 1979; Wall et al., 1980; Hopkins and Trowbridge, 1983).

Further experiments were designed to test whether any meeting could be detected within early endosomes. The passage of ligands from endosomes to lysosomes is blocked at 20°C (Dunn et al., 1980; Marsh et al., 1983) and Griffiths et al. (1988) showed that this block occurs between peripheral early endosomes and an intermediate prelysosomal compartment (late endosome). We therefore investigated whether any meeting of apical and basolateral markers occurred at this temperature. No colocalization of the markers could be demonstrated despite the presence of gold labeled structures and HRP-labeled structures in close proximity (results not shown). These results confirm that MDCK cells possess distinct apical and basolateral early endosomes. Meeting occurs in a later compartment, after a lag >5–10 min at 37°C and subsequent to the 20°C block in the pathway.

Characterization of the Meeting Compartment

To characterize the compartment in which the basally applied and apically applied ligands colocalized, ultrathin frozen sections were prepared. The thawed sections were double-labeled with antibodies to HRP and to the cation-independent MPR.

Cryosections were prepared after a 15-min internalization of apically bound gold and basally administered HRP (see above), a 15-min internalization of apically bound cationized ferritin and basal HRP, or after a continuous incubation for 2 h at 37°C with BSA-gold in the basal medium and HRP in the apical medium. After 15 min at 37°C, gold internalized from the apical surface could be visualized within peripheral HRP-negative structures showing negligible labeling with anti-MPR antibodies (Fig. 5a). Basolaterally internalized HRP was visualized within structures in close proximity to the lateral and basal surfaces (Fig. 5b). Labeling for MPR was very low but consistently observed in these basolateral structures. In contrast, structures containing both the internalized gold and showing labeling for HRP were more heavily labeled with anti-MPR antibodies (Fig. 5c). Similar results were obtained with the two other sets of markers. Cationized ferritin bound to the apical surface of the cell colocalized with basolateral HRP after 15 min at 37°C in MPR-enriched structures (Fig. 5d). After a 2-h continuous incubation with MPR.

Figure 1. Early apical and basolateral endocytic structures in the MDCK cell. Examples of structures labeled with HRP after a 5-min apical (a, b, and c) or basolateral (d) incubation at 37°C. a and b show structures located in the apical region of the cell. HRP reaction product appears to be limited to a membrane-enclosed tubule or, more likely, cisternas whose inner membrane (arrowheads) enclose an electron-lucent lumen. c shows a typical small uncoated structure lying close to the apical surface of the cell (A). d shows a basolateral early endosomal structure. Again an electron-lucent area is surrounded by HRP reaction product. Bars, 0.1 μm.

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BSA-gold in the basolateral medium and HRP in the apical medium, meeting was again detected within structures that labeled extensively with the anti-MPR antibody (Fig. 5 e) but not within peripheral structures showing low labeling for MPR. The MPR-enriched organelles where meeting occurred varied in structure, often contained intralumenal tubulovesicular membranes, and were predominantly in a perinuclear location in the cell.

These results, using three different marker systems, have shown that MDCK cells possess two distinct sets of early en-
Figure 3. Distribution of apical and basolateral markers in the MDCK cell after 5 min at 37°C. Epon section of an MDCK cell fixed 5 min after the onset of internalization of apical membrane-bound gold and basolateral HRP. 9 nm protein A-gold was bound to the apical surface via an anti-MDCK antibody at 4°C. The cells were then warmed to 37°C for 5 min in the presence of basolateral HRP. Gold particles are apparent in a tubular structure underlying the apical surface (arrow), whereas HRP-labeled structures (arrowheads) lie close to the lateral space (L). A possible HRP-labeled coated vesicle is indicated by a small arrowhead. Note that some basolaterally labeled structures are located close to the apical surface (A). Bar, 0.2 μm.

...dosomes showing low or negligible labeling for MPR and that markers from these compartments meet in MPR-enriched late endosomes (Kornfeld and Mellman, 1989).

**Accumulation of Internalized Ligands within Putative Lysosomes**

In nonpolarized cells ligands have been shown to be transported from MPR-containing endosome structures to MPR-negative lysosomes (Griffiths et al., 1988; Geuze et al., 1988). Therefore, we investigated whether ligands would pass from the late endosomes to MPR-negative structures in this cell type. For this analysis, BSA-gold was used as an easily quantifiable and nondegradable marker of bulk flow. Note that our aim was not to define the kinetics of this step of the pathway; BSA-gold appears to be slower in its movement to the end station of the endocytic pathway as compared with HRP (Bomsel et al., 1989).

BSA-gold was internalized from the basolateral surface of the cell for 40 min at 37°C and then the cells were washed and reincubated in ligand-free medium for 40 min, or overnight at 37°C. The 40-min incubation in ligand-free medium was used to chase the gold from early endosomes to late endocytic compartments, whereas the overnight incubation was intended to chase gold to the end station of the endocytic pathway.

We first determined the percentage of the internalized gold particles present in MPR-negative and MPR-positive structures after the two chase times by immunolabeling of frozen sections. As shown in Table 1, after a 40-min chase, 75% of the total internalized gold was within MPR-positive structures (Fig. 6). After the overnight chase, however, the percentage of gold particles within the MPR compartment had decreased considerably and represented only 27% of the total internalized gold. The remainder of the gold particles was present in large aggregates in MPR-negative, electron-dense structures (Fig. 7), which we presume to be lysosomes.

To investigate in more detail the accumulation of gold within the lysosomes and to determine whether the marker reaches these organelles via the MPR-enriched late endosomes, we counted the absolute number of gold particles in the various compartments after the two incubation times.

The total number of gold particles within the cell after a 40-min uptake followed by a 40-min chase was approximately equal to that found in the cell after the overnight chase. This indicates that negligible exit of gold from the cells occurred during this period due, for example, to transcytosis or release of lysosomal contents. Using the absolute number of gold particles per cubed micron of cytoplasm, together with the results from the MPR labeling of frozen sections (above), we could show that the number of gold particles in the MPR-positive compartment decreased by ~2,300 per cell during the overnight chase, whereas gold in the MPR-negative compartment (lysosomes) increased by 2,600 gold particles per cell during the same time period. These results show clearly that most, if not all, of the gold particles that reach the lysosomes must pass through the late endosomes.

The labeled profiles after the overnight chase clearly had a greater mean number of gold particles than the structures labeled after the 40-min chase (Table 1). As only half as many labeled profiles per cytoplasmic volume were observed after the overnight chase, this suggested that the gold particles were chased from a relatively large compartment to one of a smaller volume.

Our qualitative observations indicated that the location of the late endosomes and the lysosomes differed in that the latter are predominantly in the apical part of the cell whereas the former are found to be above and around the nucleus. We therefore counted the percentage of the internalized gold particles in the basal region of the cell (arbitrarily defined as below the midpoint of the nucleus). After the 40-min chase,
Figure 4. Meeting of apical and basolateral markers in the MDCK cell after 15 min at 37°C. Epon section of MDCK cell fixed 15 min after the onset of internalization of apical membrane-bound gold and basolateral HRP (for details see Fig. 3). HRP reaction product fills a number of vesicles (arrowheads) alongside the lateral space (L). Basolaterally administered HRP and apically applied gold colocalize in structures (asterisks) close to the Golgi area (G) of the cell as shown at higher magnification and with a lighter printing in the two insets (arrows indicate gold particles). A gold particle is also evident in an ill-defined structure (possibly a grazing section of a vesicle) underlying the apical surface (arrow). A, apical surface. Bars, main figure, 0.5 μm; insets, 0.1 μm.

31% of the gold particles were present in the basal region. After the overnight chase, however, when the labeled structures were almost exclusively lysosomal by the criteria described above, only 4% of the gold particles were in basally located structures. Thus, in the MDCK cell the lysosomes are predominantly (or exclusively) located in the apical region of the cell, whereas the late endosomes are also found in basal regions.

Acid phosphatase has been shown to be present in the lysosomes and the late endosomes of normal rat kidney and baby hamster kidney cells but absent from early endosomes (Gruenberg et al., 1989; Griffiths, G., R. Matteoni, R. Back, and B. Hoflack, manuscript in preparation). We observed a similar distribution of this enzyme in the MDCK cell. Fluid-phase markers were observed in acid phosphatase-negative structures after short incubations at 37°C (5-10 min) but after longer incubations, passed into acid phosphatase-positive structures (Fig. 7). After the 40-min chase of BSA-gold, ~90% of the internalized gold particles were within acid phosphatase-positive structures and after the overnight chase this had increased to almost 100% (results not shown). Thus, both the MPR-enriched late endosomes and the lysosomes in MDCK cells contain detectable amounts of acid phosphatase.

Volume of the Endocytic Compartments in the MDCK Cell

To gain a quantitative view of the endocytic pathways in the MDCK cell, HRP was administered independently to each of the two surface domains and internalized for various times.
Figure 5. Characterization of compartments containing apically and basolaterally internalized markers on cryosections. Cryosections were double-labeled with antibodies to MPR and to HRP. Three different sizes of gold are shown in the figure; the largest gold (13-14 nm) corresponds to the basolaterally internalized marker, the medium-sized gold (9 nm) (or in Fig. 4, d, cationized ferritin) corresponds to the apically administered marker and the smallest (6 nm) represents labeling for MPR. a, b, and c show cryosections of cells after binding protein A-gold to the apical surface and internalization for 15 min at 37°C with HRP in the basolateral medium (see legend to Fig. 1). In a, gold (arrows) internalized from the apical surface (A) is evident within a putative early apical endosome, which shows no labeling with anti-HRP or anti-MPR. b shows a typical basolateral endosome, in close proximity to the lateral surface (L) which labels with anti-HRP (large gold) but has no apically internalized gold. This compartment shows low, but significant labeling with anti-MPR (small gold, arrowhead). c shows an example of a compartment where the basolateral marker (HRP, labeled with anti-HRP and 13 nm protein A-gold, small arrows) colocalizes with the apical marker (BSA-gold, 9 nm; large arrows). The compartment labels significantly with antibodies against the cation-independent mannose 6-phosphate receptor (6 nm gold, arrowheads). (d) Cationized ferritin was bound to the apical surface and internalized for 15 min at 37°C, with HRP in the basolateral medium. HRP (labeled with anti-HRP and large gold, large arrows) colocalizes with cationized ferritin (small arrows indicate an area where a small cluster of the small ferritin particles are apparent) in a structure containing MPR (arrowheads). (e) HRP was internalized from the apical surface and BSA-gold (14 nm) from the basolateral surface for 2 h at 37°C. HRP (labeled with anti-HRP and 9 nm protein A-gold, small arrows) colocalizes with internalized BSA-gold (large arrows) in compartments containing significant levels of MPR (small gold, arrowheads). Anti-HRP labeling is low compared with the labeling from the basolateral surface (compare with Fig. 4, c and d) but significantly above background. Bars, 0.1 μm.

at 37°C. Stereological analysis was then performed to determine the volume density (or volume fraction) of the labeled compartments (Table II).

After 5 min of basolateral uptake of HRP, reaction product was visualized within tubulovesicular organelles located close to the lateral and basal surfaces of the cells, as described above. The volume density of the labeled structures at this time was 0.34 ± 0.05% of the cytoplasmic volume. 2

2. The electron-lucent parts of these structures were assumed to be HRP negative and only the HRP-reactive area surrounding these regions was included in the analysis (Fig. 1 a and Griffiths et al., 1989a). However, as shown in the legend to Table III, our basic conclusions are unchanged if the electron-lucent areas of the structures are also assumed to be part of the early endosomal compartment.

A 10-min incubation resulted in a similar labeling pattern with no significant increase in the volume density of the labeled compartment (0.40 ± 0.04%). To determine whether any HRP had reached late endosomes at this time and to define more accurately the volume of the basolateral early endosome, we stained cells for acid phosphatase after internalization of HRP for 10 min at 37°C. The diffuse reaction product in the HRP-labeled structures was readily distinguishable from the electron-dense, acid phosphatase-positive structures. The HRP-labeled acid phosphatase-negative compartment, which we defined as the early endosome, had a volume density of 0.37 ± 0.05%, not significantly smaller than the total HRP-labeled compartment at this time.

After a 5-min incubation with HRP in the apical medium,
of gold particles within the MDCK Cell after a 40-min Basolateral Incubation and a 40-min or Overnight Chase at 37°C

| Time          | Estimated No. of gold particles per cell (± SEM)* | Percentage (and estimated no. per cell) of internalized gold particles† in (a) MPR +ve str | (b) MPR -ve str | No. of labeled structures per unit cytoplasmic volume (± SEM)§ | Mean no. of gold particles in labeled profile (± SEM)¶ | Percentage of gold particles in basal region of cell (± SEM)¶ |
|---------------|--------------------------------------------------|--------------------------------------------|----------------|--------------------------------------------|--------------------------------------------|---------------------------------------|
| 40-Min chase  | 4,950 ± 1,390                                    | 75 (3,710)                                 | 25 (1,240)     | 9 ± 1                                      | 7 ± 2                                      | 31 ± 14                               |
| Overnight chase| 5,270 ± 2,040                                    | 27 (1,420)                                 | 73 (3,850)     | 3 ± 1                                      | 24 ± 7                                     | 4 ± 3                                 |

* Data obtained from analysis of 27 micrographs of Epon sections, final magnification 41,000×. Sections were assumed to be of a uniform thickness of 60 nm to gain estimates for the number of gold particles internalized per cell (volume 1,163 μm³).

† Data obtained by labeling frozen sections of the same experiment with anti-MPR antibodies. Any profiles containing internalized gold were scored as MPR positive (i.e., containing three or more MPR-directed gold particles) or MPR negative. 120 structures were examined after the 40-min chase and ~60 min after the overnight chase (i.e., for each time point the distribution of over 1,600 gold particles was assessed) and the experiment was performed three times. The percentage of internalized gold particles within MPR positive structures varied by ~15% between these experiments. From the estimated number of gold particles per cell in these compartments (shown in parentheses), it is evident that the decrease in the number of gold particles in the MPR compartment (~2,300) is similar to the increase in the number of particles in lysosomes (~2,600).

§ Estimates from analysis of micrographs of Epon sections as above. The basal region of the cell was defined as the portion of the cell basal to the midpoint of the nucleus.

After staining for acid phosphatase, reaction product was evident within mostly circular or oval-profiled structures that were predominantly positioned around the cell nucleus. Internal membrane structures were sometimes apparent within these profiles, which appeared very similar to those seen in the MPR-enriched late endosomes and lysosomes. The volume density of acid phosphatase–reactive structures was 1.44 ± 0.21% of the cytoplasmic volume, not significantly different from the volume of structures labeled by basolateral HRP after a 2-h incubation followed by a 2-h chase. The total combined volumes of the late endosomes and lysosomes, defined as acid phosphatase–positive, thus appeared to be similar to the volume of those late compartments accessible to basolateral ligands. When HRP was internalized from both surfaces for 2 h and then chased for a further 2 h, the total volume density of the structures labeled in this way (1.74 ± 0.21%) was, again, not significantly different from that of the compartment labeled only from the basolateral side. This makes it unlikely that there is a major class of late structures accessible only to apically endocytosed ligands, but a subclass of structures only labeled by basolaterally administered markers may exist.

Estimates of the absolute volumes and surface areas of the endocytic compartments per MDCK cell, as well as some morphometric features of the cell, are given in Table III. From these estimates it is apparent that the basolateral early endosomes are ~3.5 times larger in volume and in surface area than the apical early endosomes. This is similar to the ratio of the areas of the two surface domains (3.8 ± 0.5:1).3

3. This ratio is significantly lower than that estimated by von Bonsdorff et al. (1985; 76:1). This difference can be explained by variations in the type of filter used as well as in the precise growth conditions, both of which can affect the final appearance of the MDCK cells (unpublished observations).
Figure 6. Late endosomes (prelysosomes) of the MDCK cell. a and b show Epon sections of typical structures labeled with internalized BSA-gold after a 40-min incubation in the basolateral medium at 37°C and a 40-min chase in marker-free medium. The labeled structures are located close to the Golgi (G) and nucleus (N). Numerous internal vesicular profiles are evident. These may not all be free vesicles but simply infoldings of the limiting membrane as indicated by the arrowhead in b. c, d, and e show cryosections from the same experiment as above labeled with anti-MPR and protein A-gold. Internalized basolaterally administered gold (large particles, arrowheads) are located within structures containing relatively high levels of MPR (small gold, arrows). Internal membranes are evident within all the labeled structures and appear as circular profiles (e.g., c and d) or as myelin-like arrays of parallel membranes (d and e). In e, the MPR and internalized gold appear to be segregated to different areas of the same structure. N, nucleus. In c, an asterisk indicates an area of the cell that most likely contained glycogen deposits. These are invariably not retained in our routine frozen sections leaving large “empty” areas. Bars, 0.1 μm.

Discussion

In the preceding paper (Bomsel et al., 1989) a combined biochemical and confocal microscopic approach was used to investigate the spatial organization of the endocytic apparatus in polarized monolayers of MDCK cells and to demonstrate meeting of the apical and basolateral pathways of endocytosis. In this paper, we used an ultrastructural approach to carry out a detailed characterization of the endocytic pathways and in particular to identify the compartment where meeting occurs. In addition, we have obtained estimates for the volumes and surface areas of the endocytic compartments. We were able to distinguish three distinct endocytic structures in the MDCK cell: peripherally located early endosomes, perinuclear late endosomes, and lysosomes.
Figure 7. Putative lysosomes of the MDCK cell. a, b, and c show typical structures labeled with BSA-gold after a 40-min incubation in the basolateral medium at 37°C and an overnight chase in marker-free medium. Large aggregates of gold particles are present within the electron-dense lumen of the labeled structures (a and b) (compare with Fig. 7, a and b). Parallel arrays of membranes were often observed (e.g., b, arrowheads). c shows an unstained section of a parallel experiment in which the cells were reacted for acid phosphatase after fixation. Despite some background cytoplasmic reaction product, the gold-labeled structure is clearly acid phosphatase-positive. d and e show cryosections labeled with anti-MPR antibodies. The electron-dense structures containing internalized gold (large gold, arrow) do not have significant levels of MPR (small gold, arrowheads). In d, an adjacent profile is evident that labels significantly for MPR but is free of the internalized gold. G, Golgi. Bars, 0.1 μm.

Table II. Summary of Volume Density Measurements

| Time | Apical medium | Basolateral medium |
|------|---------------|--------------------|
| min  | % of cytoplasmic volume ± SEM | % of cytoplasmic volume ± SEM |
| HRP  |                |                    |
| 5    | 0.02 ± 0.01   | 0.34 ± 0.05        |
| 10   | 0.11 ± 0.03   | 0.40 ± 0.04        |
| 10   | 0.10 ± 0.03   | 0.37 ± 0.06        |
| (AP) |                |                    |
| 15   | 0.18 ± 0.05   | 0.72 ± 0.08        |
| 30   | 0.32 ± 0.06   | 1.22 ± 0.10        |
| 60   | 0.66 ± 0.09   | 1.68 ± 0.22        |
| 120  | 1.07 ± 0.17   | 2.28 ± 0.21        |
| 120 plus 120 chase | 0.93 ± 0.13 | 1.78 ± 0.23 |

Acid phosphatase

1.74 ± 0.14*  
1.44 ± 0.21†

* HRP in apical and basolateral medium.
† Measurements were restricted to vesicular compartments (diameter > 50 nm) to exclude the Golgi stacks and TGN, which showed variable acid phosphatase activity as in other cell types (see Hand and Oliver, 1984; Griffiths and Simons, 1986).
‡ HRP-labeled acid phosphatase-negative structure.
### Early Endosomes

The early endosomes of the MDCK cell were labeled with fluid-phase markers after short incubations (5–10 min) at 37°C. These structures showed low or negligible labeling for MPR and did not contain detectable amounts of acid phosphatase. In agreement with the confocal microscopic results of Bomsel et al. (1989), our electron microscopic studies have shown that there are two distinct sets of early endosomes in the MDCK cell that receive material only from the adjacent surface domain. The basolateral early endosomes were located very close to the basolateral surface of the cell and the apical early endosomes were shown to lie between the Golgi complex and the apical cell surface.

Although the structure of the apical and basolateral early endosomes was fairly similar, with both containing tubular and vesicular elements, they differed somewhat in their kinetics of filling. The volume of the basolaterally labeled compartment showed no change between 5 and 10 min of incubation at 37°C as previously shown for macrophages and L cells (Steinman et al., 1976) and for baby hamster kidney cells (Griffiths et al., 1989a). In contrast, the volume of the apically labeled compartment showed a fivefold increase over the same time period. This delay in filling the apical early endosome may be indicative of a slower passage of clathrin-coated or uncoated vesicles between the apical cell surface and the apical early endosomes; after 5 min, insufficient HRP may have been delivered to these structures to reach the threshold concentration necessary for detection. Our observation of HRP-labeled uncoated vesicles underlying the apical surface after short periods of internalization is consistent with this interpretation. Whether there exists some filamentous network analogous to (although less extensive than) the terminal web of the brush border, which retards the movement of these vesicles is unknown at present.

### Late Endosomes and Lysosomes

How the apical and basolateral early endosomes are kept distinct is not clear. Recent studies have shown that one of the most striking characteristics of early endosomes in vitro is their tendency to fuse rapidly and efficiently with each other (Gruenberg and Howell, 1987; Braell, 1987; Diaz et al., 1988). Clearly, few of the apical and basolateral endosomes of MDCK cells fuse with one another in vivo or mixing of their content would be observed. This could be due to the two classes of endosomes lacking the competence to fuse with one another or, alternatively, they may simply be kept spatially separate by cytoskeletal elements.

The importance of maintaining the distinct apical and basolateral early endosomes is shown by the functional characterization of the endocytic pathways presented in the preceding companion paper (Bomsel et al., 1989). Sorting of fluid to transcytosis and recycling was shown to be mediated mainly by the early endosomes underlying the two surface domains. There was no evidence for later compartments having a major role in recycling or transcytosing the internalized marker. The separation of the endocytic pathways at the level of the early endosome thus ensures that no mixing of components from the apical and basolateral surface domains occurs before sorting takes place, and that the two sets of endosomes must only deal with incoming material from one surface.

The apical early endosomes are estimated to occupy 0.10% of the cytoplasmic volume, an absolute volume of 0.96 μm³ per cell, and to have a surface area of 53 μm² per cell. The apical endosomes are therefore approximately three to four times smaller in volume and in surface area than the basolateral early endosomes, a volume of 3.53 μm³, and surface area of 152 μm². This ratio is similar to the ratio of the surface areas of the two surface domains (apical-to-basolateral ratio 1:3.8) and to the total endocytic rate from the two domains (1:36, Bomsel et al., 1989). The size of the early endosomal compartment thus may be related to the total constitutive endocytic uptake from the surface, which in turn depends on the surface area available for endocytosis.

### Table III. Summary of Volume and Surface Area Estimates for the MDCK I Cell

|                          | Value ± SEM |
|--------------------------|-------------|
| Cell volume              | 1,163 ± 200 μm³ |
| Cytoplasmic volume       | 955 ± 184 μm³  |
| Apical membrane area     | 478 ± 102 μm²  |
| Basolateral membrane area| 1,798 ± 341 μm²|
| Basolateral/apical area ratio | 3.8 ± 0.5 μm² |
| Apical early endosome†   | 0.96 ± 0.34 μm³ |
| Absolute surface per cell| 48 ± 17 μm²    |
| Basolateral early endosome† | 3.53 ± 0.88 μm³ |
| Absolute surface per cell| 152 ± 39 μm²   |
| Late endosomes and lysosomes‡ | 13.8 ± 3.3 μm³ |
| Absolute surface per cell| 248 ± 72 μm²   |

* Calculated from the volume density measurements for acid phosphatase-negative structures labeled with HRP after a 10-min apical or basolateral incubation at 37°C. For these measurements, HRP limited to the periphery of a structure was assumed to be in a tubule surrounding an electron-lucent area (see text). If the electron-lucent area is assumed to be part of the early endosomal compartment the corrected values would be as follows: apical early endosome volume = 1.14 ± 0.40 μm³, surface area = 46 ± 17 μm²; basolateral early endosome volume = 4.49 ± 1.11 μm³, surface area = 126 ± 32 μm².

‡ Calculated from the volume density of the acid phosphatase-positive compartment.

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only one of the late endosomes is shown receiving internalized material from the two domains, but colocalization of apical markers was observed even in structures basal to the nucleus. Finally, interendosomes dominantly in the apical portion of the cell.

The late endosomes in MDCK cells are predominantly located all around the nucleus. Ligands finally accumulate within apically located MPR-negative lysosomes. This organization is unknown at present. However, preliminary observations on MDCK cells have suggested that the late endosomes may be a highly dynamic compartment. These structures move apically or basally upon changing the intracellular pH (Parton, R., G. R. Bacallao, K. Simon, and K. Prydz, manuscript in preparation), consistent with results in nonpolarized cells (Heuser, 1989).

Morphologically, lysosomes showed some similarities in structure to the late endosomes but vesicular membrane contents were rarely observed and the lumen of the lysosomes appeared more condensed and electron dense. Myelin-like arrays of membranes were, however, often observed in lysosomes. The lysosomes showed a different spatial distribution in the cell to the late endosomes, being almost exclusively located in the apical half of the cell. The functional significance of this organization is unknown at present. However, preliminary observations on MDCK cells have suggested that the late endosomes may be a highly dynamic compartment. These structures move apically or basally upon changing the intracellular pH (Parton, R., G. R. Bacallao, K. Simon, and K. Prydz, manuscript in preparation), consistent with results in nonpolarized cells (Heuser, 1989).

**Conclusions**

It is apparent that the endocytic pathway shows a very high degree of spatial organization within the MDCK cell (as summarized in Fig. 8). The two distinct sets of early endosomes, functionally defined as mediating recycling and transcytosis, are located peripherally in the cell. The pathways from these early endosomes to the lysosomes converge at the level of the MPR-enriched late endosomes or prelysosomes located all around the nucleus. Ligands finally accumulate within apically located MPR-negative lysosomes. This orga-

![Figure 8. Schematic diagram of the endocytic pathways in the MDCK cell.](image-url)
ization is in marked contrast to the organization of the endocytic apparatus of MDCK cells grown on an impermeable plastic support. Matlin et al. (1983) showed that a marker for the apical surface appeared within endocytic structures distributed throughout the whole cell after just a few minutes of internalization at 37°C. These differences may well reflect the lower degree of organization of cytoskeletal elements (particularly microtubules) within the plastic-grown cell as compared with the filter-grown cell. Filter-grown MDCK cells show a distinct pattern of microtubules, with 90% of the microtubules that run vertically through the cell having the same polarity (Bacallao et al., 1989). This specialized organization may well prove to be important in directing membrane traffic in the polarized cell during both endocytosis and exocytosis.

The calculated total uptake rates for the two surface domains of the MDCK cell (Bomsel et al., 1989) correspond to the internalization of ~120 vesicles (internal diameter, 100 nm; volume, 5 × 10^-4 μm^3) from the apical surface per minute per cell and 440 vesicles per minute from the basolateral surface. On this basis, one cell would internalize ~0.7% of the area of each plasma membrane surface per minute. Thus, an area of plasma membrane equivalent to ~60 100-nm-diam vesicles per minute per cell in each direction, it is clear that very efficient sorting mechanisms must exist to maintain the distinct composition of the two domains. Clearly, the MDCK cell system will be a useful model system to study these mechanisms.

Special thanks are due to Jean Gruenberg for numerous helpful discussions and for advice about the manuscript. We would also like to thank John Tooze for comments on the manuscript; Hilkka Virta for help with cell culture; Ruth Back for technical assistance; and Helen Fry for efficient secretarial work. Transwell filters were kindly provided by H. Lane (Costar Corp., Cambridge, MA).

R. G. Parton was a recipient of a one-year fellowship from the National Council of Science and Humanities (NAVF), Norway; and M. Bomsel was a recipient of a one-year EMBO fellowship.

Received for publication on 16 June 1989 and in revised form 18 September 1989.

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