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The Recycling Endosome of Madin-Darby Canine Kidney Cells Is a Mildly Acidic Compartment Rich in Raft Components

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We present a biochemical and morphological characterization of recycling endosomes containing the transferrin receptor in the epithelial Madin-Darby canine kidney cell line. We find that recycling endosomes are enriched in molecules known to regulate transferrin recycling but lack proteins involved in early endosome membrane dynamics, indicating that recycling endosomes are distinct from conventional early endosomes. We also find that recycling endosomes are less acidic than early endosomes because they lack a functional vacuolar ATPase. Furthermore, we show that recycling endosomes can be reached by apically internalized tracers, confirming that the apical endocytic pathway intersects the transferrin pathway. Strikingly, recycling endosomes are enriched in the raft lipids sphingomyelin and cholesterol as well as in the raft-associated proteins caveolin-1 and flotillin-1. These observations may suggest that a lipid-based sorting mechanism operates along the Madin-Darby canine kidney recycling pathway, contributing to the maintenance of cell polarity. Altogether, our data indicate that recycling endosomes and early endosomes differ functionally and biochemically and thus that different molecular mechanisms regulate protein sorting and membrane traffic at each step of the receptor recycling pathway.

INTRODUCTION

Receptor-mediated endocytosis involves sequential passage through distinct endosomal compartments. Internalized molecules first arrive in early endosomes, where a mildly acidic luminal pH favors uncoupling of ligands and receptors (Geuze *et al.*, 1983; Yamashiro and Maxfield, 1984). Ligands destined for degradation, such as low-density lipoprotein (LDL), are then forwarded toward late endosomes and lysosomes, whereas housekeeping receptors, such as LDL-receptor (LDLR) and transferrin receptor (TfR), are recycled back to the plasma membrane via recycling endosomes to undergo further rounds of internalization (Gruenberg and Maxfield, 1995). Organelles involved in degradation or recycling exhibit strikingly different characteristics. The luminal pH decreases by more than a pH unit along the degradative pathway but was shown to increase along the recycling pathway in nonpolarized Chinese hamster ovary (CHO) cells (Yamashiro *et al.*, 1984). Whereas endosomes along the degradative pathway contain numerous internal membranes, recycling endosomes consist of networks of 60-nm tubules organized around the microtubule-organizing center in some cell types. Early endosomes, which are common to both pathways, exhibit a complex cisternal, tubular, and vesicular organization. Although clear morphological differences can be observed between organelles on the two legs of the endocytic pathway, the molecular basis and the functional significance of these differences are not understood.

Segregation of ligands destined for degradation or recycling takes place with rapid kinetics (half-life < 3 min) in the early endosome (Yamashiro and Maxfield, 1987). A few sequence motifs responsible for sorting of proteins destined for late endosomes have been identified (Green *et al.*, 1994; Subtil *et al.*, 1997; Blagoveshchenskaya *et al.*, 1998; Piguet *et al.*, 1999). Formation of transport intermediates along the

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degradative pathway depends on the acidic pH of the early endosome (Clague *et al.*, 1994), on the small GTP-binding protein ARF1 (Gu and Gruenberg, 2000), and on COPI coat proteins (Whitney *et al.*, 1995; Aniento *et al.*, 1996), and their transport is facilitated by polymerized microtubules (Gruenberg *et al.*, 1989). Finally, N-ethylmaleimide sensitive factor (Robinson *et al.*, 1997) and perhaps the small GTPase rab7 (Feng *et al.*, 1995) are necessary for their docking and/or fusion with late endosomes. The molecular mechanisms responsible for receptor recycling remain unclear. Until now, no sorting signals have been identified in the cytoplasmic domain of recycling proteins. Therefore, it has been proposed that recycling receptors may be sorted by iterative fractionation (Dunn *et al.*, 1989; Mayor *et al.*, 1993). Several molecules were shown to play a role in TfR recycling, including cellubrevin, SNAP23, syntaxin13, calmodulin, the unconventional myosin myr4, EFA6, rab11bp, and the small GTPases rab4, rab11, rhoA, rhoD, and ARF6 (van der Sluijs *et al.*, 1992; Apodaca *et al.*, 1994a; Galli *et al.*, 1994; D'Souza-Schorey *et al.*, 1995; Murphy *et al.*, 1996; Ullrich *et al.*, 1996; Leung *et al.*, 1998, 1999; Prekeris *et al.*, 1998; Franco *et al.*, 1999; Zeng *et al.*, 1999; Huber *et al.*, 2000), but their precise functions along the recycling pathway are not clear.

Polarized epithelial cells, such as Madin-Darby canine kidney (MDCK) cells, present additional complexity, because endocytosis occurs from both apical and basolateral plasma membrane domains (Parton, 1991; Mostov and Cardone, 1995). Whereas distinct sets of early endosomes are associated with each plasma membrane domain, late endosomes and lysosomes are common to both pathways (Bomsel *et al.*, 1989, 1990; Parton *et al.*, 1989). More recent studies indicate that communication also exists between apical and basolateral early endosomes, including along the routes followed by the TfR and the polymeric immunoglobulin (Ig) A receptor (Hughson and Hopkins, 1990; Apodaca *et al.*, 1994b; Barroso and Sztul, 1994; Hunziker and Peters, 1998; Fialka *et al.*, 1999). Whereas TfR constitutively cycles between the basolateral plasma membrane and the early endosomal system, ligand-bound polymeric IgR is transcytosed from the basolateral to the apical cell surface (Fuller and Simons, 1986; Mostov and Cardone, 1995). Despite their different final destinations and intracellular trafficking routes, both receptors can be observed within a network of thin tubules clustered in the apical region of the cell, which can also be reached by membrane-bound markers internalized from the apical surface (Apodaca *et al.*, 1994b; Barroso and Sztul, 1994; Hunziker and Peters, 1998). These elements, which closely resemble the recycling endosomes observed in some nonpolarized cells (Yamashiro and Maxfield, 1984; Marsh *et al.*, 1995), have sometimes been referred to as the apical recycling compartment (ARC) (Apodaca *et al.*, 1994b). It is not clear whether passage through this apically located recycling endosome is obligatory for basolaterally recycling molecules or whether a specialized transcytosis compartment exists downstream of the ARC (Gibson *et al.*, 1998). Importantly, protein sorting must occur in the ARC, because both transcytosing and recycling molecules have been shown to colocalize within the same structures before being selectively transported to opposite plasma membrane domains.

To better characterize recycling endosomes in polarized MDCK cells at the molecular level, we have established a subcellular fractionation protocol to specifically isolate the compartment. We show that the protein composition of recycling endosomes is distinct from that of sorting endosomes, providing biochemical evidence that the two organelles are physically separated from each other. Our data show that the luminal pH of recycling endosomes in polarized cells is less acidic than that of sorting endosomes and suggest that this pH difference is due to the absence of functional vacuolar ATPase in recycling endosomes. Last, we show that lipids and proteins generally believed to associate into membrane microdomains are highly enriched in recycling endosomes, probably creating a unique lipid environment within recycling endosomes. We propose that this environment may contribute to protein and lipid sorting along the recycling and/or transcytotic pathways.

MATERIALS AND METHODS

Reagents

M450 sheep anti-mouse Dynabeads were from Dynal (Oslo, Norway). Fluorescent probes were from Molecular Probes Europe (Leiden, The Netherlands). Human holo-Tf conjugated with HRP, apo-Tf, HRP, filipin, and fish skin gelatin were from Sigma (Division of Fluka Chemie, Buchs, Switzerland). Immobilized pH gradient strips were from Amersham Pharmacia Biotech (Dübendorf, Switzerland). All chemical reagents were from Fluka Chemie or Merck (Dietikon, Switzerland). Tissue culture media were from Life Technologies (Basel, Switzerland).

Antibodies

The anti-myc hybridoma cell line (MYC1-9E10.2; CRL1729) was obtained from the American Type Culture Collection (Rockville, MD). Antibodies against the luminal (B3/25) and cytoplasmic (H68.4) domains of the human TfR were purchased from Boehringer Mannheim (Rotkreuz, Switzerland) and Zymed Laboratories (San Francisco, CA), respectively. Anti-ZO1 and anti-caveolin-1 antibodies were purchased from Chemicon International (Temecula, CA) and Transduction Laboratories (Basel, Switzerland), respectively. Antibodies against β1 and β2 adaptins were purchased from Sigma. Anti-peptide antibodies against p23, Rab4, and Rab7 were raised in rabbits and affinity purified as described (Rojo *et al.*, 1997). Other antibodies were generous gifts: annexin I and annexin II (V. Gerke, University of Münster, Münster, Germany), vacuolar ATPase A subunit (D. Stone, University of Texas Southwestern Medical Center, Dallas, TX), cellubrevin and rab5 (R. Jahn, Max Planck Institute, Göttingen, Germany), rab11 (R. Parton, University of Queensland, Brisbane, Australia), EEA1 (H. Stenmark, Norwegian Radium Hospital, Oslo, Norway), and calnexin (A. Helenius, Federal Polytechnical School, Zürich, Switzerland). Secondary antibodies were from Amersham Pharmacia Biotech or Dianova (Hamburg, Germany).

Cells

MDCK II cells were stably transfected with the pCB6 plasmid containing the human TfR with (m-hTfR cells) or without (hTfR cells) a single myc tag at its cytoplasmic N terminus. Transfection was carried out by the calcium phosphate method and was followed by selection with G418 (Life Technologies). To avoid loss of expression, cells were not used for more than 8–10 passages. Cells were maintained as described (Bomsel *et al.*, 1989). Unless indicated otherwise, cells were seeded at high confluence onto prewet polycarbonate filters (Corning Costar Europe, Badhoevedorp, The Netherlands) and used after 4 d in culture with daily medium changes.

Labeling Conditions

To label the plasma membrane, the basolateral side of the cells was incubated on ice with 50 μg/ml Tf-HRP in internalization medium (IM) (of G-MEM, 10 mM Hepes pH 7.4, 5 mM glucose) containing 2 mg/ml BSA (IM/BSA). Unbound label was removed by two washes with ice-cold PBS+/BSA (5 mg/ml BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$). To label recycling endosomes, Tf conjugates (HRP or rhodamine; 50 μg/ml) were prebound on ice and then internalized at 37°C for 10 min in IM/BSA. Residual plasma membrane label was removed by an ice-cold deferoxamine mesylate wash as described (Jing *et al.*, 1990). Sphingomyelin–BODIPY (4,4-difluoro-4-bora-32,42-diaza-s-indacene) (5 μM) was added to both sides of the filters for 30 min at 37°C simultaneously with continuous Tf–rhodamine (25 μg/ml) uptake. To label apical early endosomes, dextran–Oregon Green (10 kDa dextran; 3 mg/ml in IM) or HRP (5 mg/ml in IM) was added to the cells from the apical side for 10 min at 37°C. Noninternalized label was removed by three washes with PBS+/BSA. To label late endosomes, cells were incubated with HRP (5 mg/ml) at 37°C for 15 min and the label was chased for 30 min in IM/BSA. When cells were labeled with HRP or Tf-HRP, enzymatic activity was measured as described (Gruenberg *et al.*, 1989).

Fluorescence

For fluorescence experiments, cells were grown on either glass coverslips or 12-mm Transclear Costar filters. When appropriate, filter-grown cells were labeled with lysine-fixable endocytic tracers before fixation. In this case, ice-cold 3% paraformaldehyde (PFA) was added to the cells and fixation was completed after 20 min at room temperature. For immunofluorescence experiments, cells were fixed with either 3% PFA for 20 min at room temperature or with methyl alcohol for 4 min at −20°C. PFA autofluorescence was quenched with 50 mM NH_4Cl, and the cells were permeabilized with 0.05% saponin during incubation with the primary antibody. Fish skin gelatin (0.2%) was used to block unspecific binding. Cholesterol was labeled with filipin (50 μg/ml) after PFA fixation as described (Kobayashi *et al.*, 1999). Antibodies were added to the apical side of whole filters. Filters were cut out from their supports, mounted onto microscope slides in Mowiol (Calbiochem, La Jolla, CA), and observed with a confocal laser scanning microscope as described (Rojo *et al.*, 1997). When cells were labeled with sphingomyelin–BODIPY, the filters were mounted onto microscope slides in ice-cold PBS+ and observed without previous fixation.

Immunoelectron Microscopy

MDCK cells were perforated and labeled exactly as described previously (Ikonen *et al.*, 1996). Briefly, MDCK cells grown on filters were perforated with the use of nitrocellulose filters applied to the apical surface. The opened cells were then labeled with anti-caveolin-1 antibodies (Dupree *et al.*, 1993) followed by 10-nm protein A–gold (University of Utrecht, Utrecht, The Netherlands) and embedded in Epon. Cells were sectioned perpendicular to the filter support. Sections were examined on a JEOL (Tokyo, Japan) 1010 microscope.

Subcellular Fractionation

All experiments were carried out with m-hTfR and hTfR cells in parallel. Cells grown on 75-mm Costar filters were scraped with a rubber policeman, and postnuclear supernatants were prepared as described (Bomsel *et al.*, 1990). The postnuclear supernatants (4 mg/ml, 500 μl) were precleared by centrifugation at 13,000 rpm for 5 min and then incubated with 9E10 culture supernatant (0.05 mg/ml) for 30 min on a rotating wheel at 2 rpm. Unspecific binding was reduced by a 15-min incubation with 0.3 M KCl. Salt washes were omitted when assaying for the presence of EEA1 in the isolated fractions. Endosomal membranes were then sedimented through a 20% sucrose cushion onto a 35% sucrose cushion at 150,000 × *g* in a swing-out ultracentrifuge rotor. For immunoisolation, the 20/35% sucrose interface was collected and membranes (25 μg) were incubated with anti-mouse Dynabeads (50-μl slurry) in PBS containing 5 mg/ml BSA for 2 h on a rotating wheel at 2 rpm. Fifty percent of the input material for the immunoisolation, referred to as "IN," and the unbound material after immunoisolation, referred to as "UB," were sedimented at 150,000 × *g* for 30 min and resuspended in sample buffer. The beads were washed with PBS containing 5 mg/ml BSA and 0.3 M KCl, and the final bead pellet was taken up in sample buffer ("B").

pH Measurements

Subconfluent m-hTfR cells, grown on glass coverslips, were labeled either by preincubation with Tf-FITC (50 μg/ml) at 4°C followed by internalization at room temperature for 5 min or by continuous internalization with Tf-FITC (25 μg/ml) for 20 min at 37°C. Endosomal pH was measured by ratio fluorescence imaging as described (Piguet *et al.*, 1999) with the use of a Zeiss Axiovert S100 TV fluorescence microscope and a 100×, 1.3 numerical aperture oil-immersion objective (Carl Zeiss, Feldbach, Switzerland). Coverslips were inserted into a perfusion chamber (Medical Systems, Greenvale, NY) at room temperature in 1 ml of IM and imaged with a 12-bit, cooled charge-coupled device interlined camera (Visitron Systems, Puchheim, Germany) controlled by MetaMorph/Metafluor software (Universal Imaging, West Chester, PA). Images were acquired for 1 s a wave length (λ) = 490 ± 6 nm and for 2 s at λ = 440 ± 6 nm with the use of a DeltaRam monochromator (PTI, Monmouth Junction, NJ), a 510 DRLP dichroic mirror, and a 535 ± 25 nm emission filter (Omega Opticals, Brattleboro, VT). Calibration and image processing were performed as described previously (Demaurex *et al.*, 1998).

Lipid Analysis

Cells were metabolically labeled overnight with [^{32}P]orthophosphate (0.5 mCi/filter; Amersham Pharmacia Biotech) or [^{14}C]acetate (50 μCi/filter; Amersham Pharmacia Biotech), and purified endosomal fractions were prepared. Lipid extraction was carried out as described (Kobayashi *et al.*, 1998b). Phospholipids were separated by two-dimensional chromatography on 10 × 10 cm silica gel 60 HPTLC plates (Merck) in chloroform:methanol:32% ammonia (65:35:5, vol/vol) for the first dimension and chloroform:acetone:methanol:acetic acid:water (50:20:10:12.5:5, vol/vol) for the second dimension. Cholesterol was separated in one dimension by TLC. The migration was carried out in two steps, first in chloroform:methanol:32% ammonia (65:35:5, vol/vol) to a height of 6 cm, then in hexane:diethyl ether:acetic acid (16:4.2, vol/vol) to the top. Lipids were detected by autoradiography and quantitated with a PhosphorImager with the use of the Molecular Imager System (Bio-Rad Laboratories, Glattbrugg, Switzerland).

Electrophoresis

Bidimensional electrophoresis was carried out as described (Pasquali *et al.*, 1997). Cells were labeled metabolically overnight with [^{35}S]Met (0.5 mCi/filter; New England Nuclear Life Science Products, Brussels, Belgium), and purified endosomal fractions were prepared. Samples were taken up directly in 400 μl of isoelectric focusing buffer and loaded in-gel for 8 h at room temperature with the use of 18-cm immobilized pH gradient strips with nonlinear isoelectric point gradients from 3.5 to 10. The first dimension was run for 65 kV/h at 15°C. The second dimension was run on 9–16% SDS-polyacrylamide gels at 6°C. The gels were treated with Entensify (New England Nuclear Life Science Products) and dried, and proteins were revealed by autoradiography.

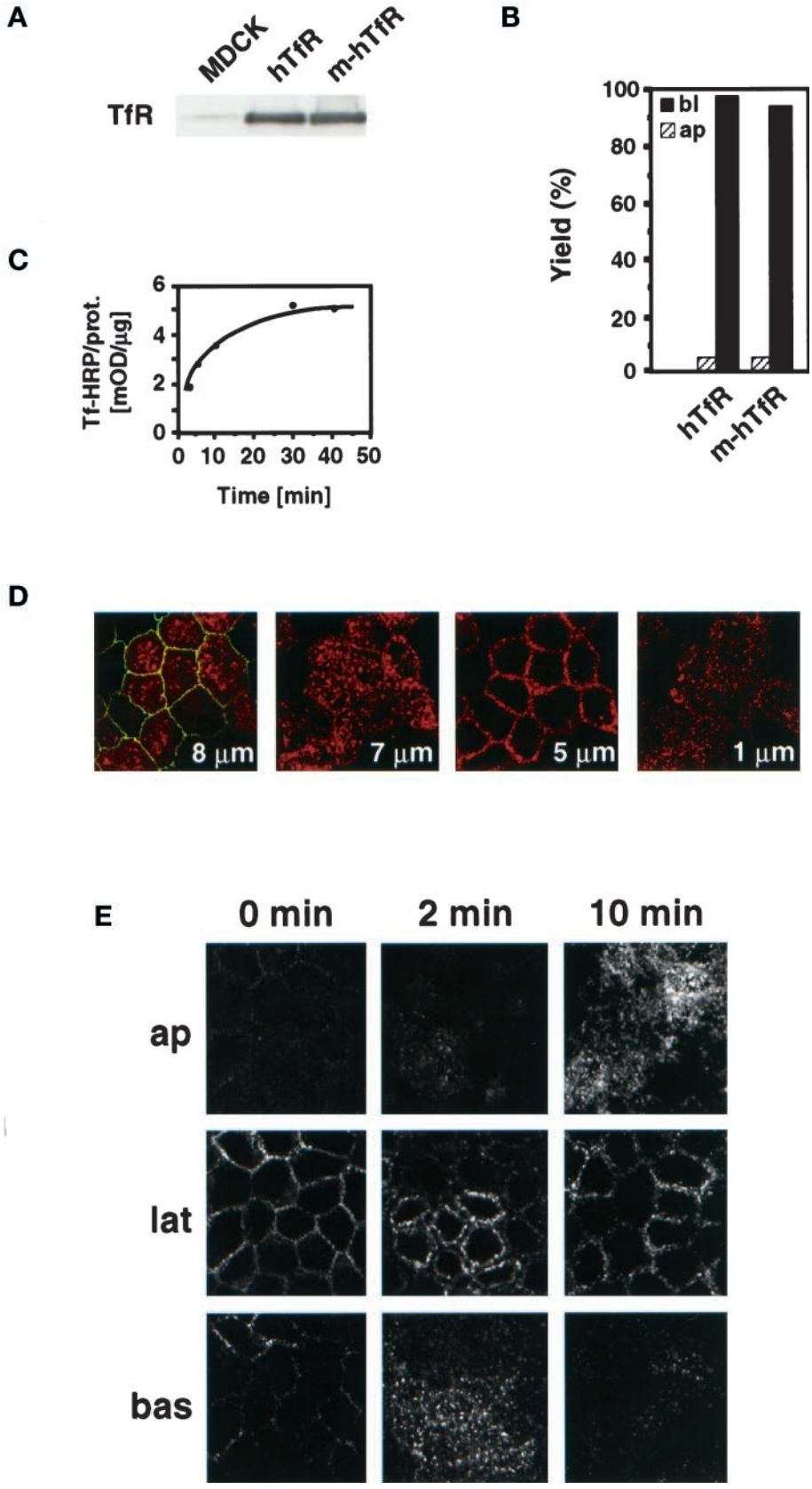


Figure 1. Characterization of the hTfR-expressing MDCK cell lines. (A) Total cell extracts prepared from untransfected MDCK cells and cells expressing hTfR or m-hTfR were analyzed by SDS-PAGE and Western blotting with the use of antibodies against hTfR. (B) Cell surface distribution of TfR was measured by incubating the apical (ap) or basolateral (bl) side of filter-grown hTfR or m-hTfR cells with Tf-HRP at 4°C. Results are presented as percentages of total cell-associated HRP activity. (C) Continuous Tf-HRP uptake at 37°C was measured in polarized m-hTfR cells as a function of time. Cells were incubated with Tf-HRP. At the indicated times, cell-associated HRP activity was quantified and normalized to total cellular protein. (D) TfR (red) topology in polarized m-hTfR cells was analyzed by immunofluorescence confocal microscopy with the use of antibodies against hTfR. Tight junctions (green) were identified with the use of antibodies against ZO1. The distance between the section plane and the filter is indicated on each panel. (E) Cellular distribution of endocytosed Tf–rhodamine. Tf–rhodamine was bound to the basolateral plasma membrane at 4°C and internalized at 37°C for the indicated times. Confocal microscope sections taken through the apical (ap), lateral (lat), and basal (bas) regions of the cells are shown.

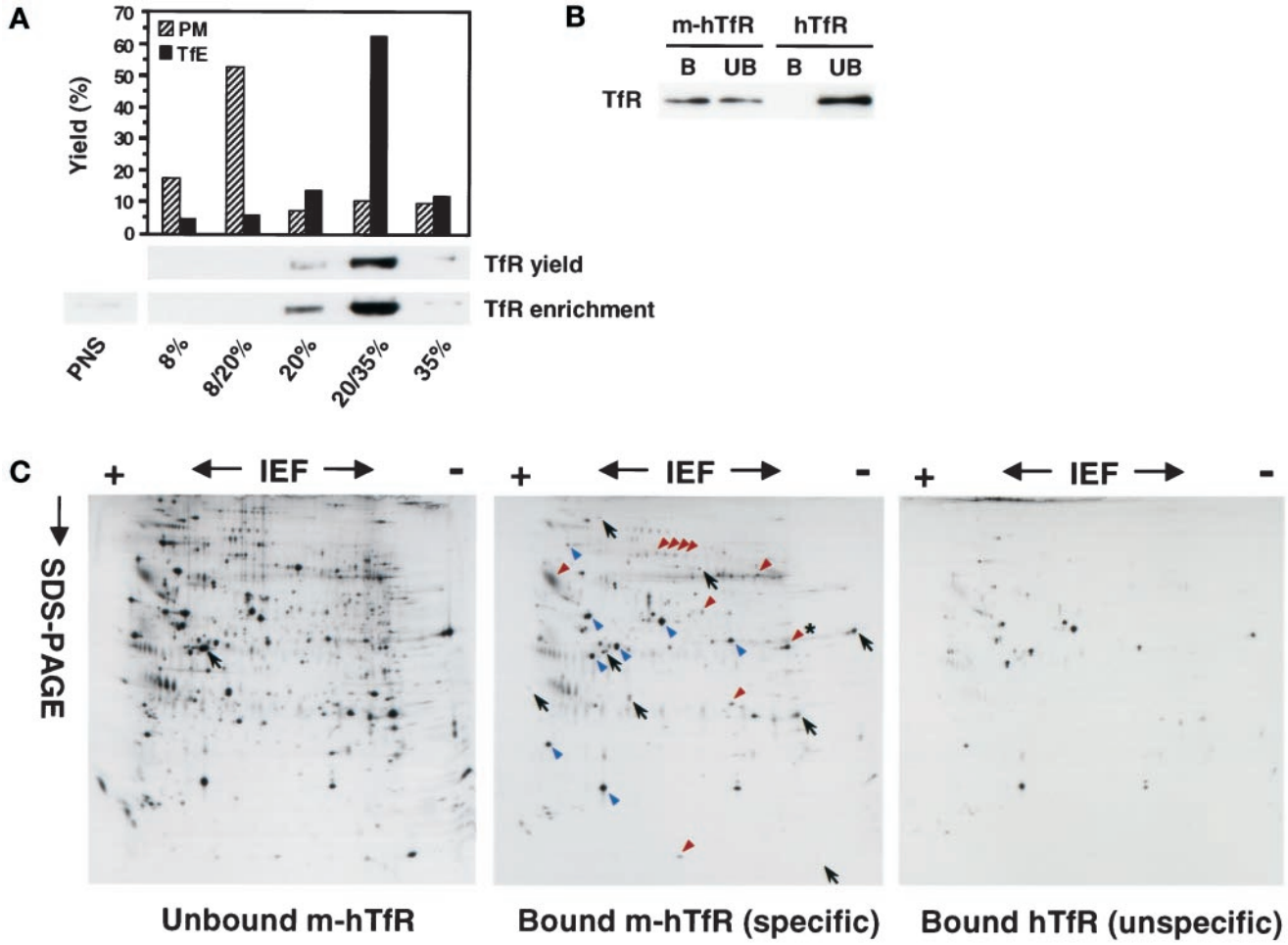


Figure 2. Purification of TfR-positive endosomes. (A) Lower panel, PNSs prepared from cells expressing m-hTfR were fractionated on a discontinuous sucrose gradient loaded with 8, 20, and 35% sucrose. After centrifugation, fractions were collected and analyzed by SDS-PAGE and Western blotting with the use of anti-TfR antibodies as in Figure 1A. Lanes were loaded with either the same amount of protein (enrichment) or 10% of the volume (yield) of each fraction. Upper panel, To label the plasma membrane (PM), Tf-HRP was bound to the surface of m-hTfR cells at 4°C. To label recycling endosomes (TfE), Tf-HRP was bound to the surface of m-hTfR cells at 4°C and internalized for 10 min at 37°C. PNSs were prepared and fractionated, and the HRP content of the fractions was quantified. (B) Membranes enriched in m-hTfR were prepared with the use of the sucrose gradient, as in A, and further fractionated by immunoisolation with the 9E10 mAb against the myc tag and magnetic beads. As a control for nonspecific binding, the whole purification procedure was carried out in parallel with hTfR cells. Beads were retrieved with a magnet, and then both bound material (B) and unbound material sedimented at 150,000 × *g* for 30 min (UB) were analyzed for TfR content by Western blotting as in Figure 1A. (C) The purification protocol was carried out as in B with the use of m-hTfR and hTfR cells that had been metabolically labeled with [^{35}S]Met, and samples were analyzed by high-resolution two-dimensional gel electrophoresis followed by autoradiography. The general protein pattern of the immunoisolated fraction [Bound m-hTfR (specific)] was compared with that of the unbound material sedimented at 150,000 × *g* for 30 min [Unbound m-hTfR] to determine which proteins were specifically immunoisolated (examples are indicated with red arrowheads). The asterisk indicates the position of flotillin-1 in the same gel system. Examples of proteins that were not isolated are indicated with black arrows. Contaminants (blue arrowheads) were determined by comparison with samples from hTfR cells [Bound hTfR (unspecific)]. The position of actin, which is commonly used for orientation in this type of analysis, is indicated on the unbound m-hTfR gel.

RESULTS

Characterization of TfR-expressing MDCK Cell Lines

To characterize endosomes along the TfR recycling pathway in polarized MDCK cells, we decided to use an immunoaffinity purification protocol. We generated stable cell lines expressing human TfR, myc-tagged at the N-terminal cytoplasmic domain (m-hTfR), to allow isolation of endosomes containing m-hTfR with the anti-myc mAb 9E10 on magnetic beads coated with anti-mouse antibodies. As a control, we also prepared stable cell lines expressing untagged human TfR (hTfR), and we carried out all experiments in parallel with cells expressing comparable levels of hTfR or m-hTfR (Figure 1A). We felt that this strategy would provide the most stringent conditions for controlling the specificity

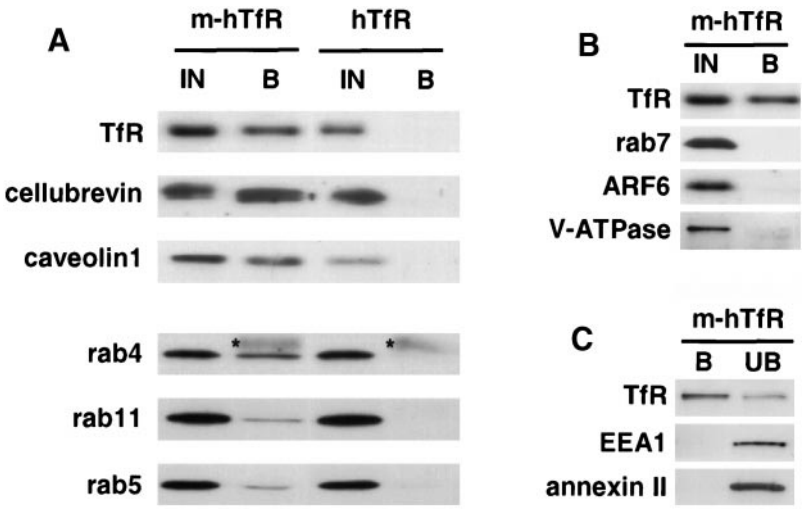


Figure 3. Protein composition of TfR-positive endosomes. Endosomes containing m-hTfR were purified, with the use of cells expressing hTfR as a control, as in Figure 2, B and C. (A and B) Immunoisolated membranes bound to the beads (B) were analyzed by SDS-PAGE and Western blotting with the use of specific antibodies against the indicated proteins. For comparison, 50% of the total fraction used as starting material for immunoisolation (IN) was also analyzed. Asterisks mark the light chain of the antibody used for immunoisolation. (C) Immunoisolated membranes (B) are compared with unbound material sedimented at 150,000 × *g* for 30 min (UB) as in Figure 2B. Analysis was as in A and B, with the use of specific antibodies against the indicated proteins.

of immunoisolation. This proved necessary, because optimization tests revealed that beads without antibodies or beads coated with irrelevant antibodies, which have been commonly used by us and others as negative controls, frequently underestimate nonspecific binding to the beads, which largely depends on the quality of the antibody preparation.

Both cell lines showed the characteristic features of polarized MDCK cells. Like parental cells, cells expressing hTfR or m-hTfR reached a height of ~12 μm, and their transepithelial resistance was >200 Ω/cm^2. The normal polarization of the cells was further confirmed by the typical distribution of ZO1, a marker of tight junctions (Stevenson *et al.*, 1986), which distributed in both cell lines as a ring around the apical portion of the cell (Figure 1D). We next analyzed the cell surface distribution of hTfR and m-hTfR in our cell lines, because endogenous cell surface TfR molecules are known to be restricted to the basolateral plasma membrane domain in MDCK cells (Fuller and Simons, 1986). Binding of human Tf conjugated to HRP (hTf-HRP) to either the apical or the basolateral plasma membrane at 4°C showed that cell surface human receptors were >95% basolateral at steady state (Figure 1B). Under our conditions, parental MDCK II cells did not bind hTf. Exogenous hTfR or m-hTfR was mostly intracellular and could be found within structures that extended throughout the apical and basolateral cytoplasm of fully polarized cells (Figure 1D). No TfR could be detected in the apical pole of the cells above the level of the tight junctions or on the apical plasma membrane. Both m-hTfR (Figure 1C) and hTfR supported hTf-HRP endocytosis, and the kinetics of the process were similar to those reported previously (Harding *et al.*, 1983). Tf–rhodamine endocytosed by cells expressing m-hTfR (Figure 1E) or hTfR distributed first to basolateral endosomes after brief incubation times and then to subapical endosomes, presumably corresponding to the ARC (Apodaca *et al.*, 1994b). These morphological and biochemical observations demonstrate that hTfR and m-hTfR were correctly localized and functional in these cell lines.

Isolation of TfR-positive Endosomes

For immunoisolation experiments, postnuclear supernatants (PNSs) were prepared from m-hTfR and hTfR cells and incubated at 4°C with the anti-myc antibody. In a second step, PNSs from these cells were fractionated on a discontinuous sucrose gradient to remove the excess free antibody but also to prepare TfR-enriched fractions for subsequent immunoisolation. The bulk of total cellular TfR (65%) was recovered at the 20/35% interface of the gradient, where the receptor was enriched ≈10-fold (Figure 2A; see also Figure 8B). Because the bulk of the receptor is in endosomes at steady state (Figure 1D), this fraction is likely to contain mostly endosomal TfR. A minor portion of TfR is present on the basolateral cell surface at steady state. Therefore, we also analyzed the distribution of the basolateral plasma membrane on the gradient and found that it was enriched at the 8/20% interface and not at the 20/35% interface (Figure 2A). In a third step, fractions recovered from the 20/35% interface were incubated at 4°C with magnetic beads coated with anti-mouse antibodies and washed. Bead-bound membranes were then recovered with a magnet and analyzed. TfR-containing membranes were immunoisolated with a 50% yield from m-hTfR cells (Figure 2B), and nonspecific binding was negligible, as determined by carrying out the same experiment with hTfR cells (Figure 2B). The total enrichment of the purification is estimated to ≈150-fold with a yield of ≈35% (see Figure 8). The immunoisolated fraction did not contain detectable levels of calnexin, an abundant protein of the endoplasmic reticulum (Trombetta and Helenius, 1998), or p23, an abundant Golgi protein (Rojo *et al.*, 1997). In addition, we did not detect in the fractions the plasma membrane-associated α and β2-adaptins and the *trans*-Golgi network-associated β1-adaptin. (Kirchhausen, 1999), although the bulk remained membrane associated during isolation. The fraction was also devoid of the small GTPase ARF6 and of annexin II (see Figure 3B and DISCUSSION). Annexin II is very abundant on the cytoplasmic face of the plasma membrane and early endosomes and tightly membrane associated (Harder *et al.*, 1997). Altogether, these observations indicate that the purified fraction was not con-

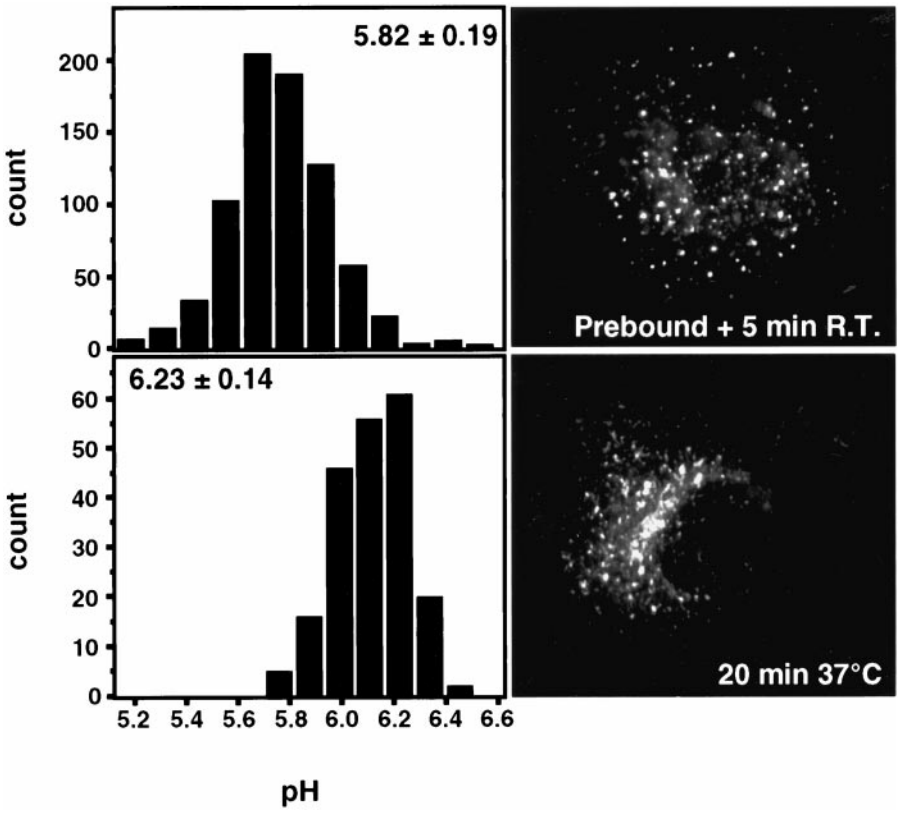


Figure 4. Recycling endosomes in MDCK cells are less acidic than early endosomes. Tf-FITC was prebound at 4°C to m-hTfR cells and then internalized for 5 min at 20°C (upper panels) or internalized continuously for 20 min at 37°C (lower panels). Typical examples of the respective labels are shown. Because of technical constraints, we used subconfluent cells grown on glass coverslips rather than on filters. The pH of individual organelles was measured by fluorescence ratio imaging of internalized Tf-FITC. The histograms show the pH distribution of 767 and 206 endosomes for the upper and lower panels, respectively, with the mean ± SD given in the left panels.

taminated to any significant extent with the plasma membrane or with biosynthetic membranes. To analyze the general protein pattern of TfR-containing membranes, cells were metabolically labeled with [^{35}S]Met before the experiment, and then immunoisolated fractions were analyzed by two-dimensional gel electrophoresis and autoradiography (Figure 2C). Only a limited subset of labeled polypeptides copurified with the TfR, confirming that the immunoisolation procedure was highly specific (the protein marked with an asterisk in Figure 2C is discussed below). Isolated proteins (red arrowheads) were highly enriched compared with the unbound material recovered after immunoisolation (black arrows) and were not detected in control samples obtained from hTfR cells (contaminants indicated with blue arrowheads).

Characterization of Purified TfR-positive Endosomes

We first analyzed our TfR-positive endosomal fractions for their content in proteins reported to regulate Tf recycling. Figure 3A shows that the v-SNARE cellubrevin, which has been implicated in Tf recycling in nonpolarized cells (Galli *et al.*, 1994) and colocalizes with the TfR by immunofluorescence (McMahon *et al.*, 1993), was isolated with the same efficiency as the TfR itself. The small GTPase rab4 also cofractionated with TfR (Figure 3A). This GTPase has been directly implicated in Tf recycling (van der Sluijs *et al.*, 1992) and colocalizes with the TfR in endosomes, but it is absent from the plasma membrane (Van Der Sluijs *et al.*, 1991). Rab11 is present on early endosomal membranes and the *trans*-Golgi network and has been proposed to function in Tf recycling (Ullrich *et al.*, 1996). In addition, rab11 was recently shown to be present on apical recycling endosomes in MDCK cells (Casanova *et al.*, 1999). As shown in Figure 3A, rab11 was coisolated with the TfR, although clearly to a lesser extent than rab4, presumably because of its broader cellular distribution. As a control, Figure 3B shows that, as expected, immunoisolated fractions did not contain the late endosomal marker rab7 (Chavrier *et al.*, 1990). Finally, the small GTP-binding protein ARF6 has also been proposed to play a role in TfR trafficking (D'Souza-Schorey *et al.*, 1995), although its precise function is unclear (D'Souza-Schorey *et al.*, 1997; Radhakrishna and Donaldson, 1997; Song *et al.*, 1998; Altschuler *et al.*, 1999). ARF6 is clearly present on the plasma membrane, but its endosomal localization is controversial (Peters *et al.*, 1995; Cavenagh *et al.*, 1996; D'Souza-Schorey *et al.*, 1998). Although tightly membrane-associated (Gu and Gruenberg, 2000), ARF6 was not detected in the fractions (Figure 3B).

Next, we analyzed the distribution of rab5, which is known to localize to the plasma membrane and early endosomes (Chavrier *et al.*, 1990) and to be involved in both internalization and early endosome docking and/or fusion (Gorvel *et al.*, 1991; Bucci *et al.*, 1992). Much like rab11, rab5 did not efficiently cofractionate with TfR during immunoisolation (Figure 3A), although the protein remained membrane-associated. These observations prompted us to test whether our fractions contained the rab5 effector protein

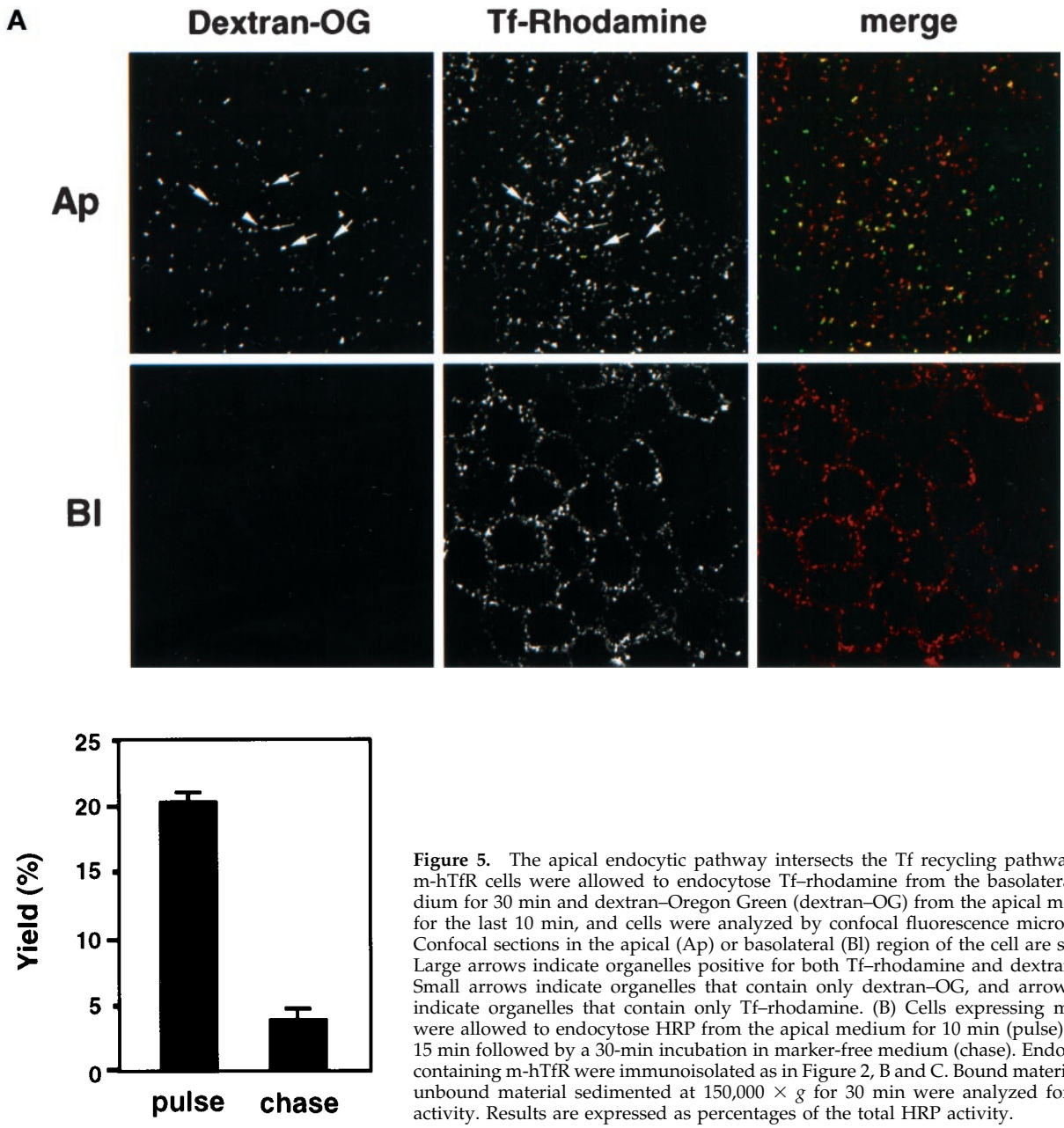


Figure 5. The apical endocytic pathway intersects the Tf recycling pathway. (A) m-hTfR cells were allowed to endocytose Tf–rhodamine from the basolateral medium for 30 min and dextran–Oregon Green (dextran–OG) from the apical medium for the last 10 min, and cells were analyzed by confocal fluorescence microscopy. Confocal sections in the apical (Ap) or basolateral (Bl) region of the cell are shown. Large arrows indicate organelles positive for both Tf–rhodamine and dextran–OG. Small arrows indicate organelles that contain only dextran–OG, and arrowheads indicate organelles that contain only Tf–rhodamine. (B) Cells expressing m-hTfR were allowed to endocytose HRP from the apical medium for 10 min (pulse) or for 15 min followed by a 30-min incubation in marker-free medium (chase). Endosomes containing m-hTfR were immunoisolated as in Figure 2, B and C. Bound material and unbound material sedimented at 150,000 $\times$ *g* for 30 min were analyzed for HRP activity. Results are expressed as percentages of the total HRP activity.

EEA1, which regulates early endosome docking and/or fusion (Simonsen *et al.*, 1998). Interestingly, we could not detect EEA1 in immunoisolated fractions enriched in TfR (Figure 3C). Although only peripherally associated with membranes, EEA1 could be sedimented from the supernatant after immunoisolation, indicating that it remained to a large extent membrane-associated (Figure 3C). We then analyzed the distribution of annexin II, which was also implicated in early endosome dynamics and is an abundant component of the plasma membrane and early endosomes, both in nonpolarized baby hamster kidney cells and in polarized MDCK cells (Gruenberg and Emans, 1993; Harder *et al.*, 1997). As shown in Figure 3C, our fractions were also devoid of annexin II, although the protein is tightly membrane-associated (Harder *et al.*, 1997). Thus, we find that TfR copurifies with proteins known to regulate Tf recycling but not with the early endosomal proteins EEA1 and annexin II.

Acidification Properties of MDCK TfR-containing Recycling Endosomes

Because endocytosed TfR is expected to transit through early (sorting) endosomes before appearing in recycling endosomes, the absence of EEA1 and annexin II in our fractions was somewhat unexpected. Therefore, we decided to make use of the pH-dependent fluorescence emission of

FITC to follow the pathway of Tf-FITC through acidic endosomes by fluorescence ratio imaging at the level of single organelles. Indeed, it is well established that early (sorting) endosomes are acidic, but recycling endosomes were reported to be less acidic in nonpolarized CHO cells (Yamashiro *et al.*, 1984). As shown in Figure 4, Tf-FITC transits soon after internalization through a peripherally located acidic compartment, presumably corresponding to early endosomes (pH = 5.8 ± 0.2). However, transit through this compartment was extremely rapid, because Tf-FITC resided in acidic endosomes only for the time required for image acquisition (i.e., ≈3 min) when internalized at 20°C. Within <2 min at 37°C, corresponding to the situation illustrated in Figure 1E, the bulk of Tf-FITC had already exited these acidic endosomes. As shown in Figure 4, after longer incubation times at 37°C, Tf-FITC distributed to a less acidic compartment (pH = 6.2 ± 0.1), indicating that recycling endosomes in MDCK cells are less acidic than early endosomes. (This pH difference was not due to a direct temperature effect on the fluorescence signal, because all pH measurements were carried out at 20°C). These experiments also show that TfR transit through acidic early endosomes is very rapid and thus that they contain little receptor at steady state. These observations agree well with our findings that TfR does not copurify with the early endosomal proteins EEA1 and annexin II (Figure 3C). TfR may be removed from early endosomes so efficiently that its levels in early endosomes remain too low for these membranes to be isolated under our experimental conditions.

It is well established that the acidic pH of endosomes depends on the action of the vacuolar (H^+)-ATPase (V-ATPase), but the mechanisms regulating vacuolar acidification are not well understood (Stevens and Forgac, 1997). As shown in Figure 3B, V-ATPase could not be detected in the recycling endosome fraction with the use of antibodies against the A subunit, which is part of the peripheral V1 domain, although it remained membrane-associated throughout the purification procedure. This was true whether endosomes were immunoisolated from MDCK cells grown on glass coverslips or on filters (Figure 3B). Altogether, our data show that recycling endosomes are enriched in proteins known to regulate Tf recycling but devoid of EEA1, annexin II, and functional V-ATPase, hence that recycling endosomes differ from early endosomes. The lack of V-ATPase is likely a direct cause of the reduced acidification of recycling endosomes in MDCK cells.

The Apical Endocytic Pathway Intersects the Tf Recycling Pathway

Although mixing of apically and basolaterally internalized fluid phase tracers cannot be observed in early endosomes (Bomsel *et al.*, 1989; Parton *et al.*, 1989), apical and basolateral recycling of membrane-bound tracers as well as transcytosis are believed to occur through a common compartment (Hughson and Hopkins, 1990; Apodaca *et al.*, 1994b; Barroso and Sztul, 1994; Hunziker and Peters, 1998; Fialka *et al.*, 1999). We investigated whether a fluid phase tracer endocytosed from the apical surface was able to reach recycling endosomes containing Tf. As shown in Figure 5A, recycling endosomes labeled with Tf–rhodamine internalized for 30 min from the basolateral surface could be reached by dextran–Oregon Green internalized from the apical surface for 10 min. We then made use of our immunoisolation protocol to quantify bulk transport of an apically endocytosed fluid phase tracer into recycling endosomes. Cells were incubated for 10 min with HRP on the apical side of the monolayer, and then recycling endosomes were immunoisolated as described above. As shown in Figure 5B, significant amounts of HRP cofractionated with TfR, because the yield of latent HRP isolation was ≈20%. In contrast, apically endocytosed HRP, which was chased for 30 min in marker-free medium and therefore reached late endosomes (Bomsel *et al.*, 1989; Parton *et al.*, 1989), did not cofractionate with TfR, as expected. Together, these data confirm that receptor molecules are more efficiently transferred to recycling endosomes than the early endosomal content (Geuze *et al.*, 1983). However, they also indicate that a significant portion of the early endosomal content is transferred to recycling endosomes, consistent with observations that at least 50% of endocytosed fluid phase tracers are regurgitated into the medium (Besterman *et al.*, 1981; Bomsel *et al.*, 1989). In previous studies, it has sometimes been difficult to detect fluid phase markers in recycling endosomes in both polarized cells (Apodaca *et al.*, 1994b; Barroso and Sztul, 1994) and nonpolarized cells (Tooze and Hollinshead, 1991), presumably because dilution of content markers within the thin tubular networks may have rendered detection difficult (Tooze and Hollinshead, 1991; Verges *et al.*, 1999). Finally, and most importantly, these data show that recycling endosomes containing the TfR can be reached by fluid phase tracers endocytosed from the apical surface and hence that the apical endocytic pathway intersects the Tf recycling pathway, in agreement with our recent observations with an in vitro transport assay (Huber *et al.*, 2000).

Raft Components in Recycling Endosomes

Next, we analyzed the lipid composition of recycling endosomes after metabolic labeling of m-hTfR cells with [^{32}P]orthophosphate or [^{14}C]acetate overnight. Recycling endosomes were prepared by immunoisolation as described above. Lipids were then extracted and analyzed by TLC on silica gel plates. As shown in Figure 6B, cholesterol and sphingomyelin as well as phosphatidylserine were highly enriched in the purified recycling endosome fraction (a typical phospholipid TLC analysis is shown in Figure 6A). To further confirm this result, we incorporated sphingomyelin–BODIPY into the plasma membrane of filter-grown m-hTfR MDCK cells and then incubated the cells at 37°C to follow the subcellular distribution of the lipid (Pagano *et al.*, 1991). As shown in Figure 7A, sphingomyelin–BODIPY colocalized intracellularly with endocytosed Tf–rhodamine in both the apical and basolateral regions of the cells, suggesting that the lipid was present in recycling endosomes. The steady-state distribution of endogenous cholesterol can easily be revealed by light microscopy with the use of filipin as a fluorescent marker (Kobayashi *et al.*, 1999). Much like sphingomyelin–BODIPY, cholesterol colocalized intracellularly with endocytosed Tf–rhodamine (Figure 7B).

Sphingomyelin and other sphingolipids can transiently associate with cholesterol, presumably forming liquid-ordered phase microdomains in the plane of the membrane (reviewed by Brown and London, 1998), which were termed rafts by Simons and collaborators (Simons and Ikonen, 1997). Accumulating data suggest the involvement of rafts in signal transduction, pathogen infection, and cell motility

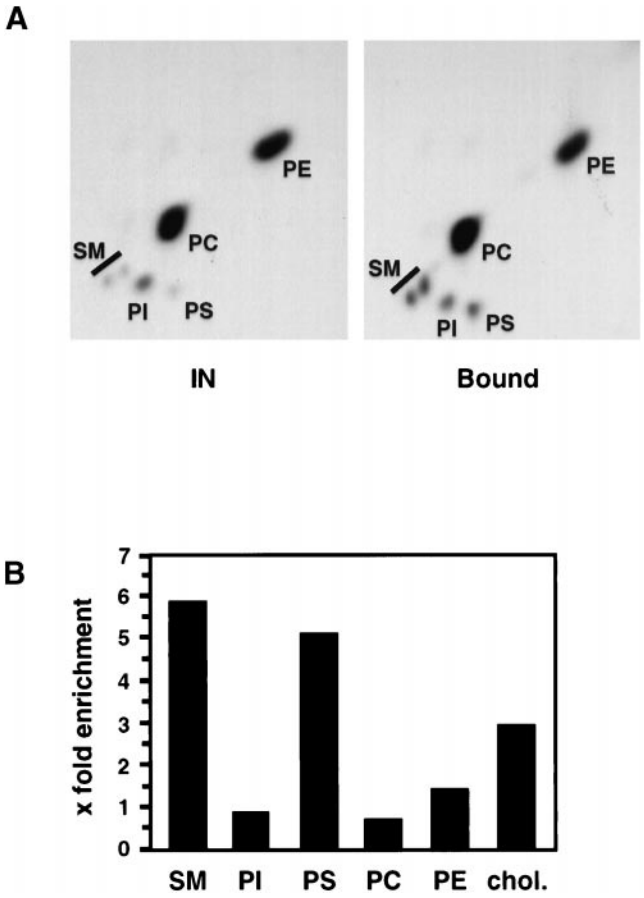


Figure 6. Raft components are enriched in purified recycling endosomes. Endosomes containing m-hTfR were purified, with the use of cells expressing hTfR as a control, as in Figure 2, B and C, from cells that had been metabolically labeled with [^{32}P]orthophosphate (for phospholipids) or [^{14}C]acetate (for cholesterol). Lipids were extracted and analyzed by two-dimensional TLC (phospholipids) or one-dimensional TLC (cholesterol). (A) Two-dimensional TLC of ^{32}P-labeled immunoisolated fractions from cells expressing m-hTfR (Bound) compared with the starting material (IN). The same amounts (dpm) were loaded in each case. Only samples from m-hTfR cells are shown in this typical experiment. (B) Radioactivity was quantified with the PhosphorImager. Indicated values correspond to the enrichment over the starting material (expressed as relative specific activity), normalized to nonspecific binding measured with the use of hTfR cells. SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; chol., cholesterol.

(Field *et al.*, 1997; Okamoto *et al.*, 1998; Abrami and van Der Goot, 1999; Manes *et al.*, 1999; Viola *et al.*, 1999) as well as in protein sorting in the *trans*-Golgi network, including in MDCK cells (Simons and Ikonen, 1997). Raft components, although present on both plasma membrane domains, are more abundant on the apical plasma membrane of MDCK cells (van Meer *et al.*, 1987). The presence of raft lipids in recycling endosomes prompted us to test whether these also contained caveolin-1. This cholesterol-binding protein is known to associate with cell surface invaginations termed caveolae, which are composed of lipid rafts, and was also shown to be present intracellularly (Dupree *et al.*, 1993; Kurzchalia and Parton, 1999). We found that caveolin-1 efficiently cofractionated with the TfR, both on the gradient (Figure 8A) and during immunoisolation (Figure 3B), yields and enrichment during purification being comparable for both proteins (Figure 8B). We were unable to localize caveolin-1 on cryosections with available antibodies. However, after preembedding labeling of permeabilized cells, anticaveolin-1 antibodies decorated a network of thin tubules with a morphology similar to that of recycling endosomes (Figure 9). Remarkably, a polypeptide specifically enriched in purified recycling endosomes (Figure 2C, asterisk) was identified by tandem-mass spectrometry with the use of the same two-dimensional gel system (M. Fivaz, F. Vilbois, and G. van Der Goot, personal communication) as the recently described protein flotillin-1, a component of lipid rafts and caveolae (Bickel *et al.*, 1997), which is particularly abundant in kidney cells (Volonte *et al.*, 1999). This polypeptide was significantly enriched, because it could not be detected even in the original PNS (Figure 2C). Altogether, these data demonstrate that raft lipids as well as caveolin-1, and presumably flotillin-1, which are known to associate with membrane microdomains, are specifically enriched in purified recycling endosomes. The recycling endosome membrane thus appears to be heterogeneous in composition, like the plasma membrane (Harder *et al.*, 1998; Abrami and van Der Goot, 1999), and to contain both raft and nonraft components (e.g., TfR).

DISCUSSION

In this paper, we show that recycling endosomes in polarized MDCK cells are enriched in proteins that regulate the TfR cycle but do not contain molecules involved in the dynamics of early endosomes. These observations suggest that different molecular mechanisms regulate membrane traffic in sorting and recycling endosomes. We also show that recycling endosomes in MDCK cells are less acidic than early endosomes, because they lack functional V-ATPase. Consistent with observations that the apical endocytic pathway intersects the basolateral Tf recycling pathway, we show that recycling endosomes are enriched in raft components, which are known to be abundant at the apical plasma membrane. Together, these data support the notion that apically and basolaterally endocytosed molecules meet in a common recycling endosome and suggest that selective partitioning into membrane microdomains may contribute to protein sorting.

Molecular Composition

Purified recycling endosomes are enriched in proteins known to regulate Tf cycling, including the v-SNARE cellubrevin and the small GTPases rab4 and to some extent rab11, in agreement with Verges *et al.* (1999). In contrast, these fractions strikingly lack the early endosomal proteins EEA1 and annexin II. Our finding that EEA1 is absent from recycling endosomes is consistent with its characteristic highly punctate and scattered pattern observed by immunofluorescence (Mu *et al.*, 1995), including in CHO cells (Gu *et al.*, 1997), in which recycling endosomes are clustered in the perinuclear region (Yamashiro and Maxfield, 1984; Marsh *et al.*, 1995). Altogether, these observations suggest that EEA1

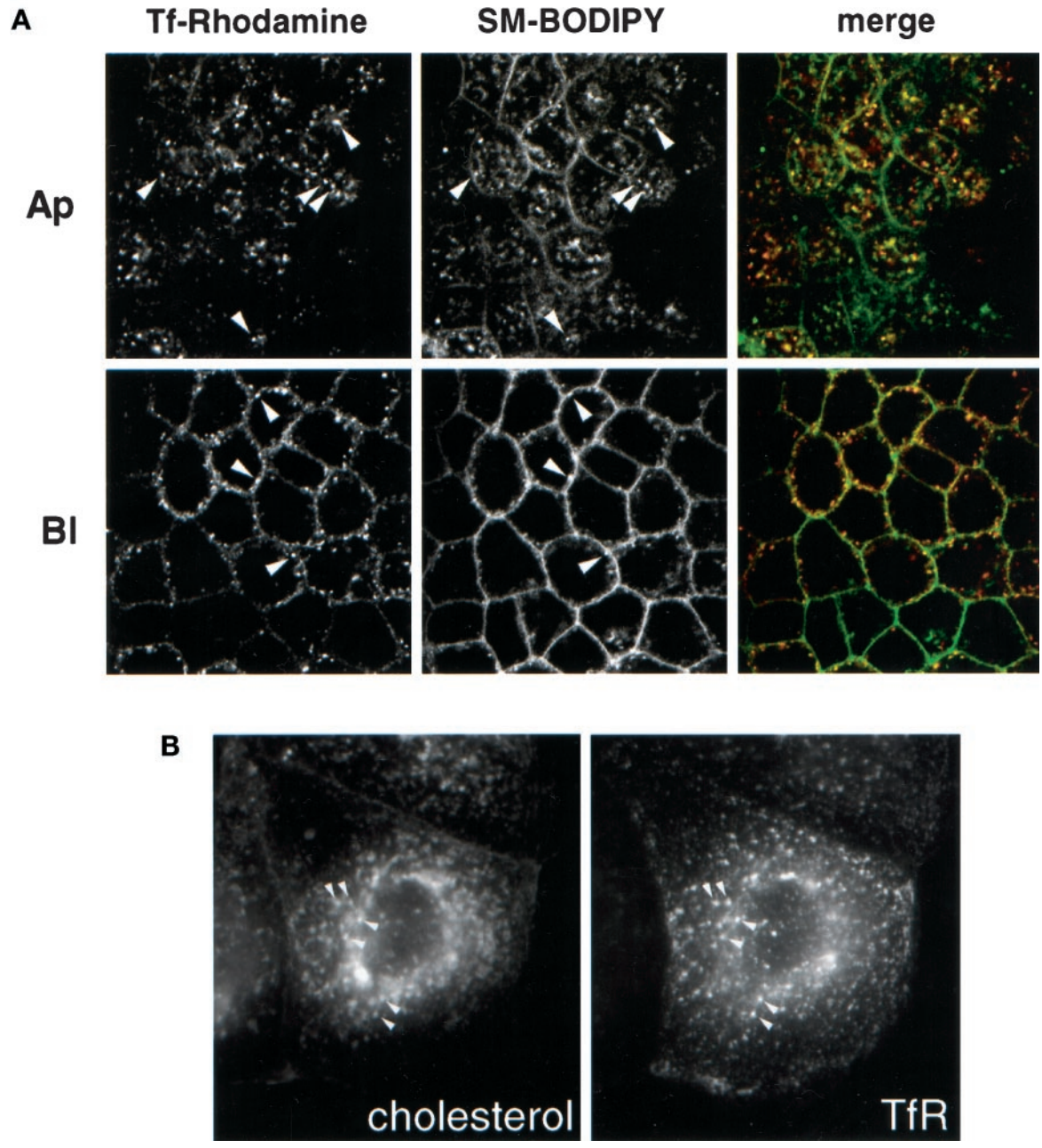
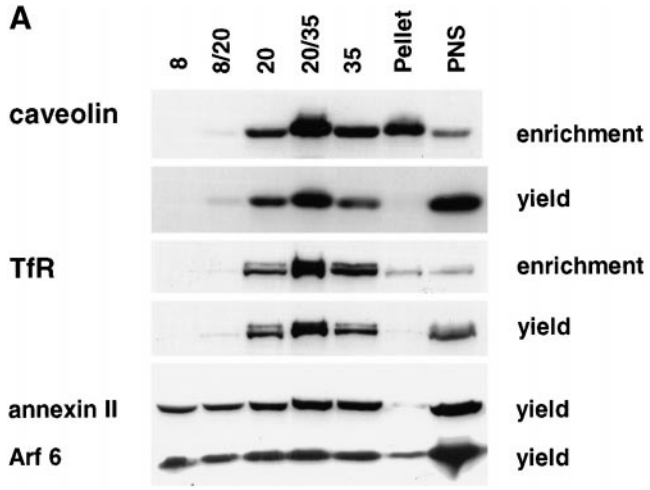


Figure 7. Raft components distribute to recycling endosomes. (A) Filter-grown m-hTfR cells were allowed to incorporate sphingomyelin (SM)–BODIPY for 30 min from both sides of the filters. Simultaneously, Tf–rhodamine was internalized from the basolateral side of the filters. Confocal sections in the apical (Ap) or basolateral (Bl) region of live cells are shown. Arrowheads indicate intracellular colocalization between sphingomyelin–BODIPY and Tf–rhodamine in both the apical and basolateral regions of the cells. (B) Cells expressing m-hTfR grown on coverslips were processed for double-label immunofluorescence analysis, with the use of antibodies against hTfR as in Figure 1C, and filipin to reveal the distribution of cholesterol. Arrowheads indicate intracellular colocalization between cholesterol and TfR.

is restricted to early endosomes, hence that its function as a rab5 effector in docking and/or fusion (Simonsen *et al.*, 1998; Christoforidis *et al.*, 1999) is also limited to these membranes, in agreement with Trischler *et al.* (1999). Because EEA1 binds to phosphatidylinositol 3-phosphate rich membranes through its FYVE finger domain, in addition to interacting with membrane-bound GTP-rab5 (Stenmark *et al.*, 1996; Simonsen *et al.*, 1998), one may speculate that phosphatidylinositol 3-phosphate itself is restricted to early endosomes. In contrast to EEA1, rab5 can be detected in recycling endosomes, although at low abundance, consistent with partial colocalization of rab5



B

	PNS [%]	PNS [x]	20/35 [%]	20/35 [x]	beads fraction [%]	beads fraction [x]
TfR	100	1	65	10	35	140
caveolin	100	1	58	9	24	120

Figure 8. Caveolin copurifies with the Tf receptor. (A) Cells expressing m-hTfR were fractionated on a discontinuous sucrose gradient loaded with 8, 20, and 35% sucrose, as in Figure 2A. Fractions were collected and analyzed by SDS-PAGE and Western blotting with the use of antibodies against caveolin or TfR as a control. Lanes were loaded either with the same amount of protein (enrichment) or with 10% of the volume (yield) of each fraction. As controls, the figure also shows the distribution of annexin II and Arf6, which were not detected in the immunoisolated fractions. (B) Amounts of caveolin and TfR were quantified by scanning the gels in a typical experiment; yields [%] and enrichment [×] are indicated, both for the 20/30% gradient interface (as in A) and for the immunoisolated fractions (as in Figure 3A).

with TfR (Chavrier *et al.*, 1990). This somewhat broader distribution of rab5 compared with EEA1 agrees with the view that rab5 may exert its functions through more than one effector (Stenmark *et al.*, 1995; Gournier *et al.*, 1998; Simonsen *et al.*, 1998).

Our observation that annexin II is not found on recycling endosomes agrees with the distribution of the protein to early endosomal elements with a characteristic tubulo-vesicular and cisternal appearance, which are labeled by fluid phase markers within 5–10 min (Emans *et al.*, 1993; Harder *et al.*, 1997). The precise function of the protein is unclear, although previous studies suggested that it is involved in endosomal membrane dynamics (Gruenberg and Emans, 1993; Harder and Gerke, 1993). Together, our observations suggest that EEA1 and annexin II act at the level of early endosomes exclusively, in contrast to cellubrevin and rab4, which regulate Tf recycling. Hence, different molecular mechanisms are likely to regulate membrane traffic in recycling and early endosomes.

Topology and Organization

We find that transit of endocytosed Tf through early endosomes is extremely rapid, because Tf exits acidic endosomes in <3 min at 37°C. Then, endocytosed Tf first appears in recycling endosomes in the basolateral cytoplasm before reaching a compartment located in the supranuclear apical portion of the cell, presumably corresponding to the ARC (Hughson and Hopkins, 1990; Apodaca *et al.*, 1994b; Barroso and Sztul, 1994). Both populations should contain large amounts of TfR at steady state and therefore are probably equally well represented in our biochemical analysis, lacking EEA1, annexin II, and functional V-ATPase, as opposed to early endosomes. Thus, recycling endosomes appear to distribute both along the basolateral membrane and close to the centrioles, which are located underneath the apical membrane in MDCK cells (Buendia *et al.*, 1990). This dual distribution appears to contrast with the exclusive pericentriolar localization of recycling endosomes in nonpolarized CHO cells (Yamashiro *et al.*, 1984). However, a similar dual distribution can be observed in neurons, in which the distribution of TfR is polarized (Parton *et al.*, 1992). In these cells, the intracellular localization of TfR is not restricted to the vicinity of centrioles, because tubular TfR-containing endosomes are abundant in the dendrites. Recent studies suggest that, in MDCK cells, the majority (>65%) of recycling to the basolateral surface occurs from basolateral early endosomes, whereas segregation of basolateral receptors from receptors intended for transcytosis takes place in apical recycling endosomes (Sheff *et al.*, 1999). Based on these and our observations, we propose that, after rapid transit through basolateral early endosomes, recycling of receptors back to the cell surface begins in proximal elements of the recycling pathway in the basolateral cytoplasm and continues in more distal elements in the apical cytoplasm, perhaps reflecting the rapid and slow recycling routes in nonpolarized cells (Mayor *et al.*, 1993; Presley *et al.*, 1993; Gruenberg and Maxfield, 1995).

Acidification Properties

Our observations that recycling endosomes are less acidic than early endosomes might help to explain the barrier function of epithelial cells, because recycling endosomes intersect apical and basolateral uptake routes. Some receptor–ligand complexes may escape dissociation in early endosomes, transit being very rapid. If recycling endosomes were acidic, these complexes might dissociate and, like any solute, free ligand molecules would be regurgitated in a nonpolarized manner to both sides of the monolayer. Our observations, however, suggest that uncoupling is unlikely to occur in recycling endosomes and hence that receptor-bound ligand molecules that escaped from early endosomes can be recycled and undergo a second round of internalization. Reduced acidification may thus contribute to limit the delivery of endocytosed ligands to the wrong side of the epithelium.

The acidic pH of endosomes depends on the action of the V-ATPase (Stevens and Forgac, 1997). Several mechanisms have been proposed to regulate the activity of this pump, including reversible dissociation of the V1 and V0 domains (Kane, 1995), disulfide bond formation at the catalytic site

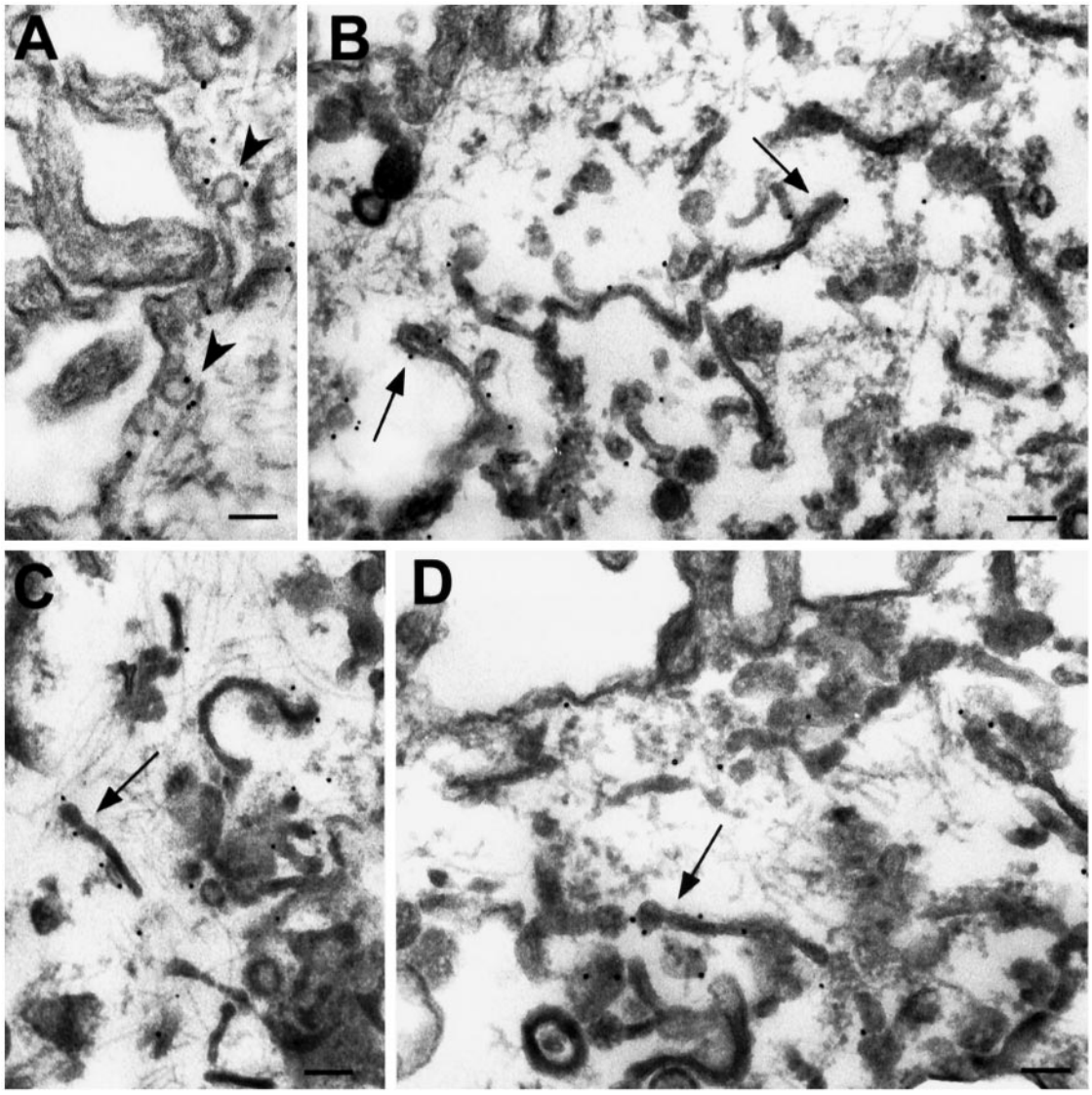


Figure 9. Immunoelectron microscopic localization of caveolin-1 in MDCK cells. Filter-grown MDCK cells were perforated with the use of nitrocellulose to remove parts of the apical surface. Cells were labeled for caveolin-1, fixed, and processed for Epon embedding. (A) Image shows an area of the lateral membrane with gold labeling associated with caveolae (arrowheads). (B–D) Images show areas underlying the apical plasma membrane. Labeling for caveolin-1 is associated with extensive tubular structures (arrows). Bars, 100 nm.

(Feng and Forgac, 1994), control of pump density (Sturgill-Koszycki *et al.*, 1994a,b), and counteraction by other pumps (Cain *et al.*, 1989; Fuchs *et al.*, 1989). Our data show that the limited acidification of recycling endosomes in MDCK cells is due to the absence of the pump, or at least the V1 domain, suggesting that pH may be regulated via V-ATPase exclusion from recycling endosomes or by regulated V0–V1 dissociation. Intriguingly, we find that the pH of recycling endosomes is nevertheless slightly acidic, perhaps resulting from the transfer of acidic content from early endosomes or from the action of residual V-ATPase molecules below the detection threshold.

Raft Components

We find that the raft lipids cholesterol and sphingomyelin are enriched in recycling endosomes. In marked contrast, late endosomes contain little free cholesterol and sphingomyelin but high amounts of the unconventional lipid lysobisphosphatidic acid (Kobayashi *et al.*, 1998b, 1999). Thus, raft lipids appear not to distribute stochastically within all endosomal membranes, raising the question of how they are sorted away from the pathway leading to degradation. Raft components may be internalized via clathrin-coated vesicles but there may also be, at least in some cell types, raft-specific

pathways for endocytosis (Parton, 1996). Caveolae, which form cell surface invaginations containing the cholesterol-binding protein caveolin, and lipid rafts apparently can be internalized, at least in some cell types (Parton, 1996; Schnitzer *et al.*, 1996a,b). Our findings that raft components are enriched in recycling endosomes suggest that internalization of caveolae, whether occurring through a specialized pathway or not, leads to endosomes.

It is attractive to speculate that the selective incorporation of raft components in recycling endosomes contributes to regulate protein/lipid sorting and trafficking. Lipids and proteins, which tend to partition within raft domains, may be preferentially incorporated within the recycling and/or transcytotic pathways. Indeed, once internalized, fluorescent derivatives of sphingolipids are rapidly returned to the cell surface much like recycling receptors in nonpolarized cells (for review, see Kobayashi *et al.*, 1998a). Moreover, glycosylphosphatidyl inositol-anchored proteins, which are associated with rafts on the cell surface, follow the same pathway as TfR after endocytosis into nonpolarized cells, albeit with slower kinetics, and their retention in endosomes depends on membrane cholesterol (Mayor *et al.*, 1998). A similar mechanism may extend to transcytosing proteins, because in small intestinal explants, dimeric IgA internalized and transcytosed via the polymeric IgR was found to associate with a detergent-insoluble membrane fraction (Hansen *et al.*, 1999). Mechanisms regulating the incorporation of raft components into the recycling pathway remain to be determined. It has been proposed that preferential packing of sphingolipids and cholesterol is facilitated by the long, saturated hydrocarbon chains of sphingolipids but also by intermolecular hydrogen bonds, especially involving the carbohydrate head groups of glycosphingolipids (Simons and Ikonen, 1997), although this issue is controversial (Ostermeyer *et al.*, 1999). One may envision that alkalinization of recycling endosomes could contribute to facilitate head group interactions, mimicking the extracellular environment, and thus the incorporation of raft components into the pathway.

Finally, proteins and lipids present in recycling endosomes must be sorted and then recycled or transcytosed to the opposite membrane domain. In the biosynthetic pathway, it was shown that sorting of apically destined molecules in the *trans*-Golgi network depends, at least in part, on N-linked carbohydrates (Scheiffele *et al.*, 1995; Gut *et al.*, 1998; Benting *et al.*, 1999). Alternatively, apical sorting was proposed to depend on selective incorporation of apically targeted proteins into raft lipid microdomains (van Meer and Simons, 1988; Lafont *et al.*, 1998, 1999). Based on our observations, we propose that a similar lipid-based sorting mechanism may operate in recycling endosomes to ensure that the proper polarized composition of each membrane domain is maintained.

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