rab4 Regulates Transport to the Apical Plasma Membrane in Madin-Darby Canine Kidney Cells*

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The small GTPase rab4 is associated with early endosomes and regulates membrane recycling in fibroblasts. Rab4 is present in epithelial cells; however, neither its localization nor function has been established in this cell type. We transfected Madin-Darby canine kidney cells with rab4, the GTPase-deficient mutant rab4Q67L, and the dominant negative mutant rab4S22N that poorly binds guanine nucleotides. Confocal immunofluorescence microscopy showed that rab4 was concentrated on internal structures at the lateral side of the cell around the nucleus. Quantitative immunoelectron microscopy revealed that the majority of rab4 was localized in the upper third of the cytoplasm. In cell surface binding experiments with 125I-transferrin, we found a redistribution of transferrin receptor from the basolateral to the apical plasma membrane in cells expressing rab4 and rab4Q67L. After accumulation of transferrin at 16 °C in basolateral early endosomes, rab4 and rab4Q67L increased the amount of apically targeted transferrin receptor. A qualitatively similar effect was obtained in control cells treated with brefeldin A. The effects of brefeldin A and rab4 on apical targeting of transferrin receptor were not additive, suggesting that brefeldin A and rab4 may act in the same transport pathway from common endosomes.

Eukaryotic cells internalize cell surface proteins and material from their environment by endocytosis. The pathway is used for the uptake of nutrients, regulation of cell surface receptors, and recycling of proteins used in the secretory pathway. Ligands are bound to receptors at the plasma membrane. The complex then enters clathrin-coated pits, is internalized in clathrin-coated vesicles, and transported to early endosomes (EEs), where sorting occurs to different compartments. The mildly acidic pH in EEs causes dissociation of several ligand-receptor complexes. Ligands are then targeted to late endosomes/lysosomes for degradation, whereas most receptors are transported via recycling endosomes back to the cell surface for reutilization (reviewed in Ref. 1). Although receptor recycling is relatively well understood at the phenomenological level, little is known about the molecular principles that regulate vesicular transport from EEs to recycling endosomes and the plasma membrane.

An additional layer of complexity in the regulation of endocytic pathways is present in polarized epithelial cells. These have biochemically distinct apical and basolateral plasma membrane domains that are separated by tight junctions. Endocytosis occurs at both poles of this cell type. Earlier studies in Madin-Darby canine kidney (MDCK) cells suggested that apically and basolaterally internalized fluid phase tracers enter distinct apical and basolateral EEs before they reach a common late endosomal compartment (2). However, more recent investigations with ligands of transcytosing and recycling receptors revealed that basolateral and apical EEs are also interconnected (3–6) and converge to the common early endosome (CE). The CE is an extensively tubularized organelle that is accessible to basolaterally internalized and recycled transferrin receptor (TfR) but also to basolaterally endocytosed polymorphic IgA receptor (pIgAR) before it reaches the apical plasma membrane (7). Fluid phase tracers enter the CE at relatively low concentrations because these have already been sorted in the basolateral or apical EEs. Recycling receptors such as TfR and the transcytosing pIgAR are most likely sorted in the CE. pIgAR is subsequently transported to the apical recycling endosome (ARE) before it reaches the apical cell surface. The ARE is typically devoid of Tf, which is transported from the CE to the cell surface and possibly apical EEs (see Ref. 8). Although little is known about the biochemical composition of the ARE, it is enriched in proteins and lipids typically found in rafts (9), whereas the luminal pH of 6.3–6.5 is considerably less acidic than that of basolateral and apical EEs (6, 9). Because sorting of TfR occurs in both EEs and the CE, it is not clear whether transit through the CE is a prerequisite for basolaterally recycling receptors (4, 10).

Testifying to the high degree of complexity of endosomal pathways is the increasing number of small GTPases that have been localized to the cytosolic surface of early endocytic organelles. For instance, rab5 is associated with basolateral and apical EEs (11), whereas its effector EE1A is predominantly associated with basolateral EEs (12), suggesting that the spatiotemporal regulation of rab5 activity may be different at these two early endosome populations. Testifying to this notion is the recent discovery that the rab5 effector Vps34p differentially regulates endocytosis at the apical and basolateral surface in WIF-B cells (13). We have been investigating the role of rab4 in transport through the early endocytic pathway (14, 15). Rab4 is localized to early endocytic compartments and transport vesicles but not to the plasma membrane (16, 17) and regulates recycling of cell surface receptors (14, 18). How rab4

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§ The abbreviations used are: EE, early endosome; ARE, apical recycling endosome; BFA, brefeldin A; BSA, bovine serum albumin; CE, common early endosome; EE1A, early endosome-associated antigen 1; MDCK, Madin-Darby canine kidney; pIgAR, polymorphic IgA receptor; Tf, transferrin, TfR, transferrin receptor; dIgA, dimeric IgA; MEM, minimum Eagle’s medium; PBS, phosphate-buffered saline; gp80, glycoprotein 80; ARF, ADP ribosylation factor 1; CHO, Chinese hamster ovary.
mediates its function is not yet clear, but the cloning and functional characterization of bifunctional effector proteins such as the rabaptins suggest that the sequential and opposite activities of rab5 and rab4 on EE membrane dynamics are coordinated through these effector proteins. Here we generated stable MDCK transfectants ectopically expressing wild type and mutant rab4. Our results show that rab4 controls transport of TR from the CE to the apical plasma membrane.

MATERIALS AND METHODS

Antibodies and Expression Constructs—Rabbit antibodies against rab4 and mouse monoclonal antibodies 9E10 and NH against the myc and influenza X31 NH epitope tags have been described previously (15, 19, 20). The rat monoclonal antibody against ZO-1 and rab4 antibodies against gp80 and EEA-1 were generously provided by Karl Mutter (University of Geneva), Claudia Koch-Brandt (Johannes Gutenberg University, Mainz, Germany), and Michael Clague (University of Liverpool), respectively. Dimeric IgA (dIgA) was generously provided by Jean-Pierre Vaerman (Catholic University of Louvain, Louvain, Belgium). Rabbit antibodies against rab11 and IgA were purchased from Zymed Laboratories Inc. and DAKO, respectively rabbit. Fluorescence-labeled secondary antibodies were from Molecular Probes (Leiden, The Netherlands) and Jackson ImmunoResearch Laboratories (Westgrove, PA). myc-tagged human TfR cDNA was excised with SacI and XbaI from mycTfr-pCB6 (21) and ligated in pcB7 (generously provided by Jim Casanova; Harvard University). rab4, rab4S22N, and rab4Q67L were released with EcoRI from corresponding pGBT9 constructs (22) and cloned in the same site of pcDNA3 (Invitrogen). NH-tagged rab4 constructs were as described previously (23).

Cell Culture and Transfection—MDCKII cells were grown in MEM (Invitrogen) and 10% fetal calf serum supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were transfected with mycTFR-pCB7 as described previously (23), and transfectants were selected in media containing 200 µg/ml hygromycin. Expression was screened by immunofluorescence microscopy after 2 weeks. Positive clones were selected for their ability to internalize and recycle, 125I-Tf and subconfluent by limited dilution. A representative clone with TR kinetics identical to those of endogenous canine Tfr (24) was transfected with rab4, rab4S22N, and rab4Q67L in pcDNA3. Double transfectants were selected in media containing 200 µg/ml hygromycin and 0.6 mg/ml G418. Colonies were screened after 2 weeks by immunofluorescence microscopy, subconfluent by limited dilution cloning, and re-evaluated by immunofluorescence microscopy. Western blot, and 125I-Tf single cycle experiments. Representative clones with reasonably uniform expression and expression levels were used for further experiments. A MDCKII transfectant expressing rabbit pIgAR was a kind gift of Walter Hunziker (University of Lausanne) (25) and was stably transfected with NH-tagged rab4 expression constructs (20) as described for experiments with filter grown cells, the filter was seeded on 6- or 24-mm polycarbonate filters with 0.4-µm pore size (Corning Inc., Acton, MA) and cultured for 4 days. Media were changed every 2 days, and 5 mM sodium butyrate was added 16–18 h before experiments to enhance expression of cytomegalovirus-driven constructs.

Single Cycle Kinetics of 125I-Tf—Cells were grown on 24-mm Transwell filters as described above, washed twice in MEM, 20 mM HEPES, pH 7.4, and 0.1% BSA (uptake medium) supplemented with 50 µM deferoxamine, and depleted from endogenous Tfr for 45–60 min at 37 °C in the same medium. 2 µg/ml 125I-Tf in uptake medium was bound at 4 °C at either the basolateral or apical side for 60–90 min. Next, the cells were washed three times with ice-cold PBS2−, transferred to a 37 °C water bath, and chased for different periods of time. After the incubation, cells were washed for 10 min with ice-cold acid wash buffer (25 mM HAOc, pH 5, 150 mM NaCl, 50 µM deferoxamine, and 0.1% BSA) and PBS2−, respectively, followed by a second cycle of washes of 5 min.

Filters were cut from the holders and counted in the gamma counter, as was done for media and washes. Results represent a percentage of the total 125I-Tf.

Distribution of Tfr between the Apical and Basolateral Plasma Membrane—Cells were grown on 24-mm Transwell filters, washed twice in uptake medium supplemented with 50 µM deferoxamine, and depleted from endogenous Tfr for 45–60 min at 37 °C in the same medium. The cells were then incubated on ice for 2 h with 2 µg/ml 125I-Tf in 0.5 ml of uptake medium at the basolateral or apical side. Non-specific binding was assayed in the presence of 100-fold excess unlabeled Tfr and was <10%. The cells were washed with PBS2− containing 0.49 mM MgCl2, 0.91 mM CaCl2, and 0.1% BSA and subsequently washed with alternating acid and neutral washes as described above. Filters were excised, and 125I was assayed in the acid washes and cells using a gamma counter.

Pulse-Chase Experiments with 125I-Tf after 16 °C Internalization—Cells were grown on 24-mm Transwell filters and depleted of endogenous Tf as described above. 1–3 µg/ml 125I-Tf was internalized at the basolateral side in 1 ml of uptake medium (without deferoxamine) for 30 min at 16 °C. The cells were put on ice, washed twice with PBS2−, washed once with MES buffer (20 mM 2-(N-morpholino)ethanesulfonic acid, pH 5, 130 mM NaCl, 2 mM CaCl2, 0.1% BSA, and 50 µM deferoxamine) followed by washed once with PBS2− (each wash was 10 min). The acid-neutral wash cycle was repeated once more, but for 5 min. Cells were then chased at 37 °C. After different periods of time, media were collected and saved for quantitation of 125I-Tf. The cells were washed twice with ice-cold medium, and cell-associated 125I-Tf was counted in the gamma counter. Results are expressed as a percentage of total 125I-Tf in the media and on the filter. For some experiments 5 µg/ml brefeldin A (BFA) was added to the medium during the internalization and chase periods.

Secretion of gp80—Cells were grown on 24-mm Transwell filters as described above and depleted in 2 ml of MEM (Sigma) lacking methionine and cysteine (labeling medium) for 30 min at 37 °C. The cells were then incubated for 60 min at 37 °C in 0.5 ml of labeling medium containing 0.49 mM MgCl2, 0.91 mM CaCl2, and 0.1% BSA, and 100 µg/ml deferoxamine. The cells were then washed on ice with MEM/0.1% BSA and chased at 37 °C. After 60 min, apical and basal media were harvested, and N-ethylmaleimide was added to 20 µM. gp80 was immunoprecipitated from the media as described previously (23) and boiled for 5 min in nonreducing sample buffer. Immunoprecipitates were resolved by SDS-PAGE on 12.5% minigels and analyzed by phosphorimaging.

Immunofluorescence Microscopy and Internalization of Fluorescence-labeled Tf—Cells grown on coverslips were subjected to immunofluorescence microscopy as described previously (26). Filter-grown cells were fixed for 5 min in 3% paraformaldehyde, 80 mM K-PIPES, pH 6.8, 5 mM EGTA, and 2 mM MgCl2, and then fixed for 10 min in 5% paraformaldehyde and 100 mM Na2O4, pH 11. The filters were washed three times in PBS and quenched in 1 mg/ml NaBH4 for 15 min. This cycle was repeated once with a fresh NaBH4 solution. Filters were cut from their inserts, permeabilized, and labeled with antibodies as described for cells grown on coverslips. Filters were mounted in 50% glycerol, 50% PBS, 0.1% NaN3, and 100 µg/ml 1,4-diazobicyclo[2.2.2]octane (Fluka, Zwijndrecht, The Netherlands). For internalization experiments, cells were washed twice with PBS2−, seeded on 6- or 24-mm polycarbonate filters with 0.4-µm pore size (Corning Inc., Acton, MA) and cultured for 4 days. Media were changed every 2 days, and 5 mM sodium butyrate was added 16–18 h before experiments to enhance expression of cytomegalovirus-driven constructs.

Immunogold Electron Microscopy—NRab4/pigAR double transfectants were grown on polycarbonate filters and fixed with 2% paraformaldehyde and 1% acrolein in 0.1 M sodium phosphate buffer, pH 7.4. After 2 h at room temperature, the cells were embedded in 10% gelatin and stored for cryoultramicrotomy as described previously (27). Ultrathin cryosections were cut perpendicular to polycarbonate filters and labeled with rabbit antibody against rab4 followed by 10 nm of protein A gold. The intracellular distribution of gold label was quantitated by counting gold particles associated with identifiable organelles on sections prepared from transfected and nontransfected control MDCK cells. To determine the labeling density per unit of membrane length, pictures were taken at ×15,000 magnification of rab4-labeled sections. A transparency displaying a squared lattice of lines that were 0.5 µm apart was put over the pictures and analyzed for intersections with the membranes of interest (28, 29). To quantify the distribution of rab4 throughout the apical and basolateral cytoplasm, we divided the entire cell into three zones as a micrograph of the nucleus, and below the nucleus. Gold particles in the three zones were counted and expressed per unit of membrane length.

Miscellaneous Methods—Human apo-Tf (Sigma) was saturated with Fe3+ as described previously (14), dialyzed against 20 mM HEPES, pH 7.8, and 150 mM NaCl at 4 °C, and stored at −80 °C. Iron-saturated Tf (500 µg) was incubated with five iodobeads (Pierce) and 1 mCi of Na125I.
Blots were probed with antibodies against rab4, c-myc, and rab11. Membrane fractions were analyzed by SDS-PAGE and Western blotting (C). Scraped into 1 ml of MEM, and resuspended in 3 ml of HB (10 mM triethanolamine, pH 7.4, 10 mM acetic acid, 1 mM EDTA, and 0.25 M sucrose). The cells were then centrifuged at 1500 rpm for 10 min at 4 °C.

To examine membrane association of the rab4 mutants, we used MDCK cells transfected with epitope-tagged rab4, NHrab4, NHrab4S22N, or NHrab4Q67L. Antibodies against the rab5 effector protein EEA1 labeled distinct punctate structures in the perinuclear area. These represented early endosomes because they also contained internalized Texas Red-Tf (red). We then compared the distribution of rab4S22N and rab4Q67L mutants with that of rab4. In cells expressing rab4S22N (Fig. 2B, green), most of the label was typically found in tubular structures close to the nucleus, whereas the scattered peripheral cytoplasmic staining seen with wild type rab4 had disappeared. In rab4Q67L transfectants (Fig. 2C, green), the GTPase-deficient mutant predominantly labeled ring-like structures around the nucleus. Consistent with these morphological alterations, we found significantly less colocalization of the mutants with endocytosed Texas Red-Tf in both cell lines.

Given the distinct labeling patterns of the two rab4 mutants, we next examined the distribution of EEA1 in rab4 transfected cells. To facilitate detection of rab4 in double labeling experiments, we used MDCK cells transfected with epitope-tagged NRhab4, NRhab4S22N, or NRhab4Q67L. Antibodies against the rab5 effector protein EEA1 labeled distinct punctate structures (Fig. 2D, green) throughout the cytoplasm in NRhab4 cells (Fig. 2D). These were similarly distributed and of the same size as those observed in nontransfected cells (data not shown) and partially colabeled with rab4 (red). In the cell lines expressing NRhab4S22N (Fig. 2F, red) and NRhab4Q67L (Fig. 2F, red), we found even less colocalization between EEA1 and the rab4 mutants. Taken together, these results suggested that rab4S22N and rab4Q67L induced distinct morphological alterations to regions of the early endosomal network in MDCK cells, which are relatively depleted of EEA1 and internalized Tf.

Endocytosis occurs from both the apical and basolateral domains of filter-grown MDCK cells, and indeed, rab5 is known to be involved in clathrin-mediated internalization from both sur-
Fig. 2. Morphological alterations of EEs in rab4S22N and rab4Q67L transfectants. Cell lines expressing non-tagged rab4 constructs were grown on coverslips and incubated for 30 min at 37°C with 25 μg/ml Texas Red–Tf (red, A–C). The cells were fixed in 3% paraformaldehyde, labeled with a rabbit antibody against rab4, and stained with fluorescein isothiocyanate–labeled anti-rabbit antibody (green, A–C). Note the co-localization of rab4 and Texas Red–Tf (A) and the morphological changes caused by the mutants rab4S22N (B) and rab4Q67L (C). MDCKII cells expressing epitope-tagged rab4 (red, D), rab4S22N (red, E), or rab4Q67L (red, F) were labeled with a rabbit antibody against EEA1 and a monoclonal antibody against the epitope tag. The NH tag was stained with Cy3–labeled goat anti-mouse antibody (D–F), and EEA1 was detected with fluorescein isothiocyanate–labeled goat anti-rabbit antibody (green, D–F).

To further define the location of rab4 at the ultrastructural level, we used immunogold electron microscopy on ultrathin cryosections tangentially cut from filter-grown MDCKII cells stably transfected with NHrab4. More than 70% of gold particles were localized to vesicles and tubulovesicular structures in the apical and lateral cytoplasm, whereas very little label was present in the basolateral cytoplasm. The distribution of rab4 was quantitated from photographs taken at low magnification. The prints were then assembled to visualize the entire cell including the filter and apical plasma membrane. We divided the cytoplasm into three zones: one above, one below, and one at the height of the nucleus. In agreement with the confocal immunofluorescence data, we found most of rab4 above the nucleus (Table I). Because the basal cytoplasm of MDCK cells coverslips were not stained with Cy3–labeled goat anti-mouse antibody and stained with fluorescein isothiocyanate–labeled goat anti-rabbit antibody (green, D–F).

Fig. 3. rab4 is concentrated at the level of the tight junction marker ZO-1. Myc–TfR/rab4 MDCKII transfectants were grown on 24-mm Transwell filters for 4 days. Cells were fixed with 3% paraformaldehyde and labeled with a rabbit antibody against rab4 and a monoclonal rat antibody against ZO-1. Rab4 was detected with fluorescein isothiocyanate–labeled goat anti-rabbit antibody (green), and ZO-1 was detected with Texas Red–labeled goat anti-rabbit antibody (red). Merged images of consecutive optical sections are shown from the basolateral side to the apical side (A–H).

TABLE I

| Region                | % gold   | % membrane length | Density |
|-----------------------|----------|-------------------|---------|
| Apical cytoplasm      | 73%      | 65.7%             | 1.1     |
| Perinuclear cytoplasm | 11.9%    | 13.2%             | 1.1     |
| Basolateral cytoplasm | 15.1%    | 21.1%             | 0.8     |

Wild Type rab4 and rab4Q67L Relocate TfR to the Apical Cell Surface—In CHO transfectants expressing high levels of rab4, we previously found that TfR was redistributed from EEs to the plasma membrane (14). In polarized epithelia, a role for rab4 has not yet been determined. We therefore started to investigate the effect of rab4 on TfR recycling in filter-grown transfectants in single cycle experiments. After prebinding 125I–Tf at the basolateral plasma membrane on ice, the cells were warmed at 37°C for different periods of time, and cell-associated 125I–Tf and 125I–Tf present in basolateral and apical media were quantitated. As shown in Fig. 4A, we found similar 125I–Tf single cycle kinetics in the wild type and mutant rab4 cell lines. We also performed these experiments with 125I–Tf that was prebound and internalized from the apical cell surface, and we obtained the same results (data not shown).

When moderate overexpression of rab4 has a limited effect on the kinetics of the TfR cycle, a single round of Tf uptake and recycling conceivably may lack the requisite sensitivity to discern the specific effects of rab4 mutants. Accordingly, we next
determined the steady-state distribution of TfR over the apical and basolateral plasma membranes. For this purpose, 125I-Tf was bound on ice at the basolateral or apical plasma membrane. As shown in Fig. 4B, we found 2 times more 125I-Tf binding at the apical side of the rab4 transfectants as compared with the nontransfected control cells or cells expressing rab4S22N. In rab4Q67L-transfected cells, we also observed an increase in TfR binding at the apical plasma membrane, although this increase was somewhat smaller than that seen in the wild type rab4 transfecant. This might be caused by the slightly lower expression level of rab4Q67L (cf. Fig. 1). Thus, overexpression of rab4 and rab4Q67L caused a redistribution of TfR from the basolateral to the apical surface of the cell.

**rab4 Enhances Apical Transcytosis of 125I-Tf from Basolateral EEs**

Because the cell surface binding assays showed an increase of 125I-Tf binding at the apical cell surface in rab4 and rab4Q67L transfectants, we investigated whether delivery of basolaterally endocytosed Tf to the apical membrane was affected by rab4. To this end, we made use of previous observations that endocytosed ligands accumulate in sorting endosomes at 16°C (32). Because it is not known whether this compartment is proximal to rab4-containing basolateral EEs, we first established the distribution of Alexa594-Tf endocytosed at 16°C with respect to rab4 in filter-grown cells using confocal microscopy. As shown in the XY sections of Fig. 5, the internalized tracer (red) localized primarily to EEs in the basolateral plasma side and incubated at 37°C. Apical and basolateral media were collected at different time periods, and the amount of 125I-Tf present is expressed as a percentage of the total. Data are the means ± S.D. of three experiments.

**Role of rab4 in Epithelial Cells**

![Diagram](image-url)
Alexa594-Tf started to fill the rab4-containing compartments within 5 min, as evidenced by Fig. 5, D–F, whereas at 30 min, Tf reached the region containing the tight junction marker ZO-1 (Fig. 5, G–I). After 60 min, a large fraction of Alexa594-Tf was chased out of the cells (data not shown). Having shown that the 16 °C compartment was proximal to rab4-containing endosomes, we next internalized 125I-Tf for 30 min at 16 °C from the basal surface. After removal of cell surface-bound 125I-Tf, cells were chased for different periods of time at 37 °C. As shown in Fig. 5J, control cells transfected with TIR recycled up to 5% of basolaterally endocytosed 125I-Tf to the apical side, whereas in rab4 and rab4Q67L transfectants, 2–3 times more 125I-Tf was transcytosed after 60 min into the apical medium. In contrast, in MDCK cells expressing rab4S22N, we found the same extent of apical transcytosis as in the control cells. Similar results were obtained when 125I-Tf was internalized at 16 °C from the apical plasma membrane (data not shown). Thus, in agreement with the binding experiments, ectopically expressed rab4 and rab4Q67L, but not dominant negative rab4S22N, perturbed polarized transport of 125I-Tf.

We also evaluated the transfer of dIgA from basolateral EEs to the apical cell surface in NHRab4/pIgAR transfectants. dIgA (50 µg/ml) was internalized for 30 min at 16 °C, and cells were washed twice to remove extracellular ligand. After different chase periods at 37 °C, cells were fixed and processed for confocal microscopy. At the end of the pulse, dIgA was accumulated in early endocytic structures with a predominantly lateral localization that contained little, if any, rab4. Upon chase at 37 °C, IgA left the lateral endosomes and moved to NHRab4-containing structures in the juxtanuclear region (Fig. 6H). Upon longer chase times, IgA exited these compartments and accumulated in subapical organelles that did not label for NHRab4. This was confirmed in immunofluorescence experiments, where the cells were labeled with antibodies against pIgAR and NHRab4. Most of the pIgAR was concentrated in this subapical compartment that excluded NHRab4 (data not shown). Thus, IgA, in contrast to Tf, transiently resided in rab4-containing endosomes, whereas Tf was seen at most chase times to colocализe with NHRab4, even in the apical cytoplasm.

Brefeldin A and rab4 Regulate TIR Transport from EEs—Previous ultrastuctural experiments showed that internalized TIR is localized to the CE that is coated with clathrin lattices and decorated with γ-adaptin-coated buds (5). In the presence of BFA, these coats are not formed, and polarized targeting of TIR in MDCK cells is lost (5). In fibroblasts, BFA was reported to cause a small decrease in the rate of TIR recycling (33). Because rab4 also reduced the polarity of TfR sorting, we next investigated whether BFA and rab4 had synergistic effects on TIR recycling. When nontransfected MDCK cells were treated with BFA, we found that 30% of basolaterally endocytosed 125I-Tf was transcytosed into the apical medium after 2 h. In cells transfected with rab4 or rab4Q67L, there was no additional effect of BFA on apical transcytosis of 125I-Tf (Fig. 7), whereas we found a slight decrease in basolateral recycling of 125I-Tf, probably due to retarded transport causing intracellular accumulation of 125I-Tf. These results suggested that rab4 and BFA may act in the same, but not parallel, TIR transport pathways.

Apical Secretion of Glycoprotein 80 Is Not Altered in rab4 Transfectants—To investigate whether enhanced recycling of 125I-Tf into the apical medium in cells transfected with rab4 and rab4Q67L was due to pleiotropic effects of rab4 expression, we next investigated the secretion of gp80. This 80-kDa sulfated protein is constitutively secreted in a polarized fashion into the apical medium of filter-grown MDCK cells (34). Transfectants were metabolically labeled for 60 min with 35S TranS label and chased for 60 min. At the end of the chase time, we immunoprecipitated gp80 from the apical and basolateral media. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and analyzed by phosphorimaging. As shown in Fig. 8, most of the gp80 molecules were apically secreted in control cells. When we quantitated the extent and
polarity of gp80 secretion in the rab4 transfectants, we found that ~75% of gp80 was apically secreted and that neither wild type rab4 nor the two mutants affected biosynthesis, maturation, and polarized targeting of gp80. Given these results, it is unlikely that the increased apical transcytosis of 125I-Tf we observed in rab4 and rab4Q67L transfectants was caused by general effects of rab4 overexpression on membrane transport.

**DISCUSSION**

Membrane transport through the endocytic pathway has been extensively investigated in the MDCK cell line, a model system for polarized epithelia. Although the basic outline of the intracellular transport pathways connecting the basolateral and apical plasma membranes has been defined, it is clear that much remains to be learned about the compartmental boundaries, the proteins that endow EEs with their specific properties, and the molecules regulating transport between these compartments. We have been focusing on rab4, a small GTPase that is involved in membrane recycling from EEAs in fibroblasts. Initially, we found that rab4 is expressed in MDCK cells; however, its function in the endocytic pathway of polarized epithelia has not yet been addressed.

Here we generated an MDCK cell line ectopically expressing TIR to facilitate the analysis of Tf endocytosis, recycling to the basolateral plasma membrane, and transcytosis to the apical cell surface. This cell line was subsequently double-transfected with rab4 cDNA or an active mutant deficient in GTP hydrolysis and an inhibitory mutant that poorly binds GDP to investigate the role of the small GTPase in MDCK cells. Our results show that rab4 is localized on early endocytic compartments or transport vesicles that are positioned distally to basolateral EEs and concentrated in the apical cytoplasm and suggest that rab4 regulates transport through or from this compartment. These conclusions are based on the following observations. First, all intracellular structures that contain rab4 are accessible to endocytosed IgA and Tf; however, its function in the endocytic pathway of polarized epithelia has not yet been addressed.

First, all intracellular structures that contain rab4 are accessible to endocytosed IgA and Tf. Second, rab4 colocalized to a limited extent with EEA1 and not with internalized Tf at 16 °C. The observations that rab4 colocalized to a limited extent with EEA1 and not with internalized Tf at 16 °C show that basolateral EEs represent only a limited subset of rab4-positive compartments. The density of the membrane-associated rab4 signal increased from the basolateral cytoplasm toward the apical cytoplasm just below the tight junction marker ZO-1. Internalized IgA did not colocalize with rab4 after longer chase periods, and most of pIgAR is in structures that are closer to the apical plasma membrane than rab4, which strongly suggest that rab4 is associated with the tubular CE domain. Our results are consistent with subcellular fractionation experiments of MDCK cells, where the small G protein was found in purified recycling endosomes (9).

What might be the function of rab4 on the CE? Important clues with respect to this question are provided by the effect of BFA on basolateral targeting of Tf. BFA causes significant changes in the morphology of endosomes in MDCK cells, although the sequential arrangement of EEs, the CE, and the ARE as well as their biochemical identity reportedly remains intact (38) in the presence of BFA. BFA and, to a somewhat lesser extent, endocytosed rab4 abrogate polarized recycling of Tf to the basolateral plasma membrane in MDCK cells, and significantly more Tf is transported to the apical plasma membrane, suggesting an effect on polarized sorting (5, 38). In agreement with this, BFA causes dispersal of γ-adaptin-containing clathrin lattices from the CE in filter-grown MDCK cells (5), whereas in CHO (21) and A431 cells (39), BFA induces extensive tubulation of endosomal membranes enriched in Tf and rab4-containing domains. We found that rab4 is primarily localized to the CE, and because the effect of BFA on polarized sorting appears to be at the level of this organelle (38), these independent observations suggested that rab4 and BFA may act in the same transport step with respect to apical Tf transcytosis in polarized MDCK cells. To test this hypothesis, we examined the effect of BFA on Tf sorting in rab4-transfected cell lines. If rab4 and BFA regulated distinct routes of Tf transport through endosomes, a synergistic effect of rab4 on BFA-mediated sorting of Tf would be anticipated. In contrast, if rab4 and BFA acted in the same transport pathway, such synergism would not be expected. This is precisely what we found and is consistent with recent data documenting a role of rab4 in the formation of TIR-containing vesicles (17). However, because BFA affects the assembly of multiple coats on different intracellular organelles, and because the association of the AP-1 subunit, γ-adaptin, with distinct compartments of

**FIG. 8.** Polarized secretion of gp80 is not affected by rab4. MDCK transfectants were grown on 24-mm Transwell filters for 4 days. Cells were depleted for 30 min in methionine- and cysteine-deficient medium, labeled with 35S Trans for 60 min at 37 °C, and chased for 60 min at 37 °C. Apical and basolateral media were collected, from which gp80 was immunoprecipitated with a polyclonal antibody. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and analyzed by phosphorimaging.
the transcytotic pathway is inhibited by BFA (39), it is clear that alternative explanations cannot be excluded. Formally, a sequential arrangement of activities of rab4 and of a BFA-sensitive target in the transcytotic pathway taken by basolaterally internalized Tf would also be consistent with the absence of synergism between rab4 and BFA to enhance Tf transcytosis. However, the latter possibility is less likely, given the predominant localization of rab4 to the CE and the observation of Dunn and co-workers (38) that the effect of BFA on polarized

Dunn and co-workers (38) that the effect of BFA on polarized
inant localization of rab4 to the CE and the observation of
of synergism between rab4 and BFA to enhance Tf transcytosis.

sensitive target in the transcytotic pathway taken by basolat-
sequential arrangement of activities of rab4 and of a BFA-
that alternative explanations cannot be excluded. Formally, a
(22, 44). Interestingly, rabaptins also bind to
ants have been identified that interact with rab4 (and rab5)
transcytosis from the CE is not known. However, rab4 effectors
transport of Tf is caused by inhibited basolateral recycling at
localization of these rab proteins in the same cell.
ious destinations in MDCK cells, as has been proposed for rab5,
and epithelial-specific rab17) has recently been evaluated.
overexpression enhances delivery of endocytosed material from
compartments in epithelial cells. Ubiquitously expressed rab5
nificance of the link between rab4, its effector proteins, and
dependent budding events from endosomes. The functional sig-
and helpful suggestions.

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