Regulation of Vesicle Trafficking in Madin-Darby Canine Kidney Cells by Rab11a and Rab25*

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Polarized epithelial cells maintain the polarized distribution of basolateral and apical membrane proteins through a process of receptor-mediated endocytosis, sorting, and then recycling to the appropriate membrane domain. We have previously shown that the small GTP-binding proteins, Rab11a and Rab25, are associated with the apical recycling system of Madin-Darby canine kidney cells. Here we have utilized inducible expression of wild-type, dominant negative, and constitutively active mutants to directly compare the functions of Rab25 and Rab11a in postendocytic vesicular transport. We found that a Rab11a mutant deficient in GTP binding, Rab11aS25N, potently inhibited both transcytosis and apical recycling yet failed to inhibit transferrin recycling. Similarly, expression of either wild type Rab25 or the active mutant Rab25S21V inhibited both apical recycling and transcytosis of IgA by greater than 50% but had no effect on basolateral recycling of transferrin. Interestingly, the GTPase-deficient mutant Rab11aS20V inhibited basolateral to apical recycling of IgA, but had no effect on either apical or basolateral recycling. These results indicate that neither Rab11a nor Rab25 function in the basolateral recycling of transferrin in polarized Madin-Darby canine kidney cells, consistent with recent morphological observations by others. Thus, transferrin receptors must be recycled to the plasma membrane prior to sorting of apically directed cargoes into Rab11a/Rab25-positive apical recycling endosomes.

Eukaryotic cells continually take up fluid, solutes, and macromolecules through endocytosis. After internalization, these materials are either recycled to the cell surface or transported to later endocytic compartments. Separation of recycling receptors from lysosomally targeted ones takes place in the sorting endosomes, which are located in the periphery of the cell (1).

Recycling receptors (e.g. transferrin receptor) subsequently enter a tubular and pericentriolar recycling endosome, where they are repackaged and transferred back to the plasma membrane (2).

The endocytic pathway of polarized epithelial cells exhibits an additional level of complexity because these cells maintain distinct apical and basolateral membrane domains (3–5). Studies in polarized MDCK1 cells have shown that fluid-phase markers internalized from the apical or basolateral surface of the cell accumulate in distinct apical or basolateral early endosomes before accumulating in common late endosomes (6, 7). However, electron microscopy studies indicate that transferrin internalized from the basolateral side of the Caco-2 cell is found in an apical endosomal compartment that is also accessible to apical endocytic markers (8, 9). In polarized MDCK cells, polymeric IgA receptor (pIgR) internalized from the basolateral surface is first delivered to basolateral sorting endosomes and then transported to apical tubular endosomal compartments before being delivered to the apical plasma membrane, and these endosomes are also accessible to apically internalized IgA (10–13). These results suggest the existence of apical recycling endosomes that receive cargoes from both apical and basolateral early endosomes. It is believed that recycling receptors and transcytosing markers are sorted in these apical recycling endosomes, but the exact nature of these compartments and the way(s) in which they interact with other partners in the endocytic pathway remain obscure.

Interestingly, the apical recycling endosome in polarized cells shares similar features with the recycling endosome in nonpolarized cells: 1) it has a tubulovesicular morphology; 2) it is located close to the centrosome; 3) it is dependent on the integrity of microtubules; and 4) it is accessible to membrane-bound but not fluid-phase endocytic markers (10, 14). These similarities have suggested that the apical recycling endosome is the counterpart of the recycling endosome of nonpolarized cells. It is possible that polarized cells would not require additional endosomal compartments for the polarized sorting of membrane constituents. Instead, the recycling endosome could be modified to function in polarized cells through the expression of polarized cell-specific regulatory proteins (15).

Rab proteins are Ras-like small GTPases, which are involved in regulating various aspects of the membrane trafficking process. More than 50 Rabas are present in distinct membrane compartments in mammalian cells. Cycles of GTP binding and hydrolysis of these proteins are linked to the recruitment of the GTP-bound state of Rab proteins (16). These hydrolysis events are thought to be important in the regulation of membrane protein sorting and membrane recycling events (16).

Interestingly, expression of the constitutively active Rab25S21V mutant inhibited apical recycling of apical endocytic markers (8, 9). Transcytosing IgA in MDCK cells is also inhibited by the active Rab25 mutant (14). These results suggest that either Rab11a or Rab25 is involved in the endocytic recycling of apical endocytic markers.

In conclusion, our results indicate that neither Rab11a nor Rab25 is involved in basolateral recycling of IgA. Rab11a appears to be involved in basolateral recycling of transferrin, with the active Rab11a mutant Rab11aS25N inhibiting both apical and basolateral recycling of IgA. Rab25 appears to be involved in apical recycling of IgA, with the active mutant Rab25S21V inhibiting both apical and basolateral recycling of IgA. These results suggest that both Rab11a and Rab25 are involved in the endocytic recycling of apical endocytic markers, with Rab11a involved in basolateral recycling and Rab25 involved in apical recycling of IgA.
specific effectors on cellular membranes. The active GTP-bound form is then thought to regulate membrane vesicle docking with target membrane surfaces (for review see Refs. 16–18). A subset of Rab proteins participates in the endocytic pathway. For example, Rab5 regulates transport from plasma membrane to early endosome and homotypic fusion between early endosomes (19); Rab7 functions in transport from early endosome to late endosome and lysosome (20, 21); and Rab11a modulates transport through recycling endosomes (22). Many Rab proteins in the endocytic pathway are expressed ubiquitously. However, Rab17 and Rab25 are only found in polarized epithelial cells. Rab17 was identified in the epithelial cells of kidney and lung during cell polarization (23). In polarized Eph4 cells, Rab17 was localized to the apical endosomal membranes, and Rab17 mutants that were defective in either GDP or GTP binding were unable to transcytose transferrin. Rab25 was found in a pericentriolar endosomal structure that was accessible to dimeric IgA internalized from the apical or basolateral side of the cell. Rab25 was located in an apical pericentriolar endosomal compartment that depended on intact microtubules for its integrity. Moreover, this compartment was accessible to IgA internalized from either the apical or basolateral side of the cell surface. Interestingly, a closely related Rab, Rab11a, colocalized with Rab25 in this subapical compartment. Because Rab11a is known to function in the recycling endosome to regulate membrane trafficking in nonpolarized cells, it is possible that the ubiquitous role of Rab11a in plasma membrane recycling applies to polarized MDCK cells. Introduction of Rab25 in polarized cells such as MDCK may provide an additional check point in the trafficking pathway. Our observation that overexpression of Rab25 inhibits IgA transcytosis and apical recycling supports this view (14).

Whereas our previous studies suggested that Rab11a and Rab25 both were associated with the apical recycling system in MDCK cells, the relative contributions of Rab11a and Rab25 to plasma membrane recycling in polarized MDCK cells have not been studied. In this study we inducibly expressed Rab11a and Rab25 along with their corresponding constitutively active and dominant negative mutants and examined their effects on basolateral recycling of transferrin as well as transcytosis and apical recycling of polymeric IgA. We demonstrate that mutants of Rab11a deficient in GTP binding fail to inhibit basolateral recycling of transferrin, yet have pronounced effects on apical recycling and transcytosis of polymeric IgA. Somewhat surprisingly, the GTPase-deficient form of Rab11a also inhibited transcytosis but had no effect on apical or basolateral recycling. Wild type Rab25 and GTPase-deficient Rab25 behaved similarly, with both potently inhibiting apical recycling and transcytosis, without affecting transferrin recycling. These studies demonstrate that both Rab11a and Rab25 function at a late stage in the transcytotic and apical recycling pathways, beyond the point at which transferrin has been sorted away from IgA. This pattern of sorting is clearly different from the situation in nonpolarized cells in which Rab11a is present on transferrin-containing compartments and regulates transferrin recycling.
were incubated with purified human dimeric IgA (10 μg/ml) in either the apical or basolateral medium for 30 min at 37 °C in Hanks’ buffered saline containing calcium, magnesium, and 0.2% bovine serum albumin (Hanks’ bovine serum albumin), washed three times quickly in the same medium, and fixed for confocal microscopy as described above.

RESULTS

GTPase-deficient Mutants of Rab11a and Rab25—Most previous studies of Rab protein function, including those involving Rab11a, have utilized WDTAGQE to WDTAGLE mutations in the P3 GTP binding motif to generate putative dominant active proteins (31, 32). However, we have previously shown that Rab11aQ70L exhibits normal GAP-stimulated GTPase activity (14). Additionally, Rab25, which has WDTAGLE as its wild type sequence, is also an active GTPase (26). We therefore sought to construct other mutations in Rab11a and Rab25, which would inhibit their GTPase activities. Fig. 1 demonstrates that Rab11aS20V and Rab25S21V both display the characteristics of dominant active proteins. Both display specific binding of GTP, albeit at a somewhat reduced capacity (Fig. 1, A and B). However, neither mutant shows any significant intrinsic GTPase activity and the mutations abolish GAP-stimulated GTPase activity (as assayed in the presence of gastric cytosol, Fig. 1, C and D).

Expression of Rab11a and Rab25 Mutants in MDCK Cells—To compare the roles of Rab11a and Rab25 in MDCK cells, we prepared stably transfected cell lines overexpressing Rab11a wild type, the dominant negative Rab11aS25N, nor Rab11aS25N, nor Rab11aS20V, or Rab25S21V or MDCK cells infected with adenovirus constructs for wild type (wt) Rab25 or Rab25T26N were cultured in the absence (−) or presence (+) of 20 ng/ml doxycycline. Total cellular proteins were resolved on SDS-polyacrylamide gel electrophoresis gels, and Western blots were performed to detect Rab11a in cells transfected with Rab11a constructs (upper panel) or Rab25 in cell lines transfected with Rab25 constructs (lower panel). (Fig. 2). However, lowering of tetracycline to 0.2 ng/ml elicited a prominent expression of the Rab proteins (Fig. 2). All six recombinant proteins were expressed at similar levels.

Transferrin Trafficking in MDCK Cells—To investigate the effects of Rab protein expression on basolateral recycling, we studied the trafficking of 125I-canine transferrin in the four stable cell lines as well as in plgR-transfected MDCK cells infected with adenovirus coding for wild type Rab25 or Rab25T26N. Cells cultured on permeable filters supports were allowed to internalize 125I-transferrin from the basolateral medium in polarized cells for 30 min. Release of 125I-transferrin into the apical and basal media was then followed over a 60-min time course to assess transcytosis and basolateral recycling, respectively. As shown in Fig. 3, neither wild type Rab11a, the GTP binding-deficient mutant Rab11aS25N, nor...
GTPase-deficient Rab11aS20V had any effect on either basolateral recycling or transcytosis of transferrin (Fig. 3A). This result was somewhat surprising in that previous observations in nonpolarized cells (22, 31) indicated that Rab11aS25N potently inhibited transferrin recycling. To confirm these findings we also infected polarized MDCK cells with a tet-regulated adenovirus encoding a second GTP binding-deficient mutant, Rab11aN124I(30). In agreement with the findings described above, no effect on transferrin basolateral recycling was observed in cells expressing Rab11aN124I (data not shown). It should be noted, however, that unattenuated expression of either Rab11aS25N or Rab11aN124I did cause a decrease of tight junction integrity leading to some leakage of transferrin through the paracellular space (data not shown). Under standard assay conditions (0.2 ng/ml doxycycline) tight junction integrity typically remained intact as determined by measurements of transepithelial resistance. As occasional leakiness was observed even in the presence of doxycycline, only monolayers that retained transepithelial resistance similar to that of controls were used. Similarly, none of the Rab25 constructs had any effect on trafficking of transferrin (Fig. 3). Similar results were observed using a shorter (10 min) pulse of transferrin (data not shown). These data suggest that, in polarized cells, neither Rab11a nor Rab25 participate in basolateral recycling of transferrin.

Effects of Rab11a and Rab25 Mutants on Apical IgA Recy-
To assess apical membrane recycling and basolateral transcytosis, we studied the transport of polymeric IgA. To investigate apical membrane recycling and basolateral transcytosis, we studied the transport of polymeric IgA. To assess apical recycling, $^{125}$I-dimeric IgA was internalized from the apical surface for 30 min at 37 °C. Cells were then cooled to 4 °C and washed, and surface-bound ligand removed by incubation with trypsin (10 μg/ml) for 1 h at 4 °C. Trypsinization was stopped by washing with cold medium containing soybean trypsin inhibitor (50 μg/ml). Filters were then placed in warm medium, and ligand was recovered from the apical (recycled) and basolateral (transcytosed) medium at the times indicated. Data are the average of two determinations and are expressed as a percentage of total internalized IgA. All data represent trichloroacetic acid precipitable counts (typically 97% of internalized ligand was trichloroacetic acid precipitable) and are representative of three separate experiments.

**FIG. 4.** Effects of Rab11a and Rab25 constructs on apical recycling of polymeric IgA. MDCK monolayers stably expressing the pIgR and tetracycline-regulated transactivator along with constructs for wild type Rab11a, Rab11aS25N, Rab11aS20V, wild type Rab25, Rab25T26N, or Rab25S21V were cultured 24 h in the absence (circles) or presence (squares) of 20 ng/ml doxycycline. To assess apical recycling, $^{125}$I-dimeric IgA was internalized from the apical medium for 30 min, and then the appearance of $^{125}$I-IgA in the apical and basolateral media was determined over a 60-min time course. As previously noted (14), little apical to basolateral transcytosis was observed in MDCK cells, and none of the transected constructs altered this pattern (Fig. 4). Whereas neither Rab11a nor Rab11aS20V altered apical recycling, the dominant negative Rab11aS25N did decrease recycling by 25%. In contrast, wild type Rab25 and Rab25S21V strongly inhibited apical recycling by 35 and 50%, respectively (Fig. 4). As previously reported (14), the GTP binding mutant Rab25T26N did not alter apical recycling or apical-to-basolateral transcytosis. These data suggest that both Rab11a and Rab25 are involved in the process of apical recycling.

**FIG. 5.** Effects of Rab11a and Rab25 constructs on transcytosis of polymeric IgA. MDCK monolayers stably expressing the pIgR and tetracycline-regulated transactivator along with constructs for wild type Rab11a, Rab11aS25N, Rab11aS20V, wild type Rab25, Rab25T26N, or Rab25S21V were grown in the absence (circles) or presence (squares) of 20 ng/ml doxycycline. To assess transcytosis, cells were loaded with $^{125}$I-dimeric IgA from the basolateral medium for 10 min at 37 °C. Filters were then washed; fresh medium was added to apical and basolateral chambers and incubated for the time points indicated. Data are the average of two determinations and are expressed as a percentage of total internalized cpm. Apical medium (containing transcytosed ligand, filled symbols) and basolateral medium (recycled ligand, open symbols) were harvested at each time point. Data are the average of two determinations and are expressed as a percentage of total internalized IgA. All data represent trichloroacetic acid precipitable counts (typically 97% of internalized ligand was trichloroacetic acid precipitable) and are representative of three separate experiments. The mean (± S.D.) internalized cpm for three transcytosis experiments were: WTRab11a-dox, 5148 ± 70; WTRab11a-dox, 5205 ± 85; Rab11aS25N-dox, 4985 ± 91; Rab11aS25N-dox, 4728 ± 103; Rab11aS20V-dox, 5887 ± 78; Rab11aS20V-dox, 6363 ± 156; WTRab25-dox, 5743 ± 73; WTRab25-dox, 6212 ± 180; Rab25T26N-dox, 4751 ± 86; Rab25T26N-dox, 4631 ± 150; Rab25S21V, 5024 ± 103; Rab25S21V-dox, 4611 ± 130. There were no significant differences in internalization by any cell group in the absence or presence of doxycycline.

(14), we observed little basolateral recycling of IgA in these pIgR-transfected MDCK cells. In these assays, wild type Rab11a had no effect on transcytosis (Fig. 5). However, Rab11aS20V and Rab11aS25N both inhibited transcytosis by 25 ± 1% and 29 ± 2%, respectively. Expression of Rab11aN124I in virally transfected cells elicited an even more pronounced inhibition of transcytosis (data not shown). As with apical recycling, wild type Rab25 and Rab25S20V both inhibited transcytosis by 55 ± 1% and 57 ± 1%, respectively. As previously noted, Rab25T26N had no effect on transcytosis. Similar amounts of ligand were internalized under each condition, indicating that the endocytic rate was unaffected by any of the expressed constructs. These data suggest that both Rab11a and Rab25 are involved in the regulation of basolateral to
Effects of Rab11a and Rab25 Mutants on IgA Trafficking Visualized through Confocal Fluorescence Microscopy—To elucidate further the effects of the Rab mutations on trafficking, we studied the morphological localization of polymeric IgA in comparison with overexpressed Rab proteins in cells exposed to polymeric IgA in their basal medium. To visualize the transfected Rab11a proteins, we utilized a monoclonal antibody raised against rabbit Rab11a that does not detect endogenous canine Rab11a in 4% paraformaldehyde-fixed tissues (14, 27). Fig. 6 demonstrates that there was considerable overlap of IgA staining with Rab11a in cells overexpressing wild type Rab11a protein. In contrast, in cells overexpressing Rab11S25N, whereas the transfected protein did appear to be associated with vesicles in the pericentrosomal region, the co-localization with IgA was less apparent (Fig. 5, c and d). In Rab11S20V-transfected cells, the Rab11aS20V immunoreactivity was distributed in a more diffuse punctate pattern throughout the subapical region of the MDCK cells (Fig. 6g). Still, we did observe considerable overlap of the Rab11aS20V immunostaining with IgA (Fig. 6h).

As we have reported previously, in cells overexpressing Rab25, Rab25 immunoreactivity showed extensive overlap with both endogenous Rab11a and IgA. However, whereas Rab25T26N immunoreactivity was observed dispersed throughout the cell cytoplasm, endogenous Rab11a and IgA were distributed together in a normal pattern of pericentriolar vesicles. In cells overexpressing Rab25S21V, Rab25 immunoreactivity was observed dispersed through the subapical region and in the perijunctional region (Fig. 7g). Endogenous Rab11a staining was also dispersed in the upper portion of the cell, as was the staining for polymeric IgA. Although there were areas of overlap, it was clear that much of the Rab25S21V staining was not colocalized with endogenous Rab11a.

**DISCUSSION**

Many studies of Rab protein function have been facilitated by the use of dominant negative and dominant active mutants, which are deficient in either GTP binding or GTPase activity, respectively. Earlier investigations in nonpolarized cells have examined the effects of Rab11aQ70L as a putative dominant active Rab11a mutant (22, 31). However, we have recently found that Rab11aQ70L possesses a normal GTPase activity (14). In addition, Rab25, which contains a WDTAGLE sequence found that Rab11aQ70L possesses a normal GTPase activity (14). In addition, Rab25, which contains a WDTAGLE sequence
Rab protein mutants as a prerequisite to studies in intact cells.

A thorough analysis of the function of some Rab proteins has been problematic because of the toxic effects of mutant Rabs in eukaryotic cells. It is therefore not surprising that we have been unable to produce stable cell lines overexpressing the GTP binding mutants for Rab11a and Rab25, Rab11aS25N and Rab25T26N, respectively. Previously, as well as in the present investigation, we have utilized an adenoviral transfection system in which Rab25T26N expression was controlled by a tetracycline repressible promoter system (14). In the present study, we have used a similar tetracycline repressible system to construct stable cell lines that inducibly express Rab11aS25N as well as the wild type Rab11a and the GTPase-deficient mutant Rab11aS20V. In previous studies, Rab11aS25N transiently transfected in nonpolarized cells (22) demonstrated a vesicular rather than a cytosolic distribution that at least partially colocalized with Golgi markers. We have found that Rab11aS25N expressed in polarized MDCK cells also partially localized in a vesicle pattern, but we did not observe significant overlap with Golgi markers (data not shown). Another GTP binding-deficient Rab11a mutant, Rab11aN124I, demonstrated cytosolic distribution without prominent vesicular localization.2 Both dominant negative mutants potently inhibited IgA trafficking but had little effect on basolateral recycling of transferrin. Forte and colleagues (30) have recently reported that Rab11aN124I inhibited regulated translocation of the parietal cell H/K-ATPase to the apical canaliculial membrane. Because Rab11a is a localized to H/K-ATPase-containing parietal cell tubulovesicles (28, 34), these data are consistent with a role for Rab11a in apical recycling.

The interactions of pathways regulating and coordinating trafficking to the apical and basolateral membranes in polarized cells remain controversial. Cargoes endocytosed from the basolateral membrane are sorted in the early endocytic process to separate cargoes to be directed to the lysosomes through the late endosomal pathway (e.g. low density lipoprotein) or cargoes undergoing recycling to either the basolateral or apical membranes (e.g. transferrin receptor and the polymeric IgA receptor, respectively). Recycling cargoes appear, at least in part, to traverse into a tubulovesicular sorting compartment. Thus, in MDCK cells, Hopkins and colleagues (13) have found that basolaterally endocytosed transferrin is transported into a common tubular sorting system also containing IgA receptor internalized from either the apical or basolateral plasma membrane. These data indicate that ligands destined for basolateral recycling are mixed with those undergoing transcytosis or apical recycling. The pericentrosomal position of this tubular sorting system suggested that it was analogous to the plasma membrane recycling system in nonpolarized cells (13, 35). Because the recycling endosomes in nonpolarized cells are labeled by antibodies to Rab11a, we had previously hypothesized that by analogy Rab11a would be a marker of this common tubular sorting compartment in polarized cells. However, recent data have questioned this supposition. Dunn and colleagues (12) have studied the trafficking of fluorescently labeled transferrin and IgA in polarized MDCK cells. Whereas their results support the segregation of transferrin and IgA away from the low density lipoprotein, they found that transferrin separates from IgA prior to entry into a Rab11a positive compartment. Thus, in polarized cells transferrin was not observed in endosomes labeled with antibodies to Rab11a.

In addition, we have recently found that myosin Vb is a downstream target of Rab11a (36). Expression of the carboxyl tail of myosin Vb lacking a motor domain acts as a dominant negative regulator of transport through the apical recycling system leading to accumulation of IgA but not transferrin (36). These results suggest that there are at least three critical sorting stages after endocytosis: 1) the sorting of recycling cargoes from lysosomally directed ones, 2) the trafficking of basolaterally recycling cargoes out of a common tubular sorting endosome, and 3) the movement of transcytosing and apically recycling cargoes into a more apical endosomal compartment, which contains Rab11a and Rab25 (Fig. 8).

The inability of the Rab11aS25N and Rab11aN124I mutants to inhibit transferrin recycling supports the work of Dunn and colleagues (12). We further observed that dominant active Rab11aS20V overexpression had no effect on either basolateral recycling of transferrin or apical recycling of polymeric IgA, but Rab11aS20V did significantly inhibit transcytosis of IgA. Several possibilities may explain this observation. Cycling of Rab11a may be required for maintenance of the transcytotic pathway, whereas apical recycling does not have an absolute requirement for Rab11a cycling. Morphological studies do suggest that Rab11aS20V altered the localization of the apical recycling endosomes, to a more diffuse distribution in the subapical region of the cell. Previous studies with Rab17 have suggested that cycling of Rab17 between its GDP- and GTP-bound forms is required for normal transcytotic rates (25). In any case, these studies all indicate that Rab11a may associate with different effectors at different points in the membrane recycling pathways. Some of these effectors may require cycling of the Rab11a between its GDP- and GTP-bound forms. Alternatively, it is possible that apically recycling and transcytosing cargoes are maintained in separate or segregated membranes. This separation could be accomplished either through different vesicle pools or through regional segregation within a tubulovesicular structure. Indeed, van Ijzendoorn and Hoekstra (37) have recently provided evidence that distinct sorting pathways may exist in the apical recycling endosome for different sphingolipids. Similarly, Rab11a and Rab25 may be associated with different pathways through the recycling system.

As we have previously reported, overexpression of wild type Rab25 dramatically inhibited both apical recycling and transcytosis. In this study we found that the GTPase-deficient mutant Rab25S21V was even more potent than wild type Rab25 in inhibiting transcytosis and apical recycling. As described above for the Rab11 mutants, neither wild type Rab25 nor Rab25S21V had any effect on basolateral recycling of transferrin. As reported previously, the GTP binding mutant Rab25T26N did not affect any of the three membrane-recycling parameters. It is possible that this lack of transport inhibition accrues from a release of the inhibitory function of Rab25 on apically directed transport.

All of these studies are indicative of the complexity of the apical recycling system in polarized epithelial cells. The diagram in Fig. 8 depicts two hypotheses that may explain the existing data on the roles of Rab11a and Rab25. Both hypotheses are built on the general construct that Rab11a is not present in the tubular common recycling endosomal sorting system (RE) where IgA and transferrin would still be colocalized. The model in Fig. 8A proposes that Rab11a regulates the transport of apically directed cargoes out of the common RE to the apical recycling endosome (ARE). Hopkins and co-workers (35) have proposed a mechanism for sorting within the RE in which basolaterally recycling proteins are selectively removed by clathrin-mediated budding, whereas the remaining endosomal membranes are enriched in transcytosing (or, presumably, apically recycling) cargo and constitute what has come to be known as the ARE. In such a

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model, the Rab-dependent fusion machinery may not be required for formation of the ARE, rather, Rab11a, through its association with myosin Vb, may facilitate the movement of these endosomal membranes through the actin-rich terminal web. A similar transport role has been proposed for Rab6, through its association with its effector, rabkinesin-6 (38). An inability of the constitutively active mutant Rab11aS20V to release its myosin effector may underlie the observed inhibitory effect of this mutant on transport. Alternatively, Rab11a may function in a more traditional role and mediate the fusion of carrier vesicles with the ARE.

Our observation that both wild-type and constitutively active Rab25 inhibit apically directed transport suggests that Rab25 is a negative regulator of this process. In classical terms, Rab25 may regulate endosome-endosome fusion within the ARE (Fig. 8A). In this regard, it is notable that overexpression of Rab5 induces the formation of enlarged sorting endosomes and inhibits recycling presumably by stimulating endosome-endosome fusion (22). Indeed, we do observe an enlargement of ARE membranes in Rab25-expressing cells (14). It is therefore possible that endogenous Rab25 does function physiologically in a positive manner to regulate transport through the ARE. An alternative hypothesis shown in Fig. 8B suggests that Rab11a and Rab25 regulate distinct pathways through the ARE. In this model, it is more likely that these proteins would compete for common exchange factors or effectors, thus accounting for the effects of Rab25 on trafficking.

In summary, using inducible expression of Rab proteins and their mutants, we have examined the function of both Rab11a and Rab25 on vesicle trafficking in polarized MDCK cells. These studies establish quantitative evidence that Rab11a functions differently in nonpolarized and polarized cells. Whereas Rab11a appears to regulate transferrin trafficking in nonpolarized cells, in polarized MDCK cells the predominance of both quantitative and morphological data supports the interpretation that Rab11a does not regulate basolateral recycling. Rather, both Rab11a and Rab25 appear to control aspects of trafficking into or out of a discrete apical endosomal system, separate from tubular sorting endosomes. These studies sup-

![Fig. 8. Models for Rab11a and Rab25 roles in MDCK trafficking. In both models, transferrin receptor (TfR) endocytosed from the basolateral membrane and plgGR endocytosed from either the basolateral or apical membranes traffic through sorting endosomes (S.E.) into a common RE. Transferrin receptor is then recycled to the basolateral membrane out of the RE, and apically directed cargoes are trafficked into the ARE system. A. Rab11a is involved in the process of trafficking to and through the ARE, whereas Rab25 acts as a negative regulator retarding exit from the ARE. B. Rab11a and Rab25 represent separable pathways through the ARE that are potentially competitive.](http://www.jbc.org/)

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port a multilevel organization for the sorting and recycling of cargoes in polarized epithelial cells.

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