Direct Interaction between Rab3b and the Polymeric Immunoglobulin Receptor Controls Ligand-Stimulated Transcytosis in Epithelial Cells

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Introduction

Members of the rab family of small GTPases control many steps of membrane traffic. Unlike coat proteins and SNAREs, which are involved in vesicle formation and fusion, respectively, rab proteins appear to play several different roles in trafficking. The best understood role of rabs is in the regulation of tethering complexes. The prototypical rab is the yeast Sec4p, which regulates the function of the exocyst, a tethering complex that docks exocytotic vesicles at the plasma membrane (PM; Guo et al., 1999). Similarly, rab1a regulates the Golgi tethering protein p115 (Allan et al., 2000), while rab5 regulates the endosomal tethering protein EEA1 (Simonsen et al., 1998). In regulating tethering, rabs may also control SNARE assembly (Waters and Pfeffer, 1999). Rabs regulate vesicle motility, such as via rabkinasein-6 (Echard et al., 1998). Rabs also regulate trafficking in other ways; for example, in the synapse, rab3a limits fusion to one synaptic vesicle per action potential. Despite the multiple roles of rabs and the large number of rab-interacting proteins (Zerial and McBride, 2001), we are not aware of a report of the direct physical and functional interaction of a rab with a cargo protein.

The polymeric Ig receptor (pIgR) is expressed in many epithelial cells that line surfaces exposed to the outside world (Mostov and Kaetzel, 1999). The pIgR has been a preeminent model for studying traffic in polarized epithelial cells. For instance, the pIgR was used in the first demonstration of the existence of a sorting signal that was both necessary (Mostov et al., 1986) and sufficient for targeting to the basolateral (BL) surface (Casanova et al., 1991). The pIgR binds its ligand, dimeric IgA (dIgA), at the BL PM of the cell. After endocytosis, the pIgR-dIgA complex traverses several endosomal compartments and is exocytosed at the apical (AP) PM. Here, the extracellular, ligand binding domain of the pIgR is proteolytically cleaved off and released into secretions together with the dIgA. The released fragment of the pIgR is termed the “secretory component” (SC). The dIgA-SC complex forms the first specific immunological defense against infection. In the absence of dIgA binding, most of the pIgR that is endocytosed at the BL PM is recycled to that PM, though some is transcytosed to the AP PM and cleaved to the SC. Binding of dIgA to pIgR stimulates transcytosis of the dIgA-pIgR complex to the AP PM. This stimulation is thought to be a way of coordinating dIgA transcytosis with the production of dIgA by the secretory immune system. This, however, may not be the case in humans (Brandtzaeg and Johansen, 2001). We have previously reported that dIgA binding elicits a signaling cascade involving activation of p62yes, PLCγ, PKCe, and elevation of intracellular free calcium, Ca (Cardone et al., 1996; Song et al., 1994; Luton et al., 1998, 1999; Luton and Mostov, 1999). The elevation of Ca and activation of PKC promote the exocytosis of the dIgA-pIgR complex at the AP PM. Transcytosis of dIgA is therefore similar to other examples of Ca-stimulated exocytosis.

The rab3 subfamily (3a–d) is involved in Ca-stimulated exocytosis in various cell types (Darchen and Goud, 2000). Interestingly, whereas most rab3 proteins are predominantly expressed in neuronal and endocrine cells, rab3b is also expressed in epithelia (Weber et al., 1994). This prompted us to ask whether rab3b played a role in the Ca-stimulated transcytosis of dIgA and pIgR. We found that rab3b directly interacts with the cytoplasmic domain of the pIgR and controls its dIgA-stimulated transcytosis.

Results

Expression and Localization of Rab3b in MDCK Cells
To investigate the function of rab3b in polarized epithelial cells, we used MDCK cells, an established model system to study intracellular membrane traffic (Mostov et al., 2000). MDCK cells endogenously express rab3b (Figure 1A). Subfractionation experiments showed that rab3b was predominantly associated with membranes,
whereas virtually no rab3b was detected in the cytosol (Figure 1A).

Immunofluorescence (IF) studies were performed to identify the rab3b-containing membranes. As with many other rabs in MDCK cells, we were unable to localize endogenous rab3b. We therefore constructed and stably transfected myc-tagged rab3b into MDCK cells. Addition of an epitope tag to the N terminus of the rab protein does not affect its subcellular localization and activity (Sønnichsen et al., 2000). Confocal microscopy revealed that myc-rab3b localized to vesicular structures in the AP region (Figures 1B and 1B'). Occasionally, rab3b was found clustered around the centrosome (Figure 1B, arrow), typically centered below the AP surface, where many organelles and transport vesicles tend to accumulate (van IJzendoorn and Hoekstra, 1999), presumably as a result of their interaction with the cytoskeleton. Disruption of microtubules with nocodazole, however, only minimally altered the spatial organization of rab3b-positive structures as evidenced by a slightly dispersed staining pattern (Figure 1C), in contrast to the severely affected distribution of rab11a-positive recycling endosomes (data not shown; cf. Casanova et al., 1999). Brefeldin A, which induces microtubule-dependent morphological changes of endosomal and Golgi membranes, similarly did not alter the rab3b staining pattern (Figure 1D). By contrast, treatment with the actin-depolymerizing drug cytochalasin D dramatically altered the spatial distribution of rab3b, reflected by a shift of the majority of rab3b to the centrosome area (Figures 1E and 1E'), suggesting that the organization of rab3b-containing compartments is dependent primarily on actin.

IF colabeling studies with various organelle markers revealed a striking lack of overlap between rab3b and postnuclear supematant, one-fifth was subjected to SDS-PAGE. Of which was subjected to SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes and probed with a rab3b-specific polyclonal antibody (3b) or nonspecific serum (NS). To examine whether rab3b was involved in the transcytosis of pIgR, we compared the localization of rab3b with pIgR. In cells that had not been exposed to dIgA there was considerable colocalization of rab3b and pIgR, particularly in the AP region (Figure 2A). However, if the cells were first exposed to dIgA there was much less colocalization (Figure 2B). Notice in Figure 2B that although the red and green stained vesicles are extensively intermingled there is very little yellow, indicating little true colocalization. In contrast, in Figure 2A there is extensive yellow signal, indicating colocalization, at least at the light microscopy level. Thus, although rab3b is colocalized with the pIgR in the absence of dIgA-mediated stimulation of transcytosis, dIgA binding to pIgR and the subsequent stimulation of transcytosis caused the dIgA-pIgR complexes and rab3b to be present in mainly different locations.

Interaction between Rab3b and pIgR

In coimmunoprecipitation (co-IP) experiments, antibodies against endogenous rab3b or pIgR coprecipitated (co-IPed) pIgR and rab3b, respectively, in a reciprocal
manner (Figures 3A and 3B). In the typical experiment shown in Figures 3A and 3B, 17% of total rab3b co-IPed with plgR and 24% of the plgR content co-IPed with rab3b. plgR did not co-IP with rab9 (Figure 3A), typically associated with late endosomes, or the recycling endosome marker rab11a (Figure 3A), while rab3b did not co-IP with transferrin receptor (Figure 3B), suggesting that the observed interaction between rab3b and plgR was specific. Co-IP experiments using MDCK cells that express previously described deletions of the cytoplasmic domain of plgR, that is, plgR655–668 and plgR-R655-STOP, revealed that a 14 amino acid membrane-proximal segment is required for the interaction with rab3b (Figure 3D).

To address whether the rab3b-plgR interaction formed only after cell lysis, lysates of cells that separately expressed only plgR or only myc-rab3b were combined after lysis, and plgR or rab3b were immunoprecipitated. No co-IP of rab3b and plgR was observed (Figure 3C), indicating that the proteins interacted before cell lysis.

To examine the nucleotide dependency of the rab3b-plgR interaction, we transiently transfected MDCK cells with myc-rab3bWT, -Q81L, or -T36N, the latter two of which are GTP- and GDP-locked mutants, respectively (our unpublished data). As with endogenous rab3b, ~21% of total myc-rab3bWT coprecipitated with plgR (Figure 3E), suggesting that the myc tag did not affect the rab3b-plgR interaction. Of total myc-rab3bQt36N, ~37% co-IPed with plgR, whereas no myc-rab3bnt36N co-IPed with plgR (Figure 3E). IF analysis of the subcellular distribution of the rab3b mutants showed that plgR colocalized with rab3bQ81L (see below), but not with rab3bT36N, which displayed a diffuse cytosolic staining pattern (data not shown). These data suggest that, in vivo, GTP-bound rab3b is present in a complex with plgR.

To test whether the proteins interact directly, in vitro binding assays were performed with recombinant GST-rab3bWT and the purified cytoplasmic domain of plgR. As shown in Figure 4, the cytoplasmic tail of plgR directly interacted with GST-rab3b, but not with GST alone. Quantitative analyses revealed that while ~9% of the available plgR tail bound to GST-rab3b, no detectable interaction occurred between the plgR tail and either GST-rab3a, -rab9d, -rab25, or -rab5. We found that, in vitro, the cytoplasmic domain of plgR was also able to interact with recombinant GDP-bound GST-rab3b (Figure 4). Together, the data suggest that the interaction between rab3b and plgR is direct, specific, and does not require additional proteins.

**Figure 2. Comparison of Rab3b and (dIgA-Bound) plgR Distribution**

(A) MDCK cells transiently expressing rab3bWT and plgR were fixed and double stained for myc-rab3b (green) and plgR (red).

(B) The basolateral surface of MDCK cells transiently expressing rab3bWT and plgR was incubated with TxR-dIgA (in red) as described in Experimental Procedures, and fixed and stained with 9E10 antibody to visualize myc-rab3b (in green). The panels show a stack of five 1 μm x-y sections, taken from the AP PM, which were merged into a single plane. The scale bars represent 5 μm.
Figure 3. Interaction between Rab3b and pIgR

(A and B) MDCK cells stably expressing pIgR were lysed and subjected to IP.

(A) The lysate was incubated with polyclonal sheep anti-rabbit SC antibodies or nonspecific serum (NSS). The precipitate was washed extensively, subjected to SDS-PAGE, and transferred to blotting membranes. Blots were probed with antibodies against rab3b, rab9, or rab11a. The right lane of each blot (input) shows the presence of blotted proteins in one-fifth of the sample following the IP step. Quantitative analyses of the blots revealed that in these typical experiments, 17% of total rab3b co-IPed with pIgR, while nothing of total rab9 and rab11a co-IPed with pIgR.

(B) Lysates were incubated with anti-rab3b antibodies or NSS, and the blots were probed for pIgR or transferrin receptor (TfR). In this typical experiment, 24% and 0% of total pIgR and TfR co-IPed with rab3b, respectively.

(C) MDCK cells that separately expressed only pIgR or only myc-rab3b were lysed. Lysates were combined only after lysis, and pIgR or rab3b were immunoprecipitated using antibodies against pIgR or the myc epitope, respectively. Note that no co-IP of rab3b and pIgR was observed under those conditions.

(D) MDCK cells stably expressing either pIgR-WT, pIgRΔ655-668, or pIgR-R655-STOP were lysed. Rab3b was immunoprecipitated and the precipitate subsequently probed with sheep anti-rabbit SC antibodies to detect the presence of pIgR. The presence of the pIgR in each starting material was verified (not shown).

(E) MDCK cells previously transfected with the pIgR were transiently transfected with myc-rab3bWT, -rab3bQ81L, or -rab3bT36N. Polarized monolayers were lysed and pIgR was immunoprecipitated. Precipitates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with the anti-myc antibody 9E10. The presence of each rab3b construct in one-fifth of the sample following the co-IP step is shown in the bottom panel. Quantitative analyses of the blot revealed that in this typical experiment, 21%, 37%, and 0% of total myc-rab3bWT, myc-rab3bQ81L, and myc-rab3bT36N co-IPed with pIgR, respectively.

A Constitutively Active Rab3b Mutant Preserves the Interaction with pIgR in dIgA-Treated Cells

We next examined whether GTP hydrolysis of rab3b was required for the disruption of the interaction between dIgA-bound pIgR and rab3b. When cells expressing myc-rab3bQ81L were incubated with dIgA, the interaction between pIgR and rab3b mutant was unperturbed (Figure 5D). This is in contrast to cells expressing myc-rab3bWT, which completely dissociated from dIgA-bound pIgR (Figure 5D), similar to endogenous rab3b (cf. Figures 5A and 5B). Similar to nontreated cells,
rab3bT36N did not co-IP with plgR. In agreement with the co-IP data, TxR-labeled dIgA-bound plgR and myc-rab3bQ81L significantly colocalized (Figure 5E). These data suggest that GTP hydrolysis of rab3b may be required for disruption of the interaction between dIgA-plgR and rab3b.

GTP-Rab3b Inhibits Stimulated Transcytosis of dIgA-Bound plgR

To further address the functional relevance of the rab3b-plgR interaction, cells were transiently double transfected with the plgR and myc-rab3bQ81L cDNAs. Control cells were transiently double transfected with the plgR cDNA and the myc vector. Over 85% of the cells coexpressed the plgR and myc-rab3b mutant, or the myc epitope. Levels of plgR and myc-rab3bQ81L or myc expression, and the ratio in which these were expressed per filter, were reproducible between individual experiments. Surface biotinylation and subsequent transcytosis assays were performed essentially as described in Luton et al. (1998). Briefly, the BL PM of the cells was biotinylated at 17°C, washed, and incubated with or without dIgA (45 min, 37°C). plgR was then immunoprecipitated from the AP media and lysates. Immunoprecipitates were subjected to SDS-PAGE, blotted, and probed with HRP-streptavidin. Apically released SC as a percentage of total plgR is a measure of transcytosis. As shown in Figure 6, ~35% of biotinylated plgR was transcytosed in control cells, that is, cells expressing plgR and the myc tag. Binding of dIgA stimulated transcytosis in these cells by approximately a factor of 1.5, consistent with our previous observations in this system (Luton et al., 1998). Unstimulated cells expressing constitutively active rab3bQ81L transcytosed slightly less plgR (~25%) when compared to control cells that had been transfected with the myc vector lacking the rab3b gene and, importantly, binding of dIgA did not increase transcytosis (Figure 6). The data suggest that GTP-locked rab3b prevents stimulated transcytosis of dIgA-bound plgR, presumably due to its inability to dissociate from the plgR-dIgA complex.

Discussion

Direct Interaction between Rab3b and plgR

We report that rab3b directly interacts with the cytoplasmic domain of the plgR and controls its dIgA-stimulated transcytosis in MDCK cells. Thus, a rab protein can directly interact with cargo to control the trafficking of that cargo.

The interaction most likely occurs at vesicles subjacent to the AP PM (Figure 1B), where rab3b and plgR colocalize (Figure 2A). In other epithelial cells, rab3b localized to the AP domain as well, but was concentrated near the tight junctions (Weber et al., 1994), a pattern we did not typically observe in polarized MDCK cells and which may reflect the cell type studied. The transcytotic itinerary followed by dIgA bound to plgR has been studied in detail (Apodaca et al., 1994; Brown et al., 2000; Futter et al., 1998; Gibson et al., 1998; Wang et al., 2000) and includes sequential transit through BL early endosomes, common endosomes, and AP recycling endosomes (ARE), the latter of which surround the centrosome at the apex of the cell. The spatial organization of the rab3b-positive compartments is dependent on actin (Figure 1E), which plays an important role in the cytoskeleton-disrupting drugs (van Ljendoorn et al., 2000).

Rabs typically cycle between a GTP- and GDP-bound conformation, reflecting their active and inactive forms, respectively (Takai et al., 2001). Co-IP data obtained from MDCK cells expressing GTP- or GDP-restricted rab3b mutants suggest that, in vivo, plgR predominantly interacts with the GTP-bound form of rab3b (Figure 3E). Indeed, the GTP-rab3b mutant is membrane-associated, as is plgR, whereas the dominant-negative rab3b is predominantly cytosolic (data not shown). Moreover, virtually all of the endogenous rab3b in MDCK cells is associated with membranes, not the cytosol (Figure 1A), in agreement with rab3b in other cell types (Larkin et al., 2000). The rab3b region involved in binding to plgR remains to be determined. Two rabCDRs (complementarity-determining regions) are present in all rabs and have been proposed to ensure rab-specific interaction with
Developmental Cell

Figure 5. Effect of dIgA Treatment on the Rab3b-pIgR Interaction

(A and B) Polarized MDCK cells, previously stably transfected with the plgR, were treated with dIgA according to the incubation schedule described in Experimental Procedures. Cells were lysed and plgR (A) or rab3b (B) was immunoprecipitated. Proteins in the precipitates were separated by SDS-PAGE, transferred to blotting membranes, and probed with antibodies against rab3b or plgR. The right lane of each blot (input) shows the presence of the blotted proteins in one-fifth of the sample following the IP step. Note the absence of rab3b and plgR co-IP in dIgA-treated cells, in contrast to that observed in lysates from cells not treated with dIgA.

(C) MDCK cells, stably expressing plgR-WT (upper two panels) or plgR-R657A (bottom panel; Luton and Mostov, 1999), were incubated with dIgA at the BL or AP PM following pretreatment with BAPTA-AM or genistein where indicated, and plgR was immunoprecipitated. Precipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and blots were probed with antibodies against rab3b.

(D) MDCK cells, previously stably transfected with the plgR, were transiently transfected with the cDNA encoding myc-rab3bWT, -rab3bQ81L, or -rab3bT36N, and treated with dIgA as described above. The plgR was immunoprecipitated. Precipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and blots were probed with antibodies against the myc epitope (9E10) to detect the presence of myc-rab3b (mutants). The presence or absence of the myc-rab3b proteins in one-fifth of the sample following the IP step is shown in the bottom panel.

(E) IF micrographs show the distribution of myc-rab3bQ81L in accordingly transfected cells. Myc-rab3bQ81L (green) staining was compared to that of dIgA-plgR (red), essentially as in Figure 2B. In the presence of myc-rab3bQ81L, much of the rab protein and the plgR are relocalized toward the periphery of the apical region of the cell. The scale bar represents 5 μm.

other proteins. The first rabCDR is necessary for the interaction of rab3a with rabphilin3a and other effectors (Ostermeier and Brunger, 1999). Interestingly, only the second rabCDR is variable between the individual rab3 family members. Since, in contrast to rab3b, rab3a and -d do not bind plgR in in vitro binding studies (Figure 4), the second rabCDR may provide a potential domain involved in plgR binding. Future studies are needed to confirm whether this is the case. We mapped the region of plgR required for binding to rab3b to a membrane-proximal 14 amino acid residue of its cytosolic domain which, intriguingly, has been previously shown to be essential for dIgA-stimulated transcytosis of the plgR (Luton and Mostov, 1999).

Rab3b Controls dIgA-Stimulated Transcytosis

The rate-limiting step of the transcytotic pathway is the transfer from the ARE to the AP PM. Consequently, a large portion of plgR and dIgA endocytosed from the BL PM can accumulate in the ARE. Binding of dIgA to plgR at the BL PM specifically stimulates this last step, and requires dIgA-induced activation of the nonreceptor tyrosine kinase p62yes and the elevation of Ca2+ (Luton et al., 1998). Binding of dIgA to plgR abolishes the interaction between the resulting dIgA-plgR complex and rab3b (Figures 5A and 5B). The abrogation of the interaction between dIgA-bound plgR and rab3b requires tyrosine kinase activity and elevated Ca2+, as shown by a rescue of the interaction between dIgA-bound plgR and rab3b in cells that had been pretreated with a tyrosine kinase inhibitor or Ca2+ chelator, respectively (Figure 5C). Interestingly, the persistent co-IP of dIgA-bound plgR with the GTP-locked rab3b mutant (Figure 5D) may suggest that hydrolysis of GTP to GDP on rab3b is the basis of the inability of rab3bWT and dIgA-bound plgR to interact. This is supported by the observation that TxR-dIgA, bound to plgR, displays significantly more colocalization with GTP-locked rab3b (Figure 5E) when...
Rab3b Binds Directly to Cargo

Though this effect was small, it is consistent with the idea that when rab3b is unable to hydrolyze GTP, the pIgR-rab3b-GTP complex may be more likely to recycle rather than undergo non-dlgA-stimulated transcytosis. Although the precise mode of rab3 action is not known, it likely involves maintaining a delicate balance between promoting tethering, docking, and/or priming of exocytic vesicles on the one hand and the inhibition of a distal Ca-sensitive fusion step on the other hand (Darchen and Goud, 2000). Both arms of this balance appear regulated by rab3 downstream effector proteins such as Rim and Noc2 (Wang et al., 1997; Kotake et al., 1997), possibly in concert with Ca\(^{2+}\)/calmodulin which can bind directly to rab3 (Park et al., 1997; Coppola et al., 1999) and pIgR (Chapin et al., 1996).

Stimulation of pIgR transcytosis upon its binding to dlgA requires two kinds of signals (Luton and Mostov, 1999). The first is the signal mediated by tyrosine phosphorylation and elevation of Ca\(^{2+}\). However, to undergo stimulated transcytosis, the pIgR must be sensitized by binding of dlgA, specifically at the BL PM. Furthermore, R657A substitution in the cytoplasmic domain of the pIgR prevents sensitization. The molecular basis of this novel signal of sensitization has been elusive. Our data suggest that the dissociation of rab3b from dlgA-bound pIgR likely accounts for the molecular basis of sensitization. Sensitization and dlgA-mediated dissociation of rab3b both have very specific characteristics, such as a requirement for BL dlgA binding and R657A.

Though intensively studied, not much is known about how rabs function in membrane traffic. For instance, the recruitment of rabs to specific membranes is poorly understood. The binding of rab3b to the pIgR suggests at least a partial explanation for the specificity of binding of rab3b to pIgR-containing vesicles, though other mechanisms must also operate to recruit rab3b to membranes, for example in cells that lack pIgR. Our results provide a new perspective on understanding rab function. Through rabs must at some level functionally interact with cargo proteins, previously this was generally thought to be indirect. Cargo proteins were thought to interact with adaptors, which then interacted with other coat components. Recently, it was reported that the adaptor-like protein TIP47 interacts with both its M6PR cargo and with rab9 (Carroll et al., 2001). Coat proteins, in turn, were generally thought to interact in a spatial and temporal way with other components of the membrane traffic machinery, including rabs, docking factors, and SNAREs. Our results provide an unexpected direct interaction between cargo and a rab protein, and thus a novel insight into the molecular mechanism of membrane traffic.

The number of distinct rabs in the mammalian genome (~60) is much larger than that of coat proteins or SNAREs (Bock et al., 2001). Moreover, rabs interact with a surprisingly large and diverse array of other proteins. Rab5, for instance, apparently interacts with at least 20 different proteins, though only less than half that number have been identified. Our results suggest that cargo may form a new class of rab-interacting proteins, and may account for some of the unexpectedly large number of rab-interacting proteins. Some cargo are very abundant, such as pIgR, which is approximately 1% of the total
protein synthesized in rodent liver (Mostov et al., 1984), and may have developed a “private” interaction to facilitate this major traffic pathway. Future studies will have to reveal whether other examples of cargo exist that interact with specific rabs.

Experimental Procedures

Rab3b Fusions
Recombinant cDNA procedures were carried out following standard protocols. cDNAs encoding human rab3bWT, rab3bT36N, or rab3bQ81L were subcloned in BarnHI/EcoRI-digested pCMVTag3B-myc (Stratagene). All constructs were verified by DNA sequencing.

Cell Culture
MDCK cells were maintained in MEM with 5% FBS and antibiotics. MDCK cells stably expressing full-length rabbit pIgR-WT have been described elsewhere (Apodaca et al., 1994; Luton and Mostov, 1999). Confocal microscopy for the transcytosis assay, cells were subsequently incubated at 37°C for 45 min, after which the AP media was collected and cells were lysed for subsequent immunoprecipitation of pIgR. For IF and co-IP assays, cells were incubated at 37°C for 5 min, which allowed the BL endocytosed dIgA-pIgR to reach BL MDCK cells were maintained in MEM with 5% FBS and antibiotics. with soybean trypsin inhibitor containing ice-cold PBS

Recombinant Proteins
DH5α cells were transformed with cDNA encoding GST-rab3bWT and protein expression was performed according to the manufacturer’s instructions. Protein concentrations were determined and standardized before subsequent incubation with polyclonal anti-rab3b antibodies bound to protein A, or with sheep anti-SC antibodies coupled to protein G agarose beads (Aroeti and Mostov, 1994), at 4°C for 16 hr. In control experiments, non-specific rabbit or sheep serum was used. Bound proteins on the beads were analyzed by SDS-PAGE and immunoblotting using appropriate primary and secondary antibodies or HRP-conjugated secondary antibodies.

Stimulation of MDCK Cells with Dimeric IgA
Occasionally, cells were pretreated with drugs as indicated in the legends. Drugs were kept present during subsequent incubations. In other experiments, the BL PM of the cells was first biotinylated (0.3 mg/ml EZ-link sulfo-NHS-SS-biotin at 17°C; 2×15 min), followed by extensive washing at 17°C. Cells were washed with MEM/BSA (1%) and the filter insert was placed on a drop containing 300 μg/ml purified human dIgA in a humidified chamber. MEM/BSA was placed in the AP chamber. For the transcytosis assay, cells were subsequently incubated at 37°C for 45 min, after which the AP media was transferred back to the well, washed with MEM/BSA at room temperature, and incubated for an additional 15 min in MEM/BSA at 37°C. During the latter step, trypsin was included in the AP medium to prevent the reinternalization of apically delivered dIgA-pIgR. The cells were put on ice and washed with soybean trypsin inhibitor containing ice-cold PBS before use (Apodaca et al., 1994; Luton and Mostov, 1999).

Immunoprecipitation Studies
Cells were washed with ice-cold PBS and lysed on ice with 1% NP-40, 125 mM NaCl, 20 mM HEPES, 50 mM NaF, 400 μM Na vanadate, 5 mM MgCl₂, guanine nucleotides, a cocktail of protease inhibitors, and PMSF. The lysates were cleared by centrifugation, protinated rabbit or sheep serum was used. Bound proteins on the beads were analyzed by SDS-PAGE and immunoblotting using appropriate primary and HRP-conjugated secondary antibodies or HRP-conjugated streptavidin, with ECL as the detection method. IP efficiency was typically >85% in all experiments shown. Co-IP efficiencies are noted in the text and/or legends to the appropriate figures.

In Vitro Binding Studies
Reactions were performed in 20 mM HEPES (pH 7.4), 50 mM NaCl, 20 mM imidazole, 5 mM MgCl₂, and 100 μg/ml BSA. 50 pmol of GST-rab3 WT, or -rab3b, -rab25, immobilized on glutathione Sepharose beads and prepared in the GTP- or GDP-bound conformation, was incubated with 50 pmol of the purified cytosolic domain of pIgR in the presence of 100 μM GTP at 37°C. Beads were washed with reaction buffer, and GST-rab-bound pIgR was detected following SDS-PAGE and blotting with SC166 and HRP-conjugated secondary antibody. Staining of the blot with Ponceau-S indicated that equal amounts of GST-fusion protein were recovered. Control experiments in which the rab protein was omitted from the reaction and GST beads were used instead revealed that virtually no pIgR tail nonspecifically bound to the beads.

Microscopy
All samples were fixed with 4% PFA and permeabilized with saponin followed by sequential incubations with primary antibodies and appropriate Alexa488- or -594-conjugated secondary antibodies as described previously (Apodaca et al., 1994). Confocal microscopy was performed essentially as described previously (Low et al., 1996).
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