Formation of Mutually Exclusive Rab11 Complexes with Members of the Family of Rab11-interacting Proteins Regulates Rab11 Endocytic Targeting and Function*

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Several Rabs, including Rab11, regulate the traffic and sorting of proteins in the endosomal pathway. Recently, six novel Rab11 family interacting proteins (FIPs) were identified. Although they share little overall sequence homology, all FIPs contain a conserved Rab11-binding domain. Here we investigate the role of FIPs as Rab11-targeting proteins and show that the Rab11-binding domain assumes an α-helical structure, with the conserved residues forming a hydrophobic Rab11-binding patch. This hydrophobic patch mediates the formation of mutually exclusive complexes between Rab11 and various members of FIP protein family. Furthermore, the formation of Rab11/FIP complexes regulates Rab11 localization by recruiting it to distinct endocytic compartments. Thus, we propose that Rab11/FIP complexes serve as targeting patches, regulating Rab11 localization and recruitment of additional cellular factors to different endocytic compartments.

Members of the Rab GTPase family have emerged as important regulators of vesicular trafficking, with specific Rab proteins governing specific membrane trafficking steps (1). Rab1 and Rab2 seem to regulate protein transport from the endoplasmic reticulum to the Golgi (2, 3), whereas Rab6 was shown to be important for intra-Golgi transport (4). At least six Rab proteins (Rabs 4, 5, 7, 9, 11, and 15) regulate trafficking and sorting of endocytosed material between endosomes, lysosomes, and the plasma membrane (5–7). Rab11, in particular, plays an essential role in protein recycling from endosomes to the plasma membrane (6). Furthermore, Rab11 has been implicated in regulating several other membrane transport pathways, including phagocytosis (8), apical targeting in epithelial cells (9), insulin-dependent glucose transporter 4 (GLUT4) transport to plasma membrane (10), and protein transport from endosomes to the trans-Golgi network (11).

Despite considerable effort, the mechanism of Rab11 action remains to be fully understood. Cycling between GDP- and GDP-bound forms of Rab proteins is suggested to regulate the recruitment of various effectors to cellular membranes, thereby affecting the targeting and fusion of transport vesicles (12). Thus, the ability of Rabs to interact with several different effector molecules, which are localized in different trafficking pathways, could be the basis for the specific functions of Rab proteins in a variety of cellular processes. Consistent with the role of Rab11 in multiple membrane traffic pathways, several Rab11-interacting proteins have been identified. Using yeast two-hybrid experiments, it has been shown that Rab11 interacts with the myosin Vb globular tail domain, suggesting that Rab11 may regulate the membrane traffic by docking the myosin motor to transferrin receptor-containing transport vesicles (13). Another Rab11-binding protein, Rab11BP/Rabphilin-11, has also been shown to regulate transferrin receptor delivery to recycling endosomes (RE) (14, 15). In addition, we and others have recently identified six novel Rab11-interacting proteins belonging to a family of proteins known as the family of Rab11-interacting proteins (FIPs) (16–20). Although they share little overall sequence homology, all FIP proteins contain a conserved Rab11-binding domain (RBD) at the C terminus of the protein (19). Although RBD is necessary for Rab11 binding (19), the essential elements of the Rab11-binding domain remain to be defined. Furthermore, the role of FIP proteins in membrane traffic is unclear. Given that most mammalian cells express several FIP proteins, one possible role of FIP proteins could be the formation of mutually exclusive complexes with Rab11, thereby directing the recruitment of Rab11 to different membrane traffic pathways. Indeed, the finding that all FIPs seem to contain equivalent, highly conserved Rab11-binding domains is consistent with that idea. In this study, we characterized the impact of Rab11/FIP binding on endocytic traffic. We showed that the RBD assumes an α-helical structure, with the conserved residues forming a hydrophobic, Rab11-binding patch. In addition, we demonstrated that FIPs compete with each other for binding to Rab11 in vitro and form mutually exclusive complexes with Rab11 in vivo. Finally, we showed that FIP proteins regulate Rab11 localization by recruiting it to distinct membranous organelles. Thus, we propose that Rab11/FIP complexes serve as targeting patches regulating Rab11 localization and recruitment of additional cellular factors to different endocytic compartments.

EXPERIMENTAL PROCEDURES

Cell Lines, Plasmids, and Antibodies—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 mM l-glutamine. HeLa cells were transiently transfected using an electroporation procedure as described previously (19). Polyclonal rabbit anti-Rip11 antibody was described previously (19). Polyclonal rabbit anti-FIP2, anti-Eferin, and anti-Rab11 antibodies were prepared by immunization with recombinant FIP2 (490–624), Eferin (600–759), and full-length Rab11a, respectively.

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1 The abbreviations used are: RE, recycling endosomes; FIP, Rab11 family interacting protein; RBD, Rab11-binding domain; GST, glutathione S-transferase; GFP, green fluorescent protein; Tf, transferrin; TxR, Texas Red; EE, early endosome.
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**Fig. 1. Biochemical characterization of Rab11 binding domain.** A, schematic representation of FIP proteins. B and C, to determine the affinities of Rip11 (490–652), FIP2 (378–511), and Eferin (665–759) binding to Rab11a, the glutathione bead pull-down assay was done using various concentrations of recombinant Rab11a in the presence of either guanosine 5′-3-O(thio)triphosphate (GTPγS) or guanosine 5′-O-(2-thio)diphosphate (GDPβS) (B, inset). In B, plotted data are the means of at least three independent experiments.

**Biochemical characterization of Rab11 binding domain.**

1. **Expression and Purification of Proteins—**GFP fusion constructs were made by cloning Rab11a, FIP2, and Rip11a fragments into pGEX-KG (Amersham Biosciences) and transforming them into BL-21 codon + *Escherichia coli* (Stratagene). GST proteins were expressed and purified using glutathione beads as described previously (21). GST fusion proteins were eluted from glutathione column by thrombin cleavage. Soluble proteins were then repurified using a size-exclusion S200 gradient. The fractions containing the protein of interest were pooled and checked for purity by separating on SDS-PAGE, followed by Coomassie Blue staining. The molecular weight of purified proteins was determined by mass spectroscopy (University of Colorado Health Sciences Center core facilities). The molar extinction coefficient (ε280) for each protein was calculated using UV spectroscopy at 205- and 280-nm wavelengths as described previously (32). Protein concentrations were determined either by Bradford assay (55) (in vitro binding experiments) or using ε280 and absorption at 280 nm (CD experiments).

2. **In Vitro Binding Assays and CD Spectroscopy—**In vitro binding assays were performed using 25 µl of packed glutathione beads coated with 20 µg of GST-fusion protein in a final volume of 500 µl and varying amounts of soluble proteins. Reaction buffer consisted of 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM CaCl₂, 0.1% Triton X-100, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 1.5 µM pepstatin. Guanosine 5′-3-O(thio)triphosphate or guanosine 5′-O-(2-thio)diphosphate were added where indicated. Reactions were kept suspended at room temperature for 30 min, pelleted at 2000 × g for 3 min, and washed three times in 1 ml of reaction buffer without bovine serum albumin but with 100 mM guanosine 5′-3-O-(thio)triphosphate or guanosine 5′-O-(2-thio)diphosphate, as necessary. Bound proteins were eluted with 1% SDS. Proteins were separated on SDS-PAGE and stained with Coomassie Blue or immunoblotted. Gels were imaged using a Bio-Rad gel documentation system and quantified using Quantity One software (version 4.1.1; Bio-Rad).

3. **CD Spectra and thermal melts—**CD spectra and thermal melts were recorded on a Jasco J-810 spectrophotometer equipped with a thermoelectric temperature controller. Measurements were made using a 0.1-cm path-length quartz cuvette. Thermal unfolding experiments were performed by measuring the CD signal at 222 nm (1 min averaging time) in 2 °C steps. Data were converted to a fraction of folded protein by fitting the lower and upper base lines as 0 and 100% folded, respectively.

4. **Separation of Cellular Membranes Using Preformed Iodixanol Gradient—**HeLa cells were grown to 70% confluence on four 100-mm plates. Cells were then scraped off in phosphate-buffered saline, washed, and resuspended in homogenization medium (0.25 mM sucrose, 78 mM KCl, 3.87 mM CaCl₂, 10 mM EGTA, 50 mM HEPES, pH 7.2, and 4 mM MgCl₂). Cells were ruptured using 20 strokes of a Dounce homogenizer. Pellet debris and nuclei were sedimented at 1,000 × g. A 5–20% iodixanol gradient was formed using a two-chamber gradient maker, and 1,000 × g supernatants were loaded on top of the gradient. Gradient centrifugation at 90,000 × g for 18 h. Gradient was then collected in 24 0.5-ml fractions and analyzed by immunoblotting.

5. **Confocal and Deconvolution Microscopy, Image Processing, and Quantification—**For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde for 15 min. Cells were then permeabilized with 0.4% saponin, and nonspecific sites were blocked with phosphate-buffered saline containing 0.2% bovine serum albumin, 0.4% saponin, and 1% fetal bovine serum. After incubation with antibodies, samples were extensively washed and mounted in VectaShield (Vector Laboratories). Cells were imaged with inverted Zeiss Axiovert 200 M deconvolution microscope. Image processing was done using Intelligent Imaging Innovations three-dimensional rendering and exploration software. For quantitation of colocalization of transiently transfected HeLa cells, only cells expressing low amounts of protein were used. The cells were considered low expressers when the fluorescence of GFP-tagged protein was similar (no more then 2-fold higher) to the fluorescence obtained from the antibodies against the endogenous protein with which it was being costained. All images were digitally deconvolved before the analysis. The background fluorescence was calculated by imaging an empty field. The background fluorescence was then subtracted from all the images before quantitation.

6. **Transferrin (Tf)-TxA Recycling Assays—**For analysis of transferrin recycling, HeLa cells were plated on collagen-coated glass coverslips and grown to 60% confluence. Cells were washed with phosphate-buffered saline and incubated for 1 h at 4 °C in serum-free, HEPES-
biochemical characterization of Rab11 and FIP protein interactions—Since the identification of Rip11 (18), the family of Rab11-interacting proteins has grown to include six members: Rip11, FIP2, RCP, Eferin, FIP4, and FIP1 (Fig. 1A) (16, 18–20). Based on sequence homology, all FIPs can be divided into three classes (Fig. 1A). Class I FIPs (Rip11 and FIP2) contain a C2 domain at the N-terminal end of the protein. Class II FIPs (Eferin and FIP4) contain two EF-hand domains. Class III includes only one member, FIP1, which exhibits no homology to known protein domains. The common feature of all FIP proteins is the presence of a highly conserved, 20-amino acid motif at the C terminus of the protein, known as Rab11/25 binding domain (RBD) (19). Although this domain has been shown to be necessary and sufficient for binding to Rab11 and Rab25, it is unlikely that the RBD represents the entire Rab11- and FIP-interacting interface. Indeed, Rab11 binding to an RBD peptide is not GTP-dependent (19). Furthermore, Rab11 binding affinity to the RBD peptide is measured in the millimolar range (data not shown), suggesting that other motifs may be important for mediating Rab11 binding. Interestingly, in addition to the RBD domain, all FIPs also contain a predicted α-helix at the C terminus of the protein (Fig. 1A). To test whether these helices might be important for Rab11 binding, we created Rip11 (490–652), FIP2 (378–511), and Eferin (665–759) truncation mutants that contained only RBD domain and predicted α-helix. CD analysis of Rip11 (490–652) (Fig. 2, D, F, and G) and FIP2 (378–511) (data not shown) confirmed that the C termini of both these proteins are highly structured (melting temperature, 68 °C) and predominantly α-helical. To test whether the α-helix is required for Rip11 binding to Rab11, we incubated GST-Rip11 (490–652)-coated glutathione beads with increasing concentrations of soluble recombinant Rab11a. As shown in the Fig. 1, B and C, addition of an α-helical motif to RBD increased the relative binding of Rip11 binding to Rab11 (EC50,5 °C) but not Rab4. Furthermore, Rip11 (490–652) binding to Rab11 was GTP-dependent (Fig. 1B, inset), compared with Rip11 (630–652), which contains only the RBD domain (19). Similar binding affinities (Fig. 1B) and GTP-dependence (data not shown) were also observed for FIP2 (378–511) and Eferin (665–759).

Despite the apparent requirement of an α-helical domain for Rab11 binding, there is little sequence homology among FIP proteins outside of the RBD domain. Thus, the specificity for Rab11 is encoded within the FIP family of proteins remains unclear. One possibility is that Rab11-FIP interactions efficiently. Indeed, Rab11 does overlap with the last two heptads of the α-helical domain. Furthermore, when plotted in the α-helical conformation, all conserved hydrophobic residues of the RBD domain form a hydrophobic patch (Fig. 2A, black). Substitution of the central isoleucine (Fig. 2A, asterisk) with glutamic acid (1629E) completely abolished Rab11 binding, whereas a conservative substitution with valine (1629V) had no effect on Rip11 association to Rab11 (Fig. 2B).

The data presented above suggest that the RBD in its α-hel-
**Fig. 3.** Rab11 forms mutually exclusive complexes with FIP proteins. A–D, HeLa cells were stained for Rab11 (A–D, red), Rip11 (B, green), and Eferin (D, green). E–H, HeLa cells, transfected with Rip11-GFP (E–H, green) were either left untreated (E and F) or treated with 5 μg/ml brefeldin A (G and H) and then stained for Eferin (F and H, red). I, HeLa cells were transfected with FIP2-GFP (green) and stained for Rip11 (red). J, HeLa cells were transfected with myc-Rab11-S25N, fixed, and stained for myc (green) and Rip11 (red). Scale bars, 5 μm (A and B), 2 μm (E–J). K, glutathione beads coated with either GST-Rip11 (490–652) (top) or GST-Eferin (665–759) (bottom) were incubated with recombinant Rab11a in the presence of varying concentrations of Rip11 (490–652). Bound Rab11a was visualized by immunoblotting. L, Rip11 (left) or Rab11 (right) were immunoprecipitated from HeLa cell Triton X-100 lysates. Precipitates were then analyzed for the presence of Rip11, Rab11, Eferin, and FIP2 by immunoblotting.

**FIP Proteins Form Mutually Exclusive Complexes with Rab11**—Because Rip11 interacts with Rab11 via RBD domain with 1:1 stoichiometry, we investigated the possibility that various FIPs form mutually exclusive complexes with Rab11. To test this, we measured Rab11 binding to GST-Rip11 (490–652) and GST-Eferin (665–759) in the presence of increasing concentrations of soluble Rip11 (490–652). As shown in Fig. 3K, soluble Rip11 (490–652) inhibited Rab11 binding to Rip11 and to Eferin in a concentration-dependent manner, suggesting that FIP proteins compete for Rab11 binding.

That, however, does not discount the possibility that individual Rab11/FIP complexes may oligomerize, thus forming large protein complexes that include several different FIPs. Indeed, Rip11, as well as some other FIP proteins, have been shown to form dimers in a Rab11-independent manner (16). To address that possibility, we immunoprecipitated Rip11 from HeLa cells and immunoblotted precipitants for the presence of Eferin protein. As shown in Fig. 3L, neither Eferin nor FIP2 coprecipitated with Rip11 protein complex (left). Rip11 and Eferin did coprecipitate with Rab11, suggesting that both FIPs do bind Rab11 in vivo. These data indicate that FIP2, Eferin, and Rip11 form mutually exclusive complexes with Rab11.

Considering the fact that FIPs compete with each other for binding to Rab11, they would be expected to “share” cellular Rab11. Indeed, myc-Rab11a only partially colocalized with any individual FIP, such as Rip11 (36.5 ± 1.39%) and Eferin (22.73 ± 0.32%), in transiently transfected HeLa cells (Figs. 3, A–D, and 4G). That colocalization was dependent on Rab11 activity, because a dominant-negative Rab11a mutant (S25N) showed very little colocalization with either Rip11 (4.34 ± 2.98%) or Eferin (6.77 ± 1.98%) (Figs. 3J and 4G). To determine whether various FIPs are localized to the same cellular membranes, we transfected HeLa cells with Rip11-GFP and immunostained them with anti-Eferin antibodies. As shown in Fig. 3, E and F, Rip11 and Eferin localized to different organelles with very little overlap between them (8.1 ± 4.3%). Thus, Rip11 and Eferin seemed to play roles in distinct Rab11-dependent membrane traffic pathways. Consistent with that, brefeldin A treatment resulted in tubulation of Rip11-containing organelles, but had little effect on Eferin-containing membranes (Figs. 3, G and H). Interestingly, not all FIPs were segregated onto distinct endocytic compartments. Although Rip11 and FIP2 also formed mutually exclusive complexes with Rab11 (data not shown), they did exhibit some overlap (54 ± 6.2%) (Fig. 3J).

Immunofluorescence analysis of FIP proteins suggests that they may be localized to overlapping but distinct subpopulations of endosomes. To test that, membranes from HeLa cells were separated using preformed linear 5–20% iodixanol gra-
ent (Fig. 4A) and immunoblotted for the presence of Rip11, FIP2, and Eferin (Fig. 4B). Consistent with immunofluorescence microscopy data, all FIP proteins were present in the fractions containing endosomal membranes and displayed overlapping but distinct distribution patterns.

**FIP Proteins Regulate Rab11 Targeting to Endocytic Membranes**—Given the fact that FIPs compete with each other for binding to Rab11, it would be expected that overexpression of any FIP should increase its colocalization with Rab11 GTPase. Indeed, transient expression of Eferin-GFP (Fig. 4, C and D) or Rip11-GFP (Fig. 4, E and F) significantly increased their colocalization with Rab11 (Fig. 4G). Furthermore, a similar effect was also obtained by overexpressing GFP-Rip11 (490–653) (Fig. 4G).

Interestingly, overexpression of Rip11 (490–653) caused extensive tubulation of Rab11-positive endocytic membranes (Fig. 5, A and B). Consistent with the mutually exclusive complex formation of FIPs with Rab11, those tubules were devoid of Eferin (Fig. 5C) and FIP2 (Fig. 5D). Thus, overexpression of Rip11 and Eferin, and especially Rip11 (490–653), seemed to sequester most of the Rab11 away from other FIP proteins. To our surprise, overexpression of GFP-Rip11 (490–653) did not change the localization of endogenous Eferin and FIP2. Despite that, Eferin and FIP2 showed very little colocalization with Rab11 (Fig. 5G); they were still localized to punctuate membranous structures (Fig. 5, C and D). Thus, membrane association of FIP2 and Eferin seemed to be independent of Rab11 binding, suggesting that Rab11 was not involved in recruiting FIP proteins to their corresponding endocytic compartment.

To test whether Rab11 was required for FIP binding to membranes, we transfected HeLa cells with GFP-Rip11 (490–653)-I629E construct. Because the I629E mutation eliminates Rip11 binding to Rab11 (Fig. 2B), the localization of Rip11 (490–653)-I629E would be independent of Rab11 association. As shown in the Fig. 5F, GFP-Rip11 (490–653)-I629E did localize to Rab11-positive structures in the cell periphery but had no effect on Rab11 distribution (Fig. 5E). Furthermore, whereas 1629E mutation inhibited coprecipitation of GFP-Rip11 (490–653) with Rab11, GFP-Rip11 (490–653)-I629E mutants were still capable of binding to cellular membranes (Fig. 5H). We must note, however, that the 1629E mutation increased the amount of GFP-Rip11 (490–653) present in the cytosol (data not shown; Fig. 5, F and H). Thus, whereas Rip11 (490–653) can be targeted to membranes independently of Rab11, Rab11 might be required to stabilize Rip11 association with membranes.

Our data suggested that FIP proteins might play a role in targeting Rab11 to different endocytic compartments by com-
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The key step in understanding endocytic transport is characterization of interactions between Rab GTPase and its binding partners. The identification of multiple Rab11 binding proteins presents a challenge in determining how Rab11 regulates protein transport between plasma membrane and endocytic compartments. One possibility is that several Rab11-interacting proteins form a large protein complex that is involved in coordinating the sequential transport of cargo through various endocytic domains. Our data, however, suggest the existence of several distinct Rab11-containing complexes. The finding that FIP family proteins form mutually exclusive complexes with Rab11 suggests that FIPs play an essential role in regulating the assembly and cellular localization of Rab11-targeting patches.

Rab11 has been implicated in the regulation of multiple membrane traffic pathways, including phagocytosis (8), insulin-dependent GLUT4 trafficking (10), apical transport in epithelial cells (9), and EE-to-trans-Golgi network transport (11). Thus, it is tempting to speculate that different Rab11/FIP complexes may regulate distinct membrane transport steps/pathways. Consistent with this, various FIPs have different tissue expression patterns. For instance, Rip11 is enriched in kidney epithelial cells (18), whereas FIP4 seems to be expressed almost exclusively in brain tissue (25). In addition, we demonstrated here that Rip11, Eferin, and FIP2 had an overlapping but distinctly different cellular distribution patterns. Finally, previous studies implicated the involvement of FIP1, Rip11, and FIP2 in different membrane transport pathways (18, 20, 26).

The classical Rab effector proteins are recruited to appropriate plasma membranes through binding to Rab GTPases (1). Surprisingly, our data revealed that FIP proteins did not require Rab11 for membrane binding. Indeed, overexpression of a dominant-negative Rab11 mutant (Fig. 3 J) or Rip11 (490–653) (Fig. 5, C and D) had no effect on membrane binding of endogenous FIP2, Rip11, and Eferin. Furthermore, the Rab11 binding mutant, Rip11 (490–653)-I629E, was still localized to

competing with each other for binding to Rab11. Thus, overexpression of Rip11 (490–653) would be expected to inhibit Rab11-dependent membrane traffic by sequestering Rab11. To test this, we took advantage of a well characterized transferrin (Tf) recycling assay (22). Endocytosed Tf is sequentially transported through Rab5, Rab4, and Rab11 compartments before being delivered to the plasma membrane (23, 24). The Tf transport throughout different recycling compartments can be followed by using Texas Red-conjugated Tf (Tf-TxR). First, Tf-TxR was bound to plasma membrane Tf receptors by incubating HeLa cells with Tf-TxR at 4 °C for 1 h. Tf-TxR internalization was then initiated by shifting cells to 37 °C. After 5 min, the majority of Tf-TxR was localized to large peripheral endosomes (Fig. 6A). These compartments also stained for EEA1 (data not shown), thus confirming their identity as early endosomes (EE). After 20 min of incubation at 37 °C, some of the Tf-TxR moved to perinuclear compartment (Fig. 6B). This compartment stained for Rab11, and therefore represented RE. Finally, after 60 min of incubation at 37 °C most of the Tf-TxR moved to RE, with trace staining left on EE (Fig. 6C).

To test whether Rip11 (490–653) had any effect on Tf traffic, we transfected HeLa cells with GFP-Rip11 (490–653) (Fig. 6E, green, and G, green) and then followed Tf-TxR transport through EE and RE. After 5 min of internalization, Tf-TxR could be detected in peripheral EE, proving that Rip11 (490–653) had no effect on Tf-TxR endocytosis and delivery to EE (Fig. 6, D and E). Overexpression of GFP-Rip11 (490–653), however, did inhibit Tf-TxR delivery to RE. Even after 60 min of incubation at 37 °C, Tf-TxR remained largely confined to EE (Fig. 6F). Rip11 (490–653)-induced tubules remained largely devoid of any transferrin receptor-TxR (Fig. 6G). Rip11 (490–653) effect was dependent on Rab11 binding because GFP-Rip11 (490–653)-I629E did not inhibit Tf-TxR transport to RE (Fig. 6, H–J). Thus, overexpression of Rip11 (490–653) seemed to inhibit transport from EE to RE, presumably by sequestering Rab11 and making it unavailable for other Rab11 effectors.

**FIG. 5.** Overexpression of Rip11 (490–653) decreases Rab11 colocalization with FIP2 and Eferin. A–D, Hela cells expressing GFP-Rip11 (490–653) (A and B–D, green) were stained for Rab11 (B, red), Eferin (C, red), and FIP2 (D, red). (E and F) HeLa cells expressing GFP-Rip11 (490–653)-I629E (F, green) were stained for Rab11 (E and F, red). Scale bars, 2 μm (A–C, and E); 5 μm (D); and 1 μm (F). G, quantitation of myc-Rab11 colocalization with endogenous Rip11 and Eferin. Left two bars are from cells expressing only myc-Rab11a; right two bars are from the cells coexpressing myc-Rab11a and GFP-Rip11 (490–653). Data are presented as the means from four randomly chosen cells. H, HeLa cells were transiently transfected with either wild-type GFP-Rip11 (490–652) (wt) or GFP-Rip11 (490–653)-I629E mutant (I629E). Cells were then subjected to either Triton X-100 extraction followed by immunoprecipitation with anti-GFP antibodies (top) or subcellular fractionation to cytosol (Cyto) and membranes (Mem) (bottom). Cytosol and membrane fractions were then analyzed for the presence of GFP-Rip11 (490–652) (anti-GFP), GFP-Rip11 (490–652)-I629E (anti-GFP), or integral membrane protein syntaxin 13 (anti-syntaxin 13).
Rab11-containing endosomes (Fig. 5F), although with somewhat lower efficiency. These observations raise an interesting possibility that FIPs may play a role in targeting Rab11 to the appropriate endocytic compartments. Consistent with that, overexpression of Rip11-GFP, FIP2-GFP, or GFP-Rip11 (490–653) dramatically changed Rab11 distribution by sequestering Rab11-containing endosomes (Fig. 5D). This could result in exposure of a cystic binding site that could be used to bind the other membrane traffic regulators, such as molecular motors.

In conclusion, FIP proteins may play a role as Rab11 scaffolding proteins, in this way ensuring the targeting of Rab11 to appropriate endocytic compartments. Upon Rab11 binding, Rab11/FIP complexes may serve as targeting patches for the recruitment of additional cellular factors, such as molecular motors or ARF GTPases. In principle, this mechanism would allow cells to modify and regulate various endocytic membrane transport pathways by changing the expression levels and localization of FIP proteins.

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