Rab11-FIP2: An Adaptor Protein Connecting Cellular Components Involved in Internalization and Recycling of EGF Receptors

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Running title: Rab11-FIP2 binds to the EH domain protein Reps1
Abstract

Rab11-FIP2 is a member of a newly identified family of Rab11-binding proteins that have been implicated in the function of recycling endosomes. Here we show that Rab11-FIP2 may also be involved with the process of receptor-mediated endocytosis. First we demonstrate that Rab11-FIP2 contains an NPF motif that allows it to bind Reps1, a member of a family of EH domain proteins involved in endocytosis. We also show that Rab11-FIP2 associates with the α-adaptin subunit of AP-2 complexes, which are known to recruit receptors into clathrin-coated vesicles. Finally, we find that over-expression of Rab11-FIP2 suppresses the internalization of EGF receptors, but not transferrin receptors through binding sites that promote complex formation with Rab11, Reps1 and α-adaptin. These findings suggest that Rab11-FIP2 may participate in the coupling of receptor-mediated endocytosis to the subsequent sorting of receptor-containing vesicles in endosomes.
Background

A large body of evidence has implicated the Rab GTPases in various aspects of vesicle sorting in cells (for review see (1)). For example, members of the Rab11 family, Rab11a, Rab11b, and Rab25, have been shown to participate in sorting of vesicles in recycling endosomes. In particular, Rab11 activity has been shown to be necessary for apical recycling and transcytosis of IgA, but not for basolateral transferrin trafficking in epithelial cells (2). Like all Ras family GTPases, Rab11 proteins function as molecular switches. When in the active GTP-bound state they interact with downstream effector proteins. Recently, a family of Rab11 binding proteins have been identified that may mediate Rab11 effects in recycling endosomes. These include, Rip11, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3/efrin, Rab11-FIP4 and RCP (3-8). These proteins all share similar Rab11 binding sites within a C-terminal coiled-coil motif. They all also localize to the recycling endosomes containing Rab11. Finally, when tested all had the ability to influence sorting of vesicles through endosomes.

However, differences among members of this Rab11 binding family have emerged. For example, RCP has the capacity to bind Rab4 as well as Rab11(6). Rab11-FIP2 is distinct in that it does not bind preferentially to active Rab11, but binds to both the inactive and active forms of Rab11 equally (5). In addition, although Rip11-FIP2 is clearly present in recycling endosomes with Rab11 and affects receptor recycling(8), its subcellular distribution is not as tightly linked to Rab11 as other family members (5). How these similarities and differences contribute specific functions to individual family members in the recycling endosome remains to be determined.

Recent data have indicated that Ral GTPases, which are close relatives of Ras rather than Rab GTPases, also participate in regulating vesicle function. For example, a growing body of evidence supports the idea that Ral proteins regulate exocytosis. First, Ral proteins are expressed at high levels in synaptic vesicles of neurons and dense granules of platelets (9-11). Second, a newly identified downstream target of the Ral-GTPases is the...
exocyst complex (12-15), which in yeast regulates secretion by targeting secretory vesicles to specific exocytic sites on the plasma membrane (16). Through this effector, Ral appears to influence sorting of vesicles to the basolateral surface of polarized epithelium (14). Third, Ral activity regulates the readily releasable pool of synaptic vesicles in neurons (15) and secretion of growth hormone in PC12 cells (14).

Ral has also been implicated in receptor-mediated endocytosis through interaction with a different effector protein, RalBP1. This notion is supported by data showing that expression of constitutively activated Ral suppressed endocytosis of EGF and insulin receptors (17,18). RalBP1 can form a complex with the μ-subunit of the AP-2 complex (18) and with two closely related EGF receptor substrates Reps1 and POB1. These two proteins contain EH domains, hallmarks of proteins involved in endocytosis (19,20). In fact, the EH domain of POB1 binds epsin, a protein previously implicated in endocytosis (21). The binding between epsin and POB1 in cells correlates with the ability of cells to internalize EGF and insulin receptors [Kariya, 2000 #1245].

In this paper, we identify Rip11-FIP2 as a binding partner for the EH domain of Reps1 and demonstrate that it has the potential to influence endocytosis in addition to its previously detected function in vesicle sorting in endosomes. Rab11-FIP2 influences endocytosis through its ability to interact with Reps1, Rab11, and the adaptor complex AP-2. In this way Rab11-FIP2 may participate in coupling receptor-mediated endocytosis to the subsequent sorting of receptor-containing vesicles in endosomes.

Materials and Methods

Cell Culture and Reagents  COS-7 and NIH 3T3 cells were grown in Dulbecco’s Minimal Eagle Medium plus 10% iron-enriched calf serum and calf serum respectively. COS-7 cells were transfected by DEAE-Dextran/Chloroquine. NIH-3T3 cells were transfected with Lipofectamine Plus (Gibco). Anti-myc (9E10) was from Santa Cruz Biotechnology and anti-HA was from Covance. TexasRed-streptavidin pre-complexed to EGF-biotin (TexasRed-EGF) and TexasRed-streptavidin was from Molecular Probes.
Plasmids AK014696 cDNA provided by Dr. Yoshide Hayashizaki (RIKEN Institute of Physical and Chemical Research) all other RBD protein cDNAs provided by Dr. Osmau Ohara (Kazuka DNA Research Institute). Human Rab11 was a generous gift from M. Zerial. Reps1, Rab11-FIP2, Rab11-FIP2 ΔC2 (103-513), Rab11-FIP2 ΔR11B (1-464), Rip11, AK014696 (mRip11/RCP), KIAA0665 (efrin/Rab11-FIP3) and Rab11 were amplified by PCR with oligonucleotides containing restriction enzyme sites, DNA was digested, and cloned into pJ3H. These were then cloned into pEGFP-C1 or pDsRed1-N1 (Gibco) by restriction enzyme digestion. Rab11-FIP2 cNPF->AAA (406-408) and Rab11-FIP2 YID->AAA (480-482) were created by overlap PCR and cloned into pEGFP-C1 possessing an HA-tag. The Eps15 (a.a 1-336) and Reps1 (a.a 230-301) EH domain domains were expressed as GST fusions using the pGEX-2T vector. The Rab11-FIP2 (a.a 443-513), Rip11 (a.a 592-653), KIAA0665 (a.a 700-756), and AK014696 (a.a 579a.a.-645) RH domains were expressed using the pGEX-4T1 vector. Rab11 was expressed in a pGEX-5X1 vector.

Computer Techniques Homology searches and comparisons were done using the BLAST and PSI-BLAST. Direct sequence comparison was done using the Blosum 45 matrix. Protein domain sequence searches were done using the InterPro version 4.0 search tool. Coiled-coil detection was done using the COILS version 2.2 search engine.

Yeast Two-Hybrid Screening For screening, the Reps1 EH domain (230a.a.-301a.a.) was cloned into the pAS1-CYH2 vector and transformed into the Y190 yeast strain. Yeast expressing the Reps1 EH domain-AD fusion was then transformed with a pGAD10 rat brain Matchmaker cDNA library (Clontech) and transformations were plated onto minimal media (-Trp,-Leu,+His) to select for co-transformed yeast. Colonies were then scraped off plates, used to make a glycerol stock, and then re-plated onto minimal media (-Trp, -Leu, -His, +3-aminotriazol) to select for interactions. Colonies were then re-screened by X-gal filter assay for β-galactosidase expression and colonies that grew on –His plates and turned blue by an X-gal assay were treated as positive colonies. Using the first plating as a standard, approximately 130,000 colonies were screened. Positive
colonies were examined by restriction digest for similarity and unique digests were sequenced.

**GST Binding Assays** GST fusions were expressed in bacteria and pre-complexed to glutathione-sepharose beads in PBS/1% Triton X-100. Approximate concentrations were determined by Comassie blue staining on SDS-PAGE relative to bovine serum albumin standards. Equivalent amounts of GST protein were then incubated with transfected COS-7 cell lysates for 2 hours, washed extensively with the appropriate lysis buffer, and analyzed by SDS-PAGE. EH domain binding was examined in Lysis Buffer (20mM Hepes pH 7.5, 1% Triton X-100, 150mM NaCl, 10% glycerol, aprotinin, leupeptin, sodium benzamide, sodium orthovanadate, PMSF). RBD domain binding was examined in Lysis Buffer with EDTA (20mM Hepes pH 7.5, 1% Triton X-100, 150mM NaCl, 10 mM EDTA, 10% glycerol, aprotinin, leupeptin, sodium benzamide, sodium orthovanadate, PMSF). Rab11 binding was examined in Lysis Buffer with β-ME/MgCl$_2$ ((20mM Hepes pH 7.5, 1% Triton X-100, 150mM NaCl, 1.5 mM MgCl$_2$, 0.5 mM β-ME, 10% glycerol, aprotinin, leupeptin, sodium benzamide, sodium orthovanadate, PMSF).

**GST-Rab11GTPγS loading** Expressed GST and GST-Rab11 were complexed to glutathione-sepharose beads and loaded by washing in Buffer A (20mM Tris pH 7.5, 150mM NaCl, 0.5 mM MgCl$_2$, and 0.5 mM β-ME) twice followed by incubation in 3 volumes of Buffer B (20mM Tris pH 7.5, 150mM NaCl, 0.5 mM MgCl$_2$, and 0.5 mM β-ME, 1mg/ml BSA, 0.2mM GTPγS) at 37°C for 15 minutes. MgCl$_2$ was then added to a final concentration of 30 mM, the sample was then vortexed, and then beads were washed twice with Lysis Buffer with β-ME/MgCl$_2$ before immediate use.

**Immunoprecipitation** The anti-myc antibody was pre-complexed to protein A-sepharose beads for 1 hour then beads were incubated with transfected COS-7 cell lysates for 16 hours, washed extensively with Lysis buffer (20mM Hepes pH 7.5, 1% Triton X-100, 150mM NaCl, 10% glycerol, aprotinin, leupeptin, sodium benzamide, sodium orthovanadate, PMSF), and analyzed by SDS-PAGE.
Fluorescence Experiments COS-7 or NIH3T3 cells were plated onto poly-L-lysine coated coverslips, transfected, and prepared after two days. Coverslips were fixed by 3.7% formaldehyde in PBS for 20 minutes, then permeabilized (if antibodies were used) with 1% Triton X-100 in PBS for 20 minutes. Immunostained cells were then blocked for 30 minutes in TexasRed-EGF Binding Buffer (20mM Hepes pH 7.5, 150mM NaCl, 1% BSA), labeled with mouse anti-HA in 5%BSA/PBS, then with either anti-mouse GFP (generous gift from D. Block) or anti-mouse Alexa 488 (Molecular Probes) in 5%BSA/PBS. Coverslips were washed extensively in PBS after each step. Cells were then incubated with Hoechst 33258 for 5 minutes, washed in PBS, and placed upside-down onto slides with 50% glycerol/PBS.

Endocytosis Assays For endocytosis assays of EGF, COS-7 cells were plated onto poly-L-lysine coated coverslips, transfected and prepared after two days. Coverslips were placed at 4°C for 10 minutes overlaid with 100µl media from the originating dish. Cells were then placed in TexasRed-EGF Binding Buffer (20mM Hepes pH 7.5, 130mM NaCl, 0.1% BSA) with 200ng/ml TexasRed-EGF or TexasRed-transferrin Binding Buffer (DMEM, 20mM Hepes pH 7.5, 1mg/ml BSA) with 100nM TexasRed-transferrin at 4°C for 1hr. Following this, coverslips were placed at 37°C for 12 minutes, then returned to 4°C for 10 minutes before being washed in Acid-wash Buffer (0.2M AcOH, 0.5M NaCl pH 2.5) twice for 5 minutes each. These were then placed in TexasRed-EGF Binding Buffer for 10 minutes at 4°C before being fixed by 3.7% formaldehyde first at 4°C for 20 minutes, then at room temperature for 20 minutes (22).

Peptide binding Peptides containing the first and third NPF sequences (NPF1 and NPF3) of Rab11-FIP2, Ac-PRKKNPFEESS-NH₂, and Ac-IPDSNPFDATA-NH₂, were synthesized by solid-phase methods and purified by C₁₈ reversed-phase high performance liquid chromatography (HPLC). Identities were confirmed using matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The EH domain (residues 227-318) from the Reps1 protein was expressed and purified as described previously (23). Samples for NMR spectroscopy included 0.5 mM ¹⁵N-labeled protein, 10 mM NaCl, 2 mM CaCl₂,
0.01% NaN₃, 5 mM DTT, and 10 mM imidazole at pH 6.8. Peptides were titrated from 10 mM stock solutions into ¹⁵N-labeled EH domain and progressive changes of ¹H and ¹⁵N resonances (Δδ) were monitored with a series of ¹⁵N-HSQC spectra (23). Equilibrium dissociation constants (Kₐ) were determined from plots of Δδ versus the ratio of [peptide] to [protein] (23).

Results

Identification of Rab11-FIP2 as a binding partner for the EH domain of Reps1. We have previously identified Reps1 as an EH domain containing substrate for the EGF receptor that also has the capacity to bind to RalBP1, a downstream target of the Ral-GTPase. Since EH domains are known to be binding sites for proteins containing NPF motifs, we screened for potential binding partners for the Reps1 EH domain using the two-hybrid screen in yeast. DNA encoding the isolated EH domain of Reps1 was used as bait to probe a rat brain cDNA library. Multiple identical clones encoding a protein that was highly homologous (86% identity) to the final 269 amino acids of a human cDNA clone KIAA0941 were detected. Apparently, this Reps1-binding protein is the rat homologue of human KIAA0941. As expected, putative EH domain-binding NPF motifs were found in the Reps1-binding region of the protein (Fig. 1A). Further analysis of the KIAA0941 clone showed that it contains a C2 domain at its N-terminus and a potential coiled coil at its C-terminus that is homologous to a family of previously identified proteins (Fig. 1B).

During our analysis of this Reps1-interacting protein, others identified it as one of a newly discovered family of at least six mammalian proteins with the capacity to bind to the Rab11 GTPase. These include Rab11-FIP1, Rab11-FIP2, Rab11-FIP3 (efrin) Rab11FIP-4, Rip11 and RCP, with the Reps1-interacting protein being Rab11-FIP2. Rab11-FIP2 is most similar to Rip11 and RCP in that they all have N-terminal C2 domains, while Rab11-FIP2 is unique in containing multiple potential EH-domain binding NPF motifs.
To confirm the results from two-hybrid experiments, binding between the isolated EH domain of Reps1 and Rab11-FIP2 was assayed in vitro. The GST-Reps1-EH domain or GST alone was isolated from E. coli and bound to glutathione-sepharose beads. The beads were then incubated with cell lysates expressing HA-tagged Rab11-FIP2. Bound Rab11-FIP2 was then detected by immunoblotting with anti-HA antibodies (Fig 2A). As expected, GST-Reps1-EH but not GST alone precipitated detectable levels of Rab11-FIP2. Beads containing a fusion protein of GST plus the three EH domains of Eps15 were also used as affinity reagent in a similar experiment. However, they were found to precipitate Rab11-FIP2 much less efficiently demonstrating selectivity in the interaction between Rab-11-FIP2 and the Reps1 EH domain.

**Identification of the NPF motifs in Rab11-FIP2 involved in binding the EH domain of Reps1.**

Rab11-FIP2 contains three potential EH domain-binding NPF motifs in the region of the protein that associates with Reps1. We previously used NMR to characterize the interaction between a peptide containing the second NPF sequence (a.a. 406-408 of Rab11-FIP2), which is flanked by amino acids most consistent with EH domain binding. The central NPF peptide (NPF2) was found to bind with an affinity of 46 ± 14 µM (23). For comparison, we performed titrations using NMR to determine the affinities of peptides containing the first and third NPF sequences of Rab1-FIP2. Addition of either NPF1 or NPF3 peptides affected the same EH domain residues as the NPF2 sequence indicating that all peptides bind the same hydrophobic pocket on the surface of the domain (23). However, NPF1 and NPF3 bound much more weakly, with $K_d$ values of 423 ± 150 and 7000 ± 1600 µM, respectively. Negatively charged residues adjacent to the NPF sequence in peptides NPF1 and NPF3 probably reduce affinity because their interaction with the electronegative binding site of Reps1 is not favorable. We then tested the importance of this second NPF motif on binding of Rab11-FIP2 to the Reps1 EH domain by changing this NPF sequence in Rab11-FIP2 to AAA (Fig. 1A). Exposure of the GST-Reps1 EH domain to cell lysates containing this mutant Rab11-FIP2 revealed severely reduced binding activity (Fig. 2B).
Co-localization of Rab11-FIP2 and Reps1 in recycling endosomes Previous work has shown that Rab11-FIP2 is present in recycling endosomes proximal to the nucleus where Rab11 resides (5,8). To confirm these findings, a vector expressing GFP-Rab11-FIP2 fusion protein was transfected into NIH 3T3 cells and the localization of its encoded protein was studied by fluorescence microscopy. Our findings with GFP- Rab11-FIP2 are consistent with published ones showing extensive labeling in perinuclear vesicles that co-localized with Rab11 (Fig. 3A-C). In contrast, expression of GFP alone led to diffuse staining throughout the cell (data not shown). To test the contribution of individual domains of Rab11-FIP2 on the protein’s subcellular distribution, various mutants of Rab11-FIP2 were studied in a similar manner. Deletion of the N-terminal C2-domain of Rab11-FIP2 did not dramatically alter the localization of the protein (Fig. 3D). In addition, mutation of the NPF motif that is responsible for binding to Reps1 had little effect on the protein’s cellular distribution (Fig. 3E). However, as would be expected for a Rab11 binding protein, deletion of the Rab11-binding domain (RBD) (Fig. 3F) or mutation of the highly conserved YID to AAA within the RBD (Fig. 3G) disrupted localization of the protein to the Rab11-containing endosomal compartment. Instead the mutant protein displayed a diffuse cytoplasmic distribution. Thus, Rab11 likely serves as a docking site for Rab11-FIP2 in recycling endosomes. Since Rab11-FIP2 binds to both active and inactive Rab11 it is not clear whether this interaction is regulatable.

GFP-Reps1 was analyzed in a similar manner and was found to yield punctate staining throughout the cytoplasm (Fig. 4A), with some perinuclear staining. To test whether Reps1 and Rab11-FIP2 co-localize, they were transfected together into NIH-3T3 cells with Reps1 fused to DsRed and the cells were analyzed by confocal microscopy (Fig. 4B and 4C). The only area of consistent co-localization was in the perinuclear region. As expected, transfection of a GFP-Reps1 mutant lacking its EH domain failed to localize to this location (Fig. 4D). Thus, Rab11-FIP2 may target a fraction of Reps1 away from the cell surface to the perinuclear Rab11 containing endosomes.
Oligomerization of Rab11-FIP2 through its C-terminal coiled-coil

A common feature of the Rab11-FIP family of proteins is an α-helical coiled-coil of approximately 50 amino acids at their C-termini that includes the Rab11-binding domain (see Fig 1B). Since coiled-coils are known to form homo-and hetero-dimers, we assessed the ability of the coiled-coil at the C-terminus of Rab11-FIP2, containing the Rab11-binding domain (RBD), to homo or hetero-dimerize with Rip11/Rab11-FIP family members. First, glutathione-sepharose beads containing GST or a GST-fusion protein containing the C-terminal 60 amino acids of Rab11-FIP2 were incubated with cell lysates expressing HA-tagged Rab11-FIP2. Rab11-FIP2 bound to beads was assayed by immunoblotting with anti-HA antibodies. Rab11-FIP2 was detected on beads containing GST-coiled-coil but not GST beads alone (Fig. 5A). Furthermore, a Rab11-FIP2 mutant lacking the C-terminal coiled-coil Rab11 binding domain (RBD) did not complex with beads containing GST-coiled-coil (Fig. 5A). These results show that the C-terminal coiled-coil of Rab11-FIP2 can homo-oligomerize. Interestingly, a more modest mutation in the coiled-coil, where the highly conserved YID motif was changed to AAA, was actually more effective than wild-type protein in forming a complex with the coiled-coil domain (Fig. 5B). In contrast, this same mutation blocked Rab11 binding (Fig 5C). In these experiments GST-Rab11 bound to GTPγS was incubated with cell lysates containing either wild type or YID to AAA Rab11-FIP2. Rab11-FIP2 ΔRBD was also included as a negative control and Rip11 as a positive control. These findings raise the possibility that Rab11 binding and oligomerization may be mutually exclusive in cells and thus represent distinct mechanisms of regulating Rab11-FIP2 function.

To determine whether Rab11-FIP2 can homo-oligomerize in cells through the C-terminal coiled-coil, HA-Rab11-FIP2 or HA-Rab11-FIP2 lacking its C-terminal coiled-coil were transfected along with Myc-Rab11-FIP2 into COS7 cells. Myc- Rab11-FIP2 was immunoprecipitated and the immunecomplex was blotted with anti-HA antibodies (Fig. 6). As predicted, full-length Rab11-FIP2 but not Rab11-FIP2 lacking its coiled-coil oligomerized in cells.
Finally, we determined whether other members of this Rab11-binding protein family can homo-dimerize and how well Rab11-FIP2 heterodimerizes with other family members. Thus, lysates of cells expressing Rip11, Rab11-FIP3 and RCP were each incubated with GST, or GST fusions of the coiled-coil domains of each of the family members cited above (Fig. 7). The coiled-coil of Rab11-FIP2 clearly complexed with full length Rab11-FIP2. The coiled-coil of Rip11 also complexed with full-length Rip11. However, neither coiled-coil complexed with the full-length version of any of the other members of the family. For Rab11-FIP3, we could not detect homo-oligomerization. Moreover, RCP bound to both a full-length version of itself and to a lesser degree to a full-length version of Rab11-FIP2. Thus, while most family members have the potential to oligomerize and show some specificity in oligomerization, the specificity is not absolute.

**Influence of Rab11-FIP2 on endocytosis of EGF receptor through its EH domain and Rab11 binding domains**

A common function of many proteins that bind to EH domains is an involvement in the process of receptor-mediated endocytosis. Often overexpression of these proteins leads to inhibition of endocytosis. Thus, the effect of overexpression of Rab11-FIP2 on the uptake of EGF receptors and transferrin receptors was investigated. In these experiments, COS7 cells were transfected with either GFP or a fusion of GFP and Rab11-FIP2. Two days later, Texas-Red-EGF or TexasRed-transferrin was added to cells and allowed to bind to the surface at 4°C to prevent endocytosis. The temperature was then raised to 37°C for 12 minutes and the cells were washed and fixed. Extra-cellular EGF or transferrin was washed away and endocytosis was scored in transfected cells by the presence of detectable intracellular TexasRed staining (Fig. 8A). Under these conditions ~27% of cells transfected with GFP- Rab11-FIP2 failed to take up EGF, which was approximately 5 times the level found in control cells (~5%) transfected with just GFP. In contrast, uptake of TexasRed-transferrin was unaffected by the expression of Rab11-FIP2. GFP and GFP-Rab11-FIP2 transfected dishes showed indistinguishable numbers of cells that failed to internalize transferrin (~4-7%).
To begin to understand how Rab11-FIP2 suppressed EGF receptor uptake, the contribution of individual domains of Rab11-FIP2 was evaluated by using mutant Rab11-FIP2 in this assay (Fig 8B). First, the N-terminal C2 domain was not involved because its deletion had no significant effect on inhibitory activity. However, the EH domain-binding NPF motif was involved since changing it to AAA suppressed inhibitory activity. Thus, Reps1 binding is likely involved in endocytosis inhibition by Rab11-FIP2. Interestingly, over-expression of Rip11, which does not contain EH domain binding NPF motifs and does not bind the EH domain of Reps1, displayed low inhibitory activity that was comparable to the NPF mutant of Rab11-FIP2. We also found low inhibitory activity upon transfection of Reps1 (Fig 8B) even though the expression levels of all tested proteins were comparable (Fig. 8C).

The C-terminal coiled-coil of Rab11-FIP2 was involved since deleting it also destroyed inhibitory activity. Thus, either Rab11 binding or oligomerization was required for Rab11-FIP2 to influence endocytosis. These two possibilities were distinguished using the Rab11-FIP2 coiled-coil mutant (YID to AAA) that blocked Rab11 binding but increased oligomerization. Since this mutant also lost inhibitory activity in the endocytosis assay, Rab11 binding appears to be the important function of this domain. Overall, these findings indicate that Rab11-FIP2 suppresses endocytosis of EGF receptors through both Reps1 and Rab11 binding.

**α-Adaptin binding**

Membrane receptors internalized through clathrin-coated vesicles contain signals that target them to clathrin-coated pits. These receptors interact with AP-2 protein complexes. The α-subunit of this complex (α-adaptin) functions as a platform for many proteins implicated in endocytosis. To add support for Rab11-FIP2 participating in receptor-mediated endocytosis, the appendage domain of α-adaptin was isolated as a fusion with GST, and incubated with cell lysates expressing HA-Rab11-FIP2. GST-α-adaptin, but not GST, precipitated Rab11-FIP2 from cell lysates indicating that Rab11-FIP2 can
complex with α-adaptin (Fig. 9A). Importantly, Rip11, which only weakly influenced endocytosis, did not complex with α-adaptin in this assay (Fig. 9A).

Deletion analysis of Rab11-FIP2 showed a correlation between the ability of proteins to bind to α-adaptin and their ability to suppress endocytosis of EGF (Fig. 9B-D). For example, deletion of the C-terminal coiled-coil of Rab11-FIP2, which blocked inhibition of endocytosis, also inhibited the association of Rip11-FIP2 with α-adaptin (Fig. 8B). Mutation of the EH domain-binding NPF motif and Rab-11-binding YID motif of Rip11-FIP2, which had partial effects on endocytosis, also suppressed complex formation with of α-adaptin (Fig 9C). Lastly, deletion of the C2 domain, which had no effect on endocytosis, had no effect on α-adaptin binding (Fig 9B). Interestingly, Reps1, which modestly suppressed endocytosis was also precipitated from cells with α-adaptin, adding support to the concept that Reps1 and Rab11-FIP2 function together in cells to influence receptor-mediated endocytosis (Fig. 9D).

Discussion

Receptors are removed from the cell surface by endocytosis. Once inside cells, these proteins become associated with early endosomes from which they can be returned to the cell surface (25) or proceed to later endocytic compartments. Some components then get shuttled to lysosomes for destruction, while others proceed to the recycling endosomes from which they may return to the same plasma membrane compartment from which they came or proceed by transcytosis to the opposite cell surface. How these sorting endosomes carry out this function is poorly understood. Progress has been made recently through the study of Rab11, a GTPase that resides in the recycling endosome, and its effector proteins. Recently, a family of putative Rab11 effectors, which may regulate the recycling of components through this cellular compartment, have been identified. These proteins include, Rip11, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3/efrin, Rab11-FIP4 and RCP. Rip11, for example, has been shown to be important for protein trafficking from recycling endosomes to the apical plasma membrane(3).
Rab11-FIP2 has also been implicated, albeit indirectly, in vesicle sorting in endosomes. In particular, expression of a mutant form of Rab11-FIP2 containing its Rab11-binding domain causes tubulation of a transferrin receptor positive early endosome compartment, a phenotype that is similar to that observed upon expression of a dominant negative Rab11 protein. However, recent findings have also suggested that Rab11-FIP2 may function in a manner distinct from other Rab11-binding proteins. First, although Rab11-FIP2 is localized to recycling endosomes where Rab11 exists, co-localization is not as complete as with other members of the protein family. This is apparent when the recycling compartment is perturbed. For example, whereas Rip11 remains localized with Rab11 upon dispersal of recycling endosomes, this is not true for Rab11-FIP2. A similar phenomenon is observed when microtubules are disrupted with taxol. Secondly, unlike other family members that bind Rab11 in a GTP-dependent manner, Rab11-FIP2 binds equally to both active and inactive Rab11, implying that rather than functioning as a regulated modulator of Rab11-FIP2, Rab11 may function as a constitutive docking site for it.

In the present study, the argument that Rab11-FIP2 has unique functions is strengthened. First, biochemical data show that Rab11-FIP2 has the capacity to form a complex with cellular components involved in receptor-mediated endocytosis. In particular we show that Rab11-FIP2 binds to an EH domain containing protein, Reps1, via an NPF motif not found in other Rab11-FIP family members. EH domains were first detected on the EGF receptor substrate Eps15 and subsequently they have been found on ~50 proteins from fungi to mammals. In many cases these proteins participate, along with their NPF-containing binding partners, in a protein network that influences internalization of receptors through clathrin-mediated endocytosis. Although Reps1 has not been directly linked yet to endocytosis, substantial data implicate its close relative, POB1, in this process. Moreover, Rab11-FIP2, can form a complex with the α-subunit of AP-2, a major clathrin adaptor complex in cells.

These biochemical properties suggest that Rab11-FIP2 has the ability to influence receptor-mediated endocytosis along with its previously described influence on recycling
endosomes. This hypothesis was confirmed in experiments designed to test its effects on the uptake of EGF receptors. We found that expression of Rab11-FIP2 suppresses EGF uptake in transfected cells such that ~30% of cells transfected with Rab11-FIP2 do not internalize detectable levels of EGF. However, it is likely that a greater percentage of transfected cells are actually affected, since in the immunofluorescence assay only those cells where endocytosis is completely blocked are counted. Undoubtedly, additional cells are partially affected. Importantly, there is significant specificity in the effect of Rab11-FIP2 on endocytosis, since its expression had no effect on endocytosis of transferrin receptors. This finding is consistent with previous experiments showing that overexpression of components of the Ral signaling cascade also influence EGF receptor but not transferrin receptor uptake (17).

Although these experiments suggest an involvement of Rab11-FIP2 in EGF uptake, they do not prove that the natural role of the protein is to suppress endocytosis. It is just as likely that the protein participates positively in the process, but in these experiments overexpression of Rab11-FIP2 competes key components away from the endocytic machinery and inhibits endocytosis. In fact, one such component may be Reps1. Immunofluorescence experiments suggest that while Reps1 exists mostly in membrane components that are distinct from perinuclear recycling endosomes containing Rab11-FIP2 and Rab11, overexpression of Rab11-FIP2 concentrates Reps1 there. In such a model, Rab11-FIP2 would not influence endocytosis directly but rather indirectly through its ability to bind to Reps1. This model implies that rather than being a direct participant in the endocytic process, Rab11-FIP2 functions to couple Reps1-containing vesicles originating from clathrin coated vesicles and early endosomes to the recycling endosome. The interaction with the endocytic compartments is through its EH domain-binding region while the interaction with the recycling compartment is through its Rab11-binding site. Such a function is consistent with the fact that the inhibitory activity of Rab11-FIP2 on endocytosis is suppressed by a mutation that prevents it from binding to Reps1. In addition, Rab11-FIP2 association with α-adaptin was also found to be partially dependent upon its ability to bind to Reps1.
Interestingly, we found that the ability of Rab11-FIP2 to inhibit endocytosis and associate with α-adaptin is also suppressed by mutations that block its ability to bind to Rab11. This raises the possibility that localization of Rab11-FIP2 to the recycling endosome can, in fact, directly influence endocytosis. Alternatively, the Rab11 binding site on Rab11-FIP2 may have the capacity to bind other proteins, such as other Rab family members more directly involved in uptake of cell surface receptors. This alternate function for the Rab11 binding domain is suggested by the fact that unlike other members of the FIP family, the interaction of Rab11-FIP2 with Rab11 is not dependent upon the active state of the GTPase. However, to date, neither we nor others have found another Rab family member that binds to Rab11-FIP2.

The function of other members of the FIP family, such as Rip11, which binds specifically to the active GTP-bound state of Rab11, may be more restricted to the recycling endosome. This notion is consistent with our observation that Rip11 failed to associate with components of the AP-2 adaptor and only weakly influenced EGF receptor uptake.

Since Rab11-FIP2 binding to Rab11 is not dependent upon the activation state of the GTPase, it is unlikely that Rab11-FIP2 is a true effector of the Rab11 GTPase. As such, if the binding between the two proteins is regulated, another mechanism must be involved. We and others (7) have found that the Rab11-binding domain of Rab11-FIP2 can also function as a homo-oligomerization motif. In fact, we found that a mutation in this motif that blocks Rab11 binding actually increases the homo-oligomerization of the protein. This may mean that Rab11 binding and homo-oligomerization are mutually exclusive. If so, Rab11/Rab11-FIP2 binding may be influenced by the degree of oligomerization.

We identified Rab11-FIP2 as a binding partner of Reps1, which itself was isolated as binding partner of the Ral-GTPase effector protein RalBP1. This raised the possibility that a Reps1/Rab11-FIP2 complex could mediate the effect of Ral activity on endocytosis. However, reports on the effects of Ral activity on receptor-mediated...
endocytosis have not been consistent. Whereas one paper cited suppression of EGF and insulin uptake by activated or dominant inhibitory Ral (17), another reported suppression by activated but not dominant negative Ral (18). Remarkably, although we could detect inhibition of EGF uptake by transfection of Rab11-FIP2, we could not detect an effect of transfected activated or dominant negative Ral (data not shown). It is not clear why there is such a discrepancy, but it may be due to the fact that the former studies used cells overexpressing receptors for ligands. Since Reps1 becomes tyrosine phosphorylated by the EGF receptor and forms a complex with it through Grb2 binding, a Reps1/ Rab11-FIP2 complex may be able to influence the sorting of EGF receptors in a Ral-independent manner. This is also consistent with our observation that Rab11-FIP2 affects EGF but not transferrin receptor uptake.

These observations, along with previous ones showing that the protein can also bind to Rab11 and influence recycling endosome function, suggest that Rab11-FIP2 functions as an adaptor protein in the delivery of endocytic vesicles to Rab11-containing recycling endosomes (See Fig 10).

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References:


**Figure Legends:**

**Fig. 1** Functional domains of Rab11-FIP2 and various mutants. (A) Functional domains of wild-type and mutant Rab11-FIP2. (C2) C2 domain, (NPF) binding motif for EH domains, (RBD) Rab11-binding domain. (B) Sequence comparison of Rab11 binding domains. The amino acid sequences of the Rab11-binding domains of mammalian FIP family members are compared. An optimal layout of the predicted coiled-coil is presented above it. A and D residues that are thought to form the hydrophobic interface are underlined in the amino acid sequences. Conserved and highly homologous residues are depicted in bold. Completely conserved residues are shown as larger letters.
Fig. 2 Binding activity of the EH domains of Reps1 and Eps15. (A) COS7 cell lysates containing transfected HA- Rab11-FIP2 were probed with glutathione-sepharose beads complexed to GST, a GST fusion of the Reps1 EH domain, or a GST fusion of the three Eps15 EH domains. Beads were washed, run in SDS-PAGE, and immuno-blotted with anti-HA antibody. (B) HA- Rab11-FIP2 and the HA- NPF->AAA mutant of Rab11-FIP2 were examined for binding activity against the Reps1 EH domain as described in A. Protein expression levels in lysates are also shown.

Fig. 3 Localization of Rab11-FIP2. Proteins were expressed as GFP or DsRed fusions in NIH3T3 cells. Cells were fixed and examined by fluorescence microscopy (A-E) or confocal microscopy as described elsewhere. (A) GFP-Rab11-FIP2 (B) GFP fluorescence of cells co-transfected with GFP-Rab11-FIP2 and DsRed-Rab11 (C) DsRed fluorescence of cells co-transfected with GFP-Rab11-FIP2 and DsRed-Rab11 (D) GFP-ΔC2-Rab11-FIP2 (E) GFP-NPF->AAA Rab11-FIP2 F) GFP-ΔRBD-Rab11-FIP2 G) GFP-YID->AAA Rab11-FIP2

Fig. 4 Localization of Rab11-FIP2 and Reps1 NIH3T3 cells were transfected as described in Fig. 3. A) GFP-Reps1 B) GFP fluorescence of co-transfected GFP-Reps1 and DsRed-Rab11-FIP2 C) DsRed fluorescence of co-transfected GFP-Reps1 and DsRed-Rab11-FIP2 D) GFP fluorescence of co-transfected GFP-RepsΔEH and DsRed-Rab11-FIP2.

Fig. 5 Homooligomerization of the Rab11-FIP2 protein. (A) HA-tagged Rab11-FIP2 and its Rab11 binding-domain deletion mutant were transfected into COS7 cells and lysates were probed with glutathione-sepharose beads complexed to GST or a GST Rab11-FIP2 Rab11 binding domain fusion. Beads were then washed in lysis buffer, run on SDS-PAGE, and western blotted with anti-HA antibody. (B) HA-tagged Rab11-FIP2 and the YID mutation were examined for homo-oligomerization. Expression levels of wild type and mutant proteins in cell lysates are also shown. (C) HA-tagged Rip11, Rab11-FIP2, and Rab11-FIP2 mutants were expressed in COS7 cells and cell lysates were incubated with glutathione-sepharose beads complexed to GST or GST-Rab11-
GTPγS. Beads were immuno-blotted with anti-HA antibody. Protein expression levels of proteins in cell lysates are also shown.

**Fig. 6 Oligomerization of Rab11-FIP2 in vivo.** HA-tagged Rab11-FIP2 or its Rab11 binding-domain deletion mutant were transfected along with Myc-Rab11-FIP2 into COS7 cells and cell lysates were immunoprecipitated with anti-myc antibody and immuno-blotted with the anti-HA antibodies or anti-myc antibodies.

**Fig. 7 Hetero-oligomerization of Rab11-binding domains.** HA-FIP proteins were transfected into COS7 cells and lysates were probed with glutathione-sepharose beads complexed to either GST or GST Rab11-binding domain fusions from each of the assayed proteins. Beads were then washed in lysis buffer, run on SDS-PAGE, and immuno-blotted with anti-HA antibody.

**Fig. 8 Endocytosis of TexasRed-EGF and TexasRed-transferrin in COS-7 cells.** A) COS7 cells were transfected with GFP or a GFP-Rab11-FIP2 fusion and assayed for their ability to affect endocytosis of TexasRed-EGF or TexasRed-transferrin. The data represents transfected cells that did not internalize detectable TexasRed-EGF or TexasRed-transferrin. Values represent the average and standard error of the mean from 3 assays of 200 cells each. Significant differences between GFP and Rab11-FIP2 were confirmed using student’s $t$ test. B) COS7 cells were transfected with GFP fusions of Rab11-FIP2 mutants, Reps1, or Rip11 and examined for their ability to inhibit TexasRed-EGF internalization. C) Expression levels of transfected proteins.

**Fig. 9 Binding of Rab11-FIP2 to the appendage domain of α-adaptin.** (A) HA-tagged Rab11-FIP2 or Rip11 were transfected into COS7 cells and cell lysates were incubated with glutathione-sepharose beads complexed to GST or GST α-adaptin appendage domain fusion proteins. Beads were then immuno-blotted with anti-HA antibody. (B) Deletion mutants of the Rab11-FIP2 protein were assayed for α-adaptin binding as described in A. (C) Point mutations of Rab11-FIP2 were analyzed as in A. (D) HA-Reps1 binding to GST-α-adaptin was examined as in A.
Fig. 10. Model how Rab11-FIP2 may function in vesicle recycling through its EH and Rab11-binding domains. Rab11-FIP2 is localized to perinuclear recycling endosomes through its constitutive binding to Rab11. Its ability to bind to the EH domain of Reps1 may promote the targeting of early endosomes to the recycling endosome over other membrane compartments such as the lysosome.
Fig. 1A

Rab11-FIP2

Rab11-FIP2ΔC2

Rab11-FIP2ΔRBD

Rab11-FIP2 YID->AAA

Rab11-FIP2-NPF->AAA

RBD

NPF NPF NPF

NPF NPF NPF

NPF NPF NPF

NPF NPF NPF

AAA

RBD

NPF AAA NPF
Fig. 1B

Predicted Coiled-coil

Rab11-FIP2  YE EVLQELVKHKELLRRKDH|TRELED YIDNLLVRLMEETPSILRVPYERPSRKAGKF--
Rip11       HD ELISLL|QRERESQREHQLS|ESYIDRLLVRIMEETSPLLQIPPPK-------
RCP         HD ELIQLVLEQKT|ISKEFQVRELED YIDNLLVRMEETPNILRIPTQVGGKAGKM--
efrin/Rab11-FIP3 AE ISSVSRDELMAIQKQEEINFLQD YIDRIIVAIMETNP|IL|EVK----------
Rab11-FIP4  AE IDTASREDMEALKEQEEINFR|LQYM|DIILAILDHNPSILEIKH----------
Rab11-FIP1  HD ELIQLVLKQKET|ISKEFQVRELED YIDNLLVRMEETPNILRIPAQ|AGRRAGKM
**Fig. 2A**

Rab11-FIP2

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<tr>
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<th>GST</th>
<th>GST Reps1 EH</th>
<th>GST Eps15 EHs</th>
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**Fig. 2B**

Rab11-FIP2

NPF->AAA

| + | - | + | - | + | - |

| Lysate | GST Reps1 EH |

---
Fig. 3

A  B  C  D  E

GFP-Rab11-FIP2  GFP-Rab11-FIP2  DsRed1-Rab11  GFP-ΔC2  GFP-NPF->AAA
(with DsRed1-Rab11)  (with GFP-Rab11-FIP2)

F  G

GFP-ΔRBD  GFP-YID->AAA
Fig. 4

A. GFP-Reps1

B. GFP-Reps1 (with DsRed1-Rab11-FIP2)

C. DsRed1-Rab11-FIP2 (with GFP-Reps1)

D. GFP-Reps1ΔEH (with DsRed1-Rab11-FIP2)
Fig. 5A

Rab11-FIP2
ΔRBD

+ - + - + -
- + - + - +
— Lysate — — GST — — GST — Rab11-FIP2 RBD

Fig. 5B

Rab11-FIP2
YID->AAA

+ - + - + -
- + - + - +
— Lysate — — GST — — GST — Rab11-FIP2 RBD
Fig. 5C

Rab11-FIP2
Rab11-FIP2YID->AAA
Rab11-FIP2∆RBD
Rip11

Lysate — GST — GST-Rab11-
GTPγS
**Fig. 6**

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Legend:

- Lysate
- anti-myc IP
Fig. 7

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Fig. 8A

% of cells inhibited

GFP alone  Rab11-FIP2  GFP alone  Rab11-FIP2

TexasRed-EGF  TexasRed-transferrin
Fig. 8B

Comparison of the percentage of cells inhibited by different conditions:
- GFP alone
- Rab11-FIP2
- Rab11-FIP2 ΔC2
- Rab11-FIP2 ΔRBD
- Rab11-FIP2 YID->AAA
- Rab11-FIP2 NPF->AAA
- Rip11
- Reps1

The graph shows a significant increase in cell inhibition with Rab11-FIP2 ΔC2 compared to other conditions.
Fig. 8C
**Fig. 9A**

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--- Lysate --- GST --- α-adaptin ---

**Fig. 9B**

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--- Lysate --- GST --- α-adaptin ---

**Fig. 9C**

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--- Lysate --- GST --- α-adaptin ---
Fig. 9D

Reps1

Lysate  GST  GST α-adaptin
Fig. 10
Rab11-FIP2: An adaptor protein connecting cellular components involved in internalization and recycling of EGF receptors
Donald N. Cullis, Betsey Philip, James D. Baleja and Larry A. Feig

J. Biol. Chem. published online October 2, 2002

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