Identification and Characterization of a Family of Rab11-interacting Proteins*

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Rab11a is a small GTP-binding protein enriched in the pericentriolar plasma membrane recycling systems. We hypothesized that Rab11a-binding proteins exist as downstream effectors of its action. Here we define a family of four Rab11-interacting proteins: Rab11-Family Interacting Protein 1 (Rab11-FIP1), Rab11-Family Interacting Protein 2 (Rab11-FIP2), Rab11-Family Interacting Protein 3 (Rab11-FIP3), and pp75/Rip11. All four interacting proteins associated with wild type Rab11a and dominant active Rab11a (Rab11aS20V) as well as Rab11b and Rab25. Rab11-FIP2 also interacted with dominant negative Rab11a (Rab11aS25N) and the tail of myosin Vb. The binding of Rab11-FIP1, Rab11-FIP2, and Rab11-FIP3 to Rab1a was dependent upon a conserved carboxyl-terminal amphipathic α-helix. Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 colocalized with Rab11a in plasma membrane recycling systems in both non-polarized HeLa cells and polarized Madin-Darby canine kidney cells. GFP-Rab11-FIP3 also colocalized with Rab11a in HeLa cells. Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 also coenriched with Rab11a and H⁺K⁺-ATPase on parietal cell tubulovesicles, and Rab11-FIP1 and Rab11-FIP2 translocated with Rab11a and the H⁺K⁺-ATPase upon stimulating parietal cells with histamine. The results suggest that the function of Rab11a in plasma membrane recycling systems is dependent upon a compendium of protein effectors.

The Rab GTPase family contains more than 50 different members that are believed to have a regulatory role in the formation, targeting, and/or fusion of transport vesicles (1). Whereas the precise mechanism of action remains poorly understood, individual Rab proteins localize to distinct intracellular vesicular compartments suggesting that each Rab has a well defined functional role. For example, Rab1 and Rab2 are perinuclear in location and function in endoplasmic reticulum to Golgi translocation (2). Rab8 resides in the trans-Golgi/post-Golgi networks and regulates post-Golgi targeting to the plasma membrane (4). Rab5 on early endosomes functions in trafficking from the plasma membrane (5). The sub-apically localized Rab11 family is involved in vesicle recycling, plasma membrane recycling, and transcytosis (6–8).

Rab11a was first isolated from bovine brain membranes in 1988 (9). Since that time, the rat (10), dog (11), mouse (12), rabbit (13), and human (14) homologues have been identified. The Rab11 family now also includes two other gene products, Rab11b and Rab25 (15). Rab11a is sub-apically located in epithelial cells (16) and is involved in vesicle recycling through the pericentriolar recycling endosome (8). Rab11a colocalizes with transferrin receptor in recycling compartments of K562 cells, a human hematopoietic cell line, (17) and mutants deficient in GFP hydrolysis inhibit transferrin recycling (18). Although ubiquitous in expression, Rab11a is enriched in epithelial cells and gastric parietal cells (13, 16). In gastric parietal cells, Rab11a colocalized with the H⁺K⁺-ATPase and translocated to the secretory canaliculus upon stimulation with histamine (19). A dominant negative form of Rab11a inhibits acid sequestration in gastric glands (20) and inhibits recycling of polymeric IgA receptor and basolateral to apical transcytosis in MDCK cells (6). Recently, Rab11a was also implicated in recruiting membrane to the cell surface in macrophages for phagocytosis (21) and for cell membrane extension.

Although the associations of Rab11a and the apical recycling systems is now well established, little is known about its physiological function. Studies have identified three Rab11-interacting proteins. The first protein, Rab11-binding protein (Rab11BP) or rabphilin 11, was isolated from bovine brain (22) and rat brain (23), respectively, and interacted with the GTP-bound form of Rab11a (22). Rab11BP/rabphilin 11 localizes to the pericentriolar recycling compartment in MDCK cells and HeLa cells and participates in transferrin recycling and membrane turnover events (22–23). Myosin Vb, the second Rab11a-interacting protein, was identified from yeast two-hybrid screening of a parietal cell cDNA library (24). Myosin Vb interacts with all members of the Rab11 family: Rab11a, Rab11b, and Rab25. In MDCK and HeLa cells, myosin Vb associates with the Rab11a-containing plasma membrane recycling systems. Transfection of the tail of myosin Vb lacking the motor domain retards trafficking through the plasma membrane recycling systems (24).

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1 The abbreviations used are: MDCK, Madin-Darby canine kidney; GFP, green fluorescent protein; PCR, polymerase chain reaction; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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Yeast Two-hybrid Screening and Cloning of Full-length Rab11-FIP1—Poly(A) mRNA was isolated from rabbit gastric parietal cells and primed with oligo(dT) to construct a parietal cell cDNA library in pAD-GAL (Stratagene, La Jolla, CA) with an average clone length of 2.0 kilobase pairs. Rab11aS20V was mutated as described previously (6) and primed with oligo(dT) to construct a parietal cell cDNA library in vector pAD-GAL4 (Stratagene, La Jolla, CA) and sequenced (MCG Molecular Biology Core Facility). The Rab11-FIP2 coding sequence was amplified from the KIA0941 cDNA via PCR using a 5’ oligonucleotide with an EcoRI site (GCGGAATTCAACAGCAGACGATGCTG) and 3’ oligonucleotide with SalI site (GGCGTGCACATGTGGCTTATTAACTGTTAGAG) and ligated into pAD-GAL4 digested with EcoRI and SalI. The Rab11-FIP3 coding sequence was amplified from the KIA0665 cDNA via PCR using a 5’ oligonucleotide with an EcoRI site (GCGGATTCCTCCGGGAGCAGGCTACTGTCG) and 3’ oligonucleotide with XbaI site (GCCGCTAGCTGAGCTCCCTGTCCTAACCTTGG) and ligated into pAD-GAL4 and EGFP-C2 digested with EcoRI and XbaI. pAD-GAL4/ppt51 was prepared as described previously (26).

Yeast two-hybrid binary assays were conducted as follows. For 20 reactions, 25 μl of yeast liquid (CLONTECH) were mixed with 25 μl of 100-fold concentrated yeast from the strain Y190. The culture was grown overnight at 30 °C. Cells were harvested by centrifugation at 1000 × g. Yeast cells were resuspended in 10 ml of water and centrifuged again at 1000 × g. Yeast cells were resuspended in a 100 μM lithium acetate/Tris EDTA solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and incubated for 5 min. For each reaction, 20 μl of the lithium acetate/Tris EDTA yeast solution were added to a solution containing 20 μg of heat-denatured salmon sperm DNA. 120 μl of lithium acetate/Tris EDTA/polyethylene glycol solution (100 mM LiAc, pH 7.5, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, 40% polyethylene glycol), 200 ng of pBD-GAL4 construct plasmid, and 200 ng of pAD-GAL4 construct plasmid. The solution was mixed well and placed at 30 °C shaking for 30 min. Cells were then heat-shocked at 42 °C for 15 min. The yeast cells were pelleted at 1000 × g for 30 s; the supernatant was removed; the cells were resuspended in 100 μl sterile water, and then the cells were plated onto a tryptophan- and leucine-deficient medium. The plate was incubated at 30 °C for 3 days, and the colonies were lifted with a 70-mm filter paper disc. The discs were then processed for detection of β-galactosidase activity as detailed above. Observation of a blue color within 3 h was considered a positive result.

Antibody and Recombinant Protein Production—Rab11-FIP1(263-651) was cloned into pET-19b, and recombinant His-tagged Rab11-FIP1(263-651) was expressed and purified as described previously (16). Mouse monoclonal Rab11-FIP1 antibody was produced against recombinant Rab11-FIP1(263-651) at the University of Georgia Monoclonal Antibody Facility. Polyclonal Rab11-FIP2 antibody was produced in rabbits at Biogenex (San Ramon, CA). Rabbit polyclonal Rab11-FIP1 KIA0941 (NRQDYFYDESTN) spanning amino acids 395–406 of Rab11-FIP2 conjugated to keyhole limpet hemocyanin. Rabbit polyclonal pp75 was produced as described previously (26).

Full-length Rab11-FIP1, Rab11-FIP1(-1–615), Rab11-FIP2, Rab11-FIP2(-1–465), and Rab11-FIP3 sequences were cloned into pET-30a, and recombinant expression protein was purified as described previously (16).

Rab11a γ-32P-GTP Overlay Experiments—2.5 μg of recombinant Rab11-FIP1, Rab11-FIP1(-1–615), Rab11-FIP2, Rab11-FIP2(-1–465), and Rab11-FIP3 proteins were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose (Protran, Schleicher & Schuell). The membrane was blocked for 2 h in blockbinding buffer (5% milk and 0.1% bovine serum albumin in TBS) at 4 °C.

For Rab3a, Rab3b, or Rab11a labeling with γ-32P-GTP, 40 pmol of His-tagged Rab protein were incubated for 20 min at 30 °C in 35 μl of GT-P-charging buffer (18 mM Tris-HCl, pH 7.5, 8.1 mM EDTA, 0.9 mM dithiothreitol, 4.5 mM MgCl2, and 0.3% CHAPS) with 5 μCi of γ-32P-GTP (PerkinElmer Life Sciences). 4 μl of 100 mM MgCl2 were then added, and reactions were placed on ice. Labeled RabS were then transferred to a blockbinding buffer for 1 h. If the reaction required peptide blockade, Rab11-FIP1 peptide (KEFQVRELEDY-IDLNLVVMEEPFTNLIR) or HT-31 peptide was added to final concentration of 500 μM in the 1 ml of block/blocking buffer containing Rab11a γ-32P-GTP. The solutions with γ-32P-GTP-labeled Rab3a, Rab5, and Rab11a with or without peptide were then incubated with the membranes and 1 ml of block/blocking buffer for 2 h. Membranes were
removed and washed 5 times with block/binding buffer and 2 times with TBS. Membranes were imaged on PhosphorImaging screens (Molecular Dynamics, Sunnyvale, CA) for 16 h.

**Parietal Cell Tubulovesicle Preparation and Western Blot Analysis**—Enriched parietal cell tubulovesicle preparation was conducted as described previously (28). 20 µg of protein from each fraction were electrophoresed on 10% SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA). Membranes were blocked for 1 h in blocking buffer (TBS with 5% nonfat milk and 0.05% Tween 20). Membranes were then probed with the appropriate primary antibody in 2 ml of antibody incubation buffer (TBS with 2.5% nonfat milk and 0.05% Tween 20) for 2 h. Antibody dilutions were as follows: Rab11-FIP1 was used at 1:10,000; Rab11-FIP2 was used at 1:100; Rab11a (SH10-(16)) was used at 1:50; H′K′-ATPase was used at 1:50,000. Membranes were washed 3 times for 10 min each with blocking buffer. Membranes were then probed with appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) in 2 ml of antibody incubation buffer. All horseradish peroxidase-conjugated secondary antibodies were used at 1:3000. Membranes were washed 2 times for 10 min in TBS and developed with Super Signal Chemiluminescent Substrate (Perica) for 1 min followed by exposure to film (Kodak, Biomax-ML).

**Cell Culture and Immunofluorescence Microscopy**—HeLa cells were grown to confluence on glass coverslips. MDCK cells were grown to confluence filters. Primary rabbit parietal cells were isolated and maintained in culture for 2 days following harvesting on glass coverslips coated with Matrigel (Becton Dickinson) as described previously (30–31). Nocodazole and taxol treatment of MDCK cells was performed as described previously (7, 29). Prior to fixing, all cells were washed 2 times with PBS (150 mM NaCl and 150 mM sodium phosphate, pH 7.4). All cells were fixed for 30 min at 4°C in 4% paraformaldehyde and washed 3 times with PBS prior to storage at 4°C in PBS.

To stimulate parietal cells, media were changed 24 h after plating cells. Cells were then pretreated for 5 min with 10 µM omeprazole prior to the addition of 100 µM histamine for 30 min. Cells were then washed 3 times for 10 min with PBS and fixed as above. Transient transfection of GFP-Rab11-FIP3 into HeLa cells was conducted as described previously (32). Staining of fixed cells was conducted as follows. Cells were blocked for 20 min with donkey serum solution (0.3% Triton X-100, 20 mM sodium phosphate, 8% NaN, 16.6% donkey serum) and then incubated with appropriate primary antibodies diluted in donkey serum solution overnight at 4°C. Cells were washed 3 times for 5 min with PBS and then incubated with appropriate fluorescently conjugated secondary antibodies (donkey anti-goat Cy3, donkey anti-mouse Cy3, donkey anti-rabbit Cy3, donkey anti-mouse Cy5, or donkey anti-rat Cy5) for 2 h. Alexa488-labeled phalloidin (Molecular Probes, Eugene, OR) was included with fluorescently conjugated secondary antibodies when staining parietal cells. Cells were washed 2 times for 5 min with PBS and 2 times for 5 min with 50 mM sodium phosphate, pH 7.4. Coverslips or filters were mounted onto slides using Prolong Antifade (Molecular Probes, Eugene, OR).

HeLa cells were observed utilizing a Zeiss Axiphot equipped with a SPOT digital imaging system. MDCK cells were imaged utilizing a Molecular Dynamics confocal microscope as described previously (6). Parietal cells were imaged in confocal microscopy using single 0.3-µm optical sections.

**RESULTS**

**Dominant Active Rab11a Identifies Rab11-FIP1 from a Rabbit Parietal Cell cDNA Library**—To identify proteins that interact with Rab11a, a yeast two-hybrid screen of a rabbit parietal cell library was performed with dominant active Rab11a (Rab11aS220V). Two clones for an identical cDNA sequence were found to activate histidine production and to produce β-galactosidase. The cDNAs were 1692 nucleotides in length and 2616 nucleotides in length with open reading frames initiating for 77 amino acids and 183 amino acids, respectively. Since neither cDNA site was found, the larger cDNA clone was used to screen a UniZAP phage parietal cell cDNA library. A cDNA clone of 2900 nucleotides was isolated, and it contained a 481-amino acid open reading frame without an in-frame upstream stop codon. 5'-Rapid amplification of cDNA ends technique was then utilized to obtain further sequence coding for the remaining 171 amino acids. The assembled full-length sequence (3519 nucleotides) contained an open reading frame coding for 652 amino acids and was named Rab11-Family Interacting Protein 1 (Rab11-FIP1) (Fig. 1). The homologous human protein is located on chromosome 2 containing 5 exons with 99% identity. Northern blot analysis of parietal cell RNA demonstrated a 3.2-kilobase pair mRNA species (data not shown).

The results of the screen suggested that only the carboxy-terminal 77 amino acids were required for binding of Rab11a. We therefore studied the interaction of truncations of Rab11-FIP1 with Rab11a. Truncated forms of Rab11-FIP1 containing amino acids 263–651 or amino acids 576–651 maintained an interaction with Rab11a (Fig. 2A). However, truncations of Rab11-FIP1 that do not contain amino acids 616–651 lost their ability to interact with Rab11a (Fig. 2A). Hydrophat plot analysis of amino acids 615–651 indicated the presence of an amphipathic α-helix between amino acids 615 and 632 (Fig. 2B). Based on these results we have designated this region as the putative "Rab11 binding domain" (Rab11BD).

**Rab11-FIP1 Rab11-BD Identifies Three Human Proteins with Homologous α-Helices**—A PROSITE search with the Rab11-FIP1 amino acid sequence did not reveal any significant structural motifs. However, the amino-terminal half of the protein did contain proline-rich domains. Since Rab11-FIP1 binding to Rab11a was dependent on a region containing an amphipathic α-helix, and because such α-helices are known to be involved in protein-protein interactions (32), we hypothesized that other proteins might exist that contain a similar Rab11a-binding motif. A computer BLAST search of the human GenBank™ EST database yielded three homologous proteins with high carboxy-terminal identity. One protein (KIAA0941) contained the identical 18-amino acid amphipathic α-helix sequence as in Rab11-FIP1, and the other two proteins (KIAA0665 and KIAA0857) showed high identity in the putative Rab11 binding domain (Fig. 3A). Other than the helix, these three proteins demonstrated no significant identity with Rab11-FIP1.

KIAA0941 was first published as one of 100 cDNAs isolated from human brain which code for large proteins (33). By utilizing the PROSITE domain search engine, KIAA0941 was found to contain a putative amino-terminal C2 domain that was similar to the C2 domain present in synaptotagmin (Fig. 3B). The KIAA0941 sequence is located on chromosome 10 with 5 exons and only 15.6% overall identity to Rab11-FIP1. KIAA0857 was initially published by Nagase and colleagues (34) and first characterized as an autoantigen designated pp75 (26). This same protein was subsequently identified as Rab11-interacting protein (Rip11) because GST-Rab11a affinity assays and immunocytochemistry showed a relationship between Rab11a and Rip11 (25). The carboxy-terminal α-helix in KIAA0857 maintains 68.4% identity to Rab11-FIP1 and contains 2 other amino acids with like charge, pp75/Rip11 maintains 29.6% identity with KIAA0941 due to the presence of highly similar amino-terminal C2 domains. However, the C2 domain of pp75/Rip11 shows more similarity with the C2 domain of protein kinase C.

KIAA0665 was also first published as a cDNA isolated from human brain that codes for a large protein in vitro (35). By utilizing the PROSITE domain search engine, KIAA0665 contains a putative ezrin-radixin-moesin (ERM) motif from amino acids 469 to 692. The carboxy-terminal α-helix in KIAA0665 maintains 47.4% identity to Rab11-FIP1 and contains 4 other amino acids with like charge.

Rab11-FIP1, KIAA0941, Rab11-FIP2, KIAA0665 (Rab11-FIP3), and pp75/Rip11 Interact with All Three Members of the Rab11 Family of Small GTPases—Based on the presence of either an identical (KIAA0941) or highly similar (KIAA0665)
and pp75/Rip11) carboxyl-terminal α-helix, we sought to determine whether these proteins could interact with Rab11a. The pAD-GAL4 constructs of Rab11-FIP1, KIAA0941, KIAA0665, and pp75/Rip11 were used in yeast two-hybrid binary association assays to determine whether the proteins interacted with Rab11 family members cloned into pBD-GAL4. Fig. 4 demonstrates that Rab11-FIP1, KIAA0941, KIAA0665, and pp75/Rip11 interacted with Rab11a and the dominant active form of Rab11a (Rab11S20V). In addition, all four interacted with Rab11b and Rab25. Therefore, Rab11-FIP1, KIAA0941, KIAA0665, and pp75/Rip11 represent a family of interacting proteins that bind Rab11a, Rab11b, and Rab25. We therefore have designated KIAA0941 as Rab11-Family Interacting Protein 2 (Rab11-FIP2) and KIAA0665 as Rab11-Family Interacting Protein 3 (Rab11-FIP3). Interestingly, Rab11-FIP1 and Rab11-FIP3 showed no interaction with either dominant negative Rab11a or myosin Vb. Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, and pp75/Rip11 showed no interaction in yeast two-hybrid binary assays with Rab2, Rab3a, Rab3b, Rab5, or the dominant active form of Rab8 (Rab8Q67L).

Rab11 γ-35S-GTP Overlays of Rab11-FIP Proteins—To confirm the results obtained from yeast two-hybrid analysis, Rab11a overlay experiments were conducted. Recombinant Rab11-FIP1, Rab11-FIP2, and Rab11-FIP3 were immobilized on nitrocellulose membranes. These membranes were probed with γ-35S-GTP-labeled Rab11a. Fig. 5 shows γ-35S-GTP-labeled Rab11a binding to Rab11-FIP1, Rab11-FIP2, and Rab11-FIP3. To show that the Rab11a binding was specific, Rab11-FIP proteins were overlaid with γ-35S-GTP-labeled Rab3a and Rab5, and no binding to Rab11-FIP1, Rab11-FIP2, or Rab11-FIP3 was detected (Fig. 5). Based on initial two-hybrid binary assays, amino acids 615–651 of Rab11-FIP1 were necessary for Rab11a binding. To provide evidence of a Rab11a-binding motif, mutant constructs of Rab11-FIP1 and Rab11-FIP2, each with the carboxyl-terminal α-helix deleted (Rab11-FIP1-(1–615) and Rab11-FIP2-(1–465)), were expressed. γ-35S-GTP-labeled Rab11a did not bind recombinant Rab11-FIP1-(1–615) or Rab11-FIP2-(1–465). γ-35S-GTP-labeled Rab3a and Rab5 also failed to bind Rab11-FIP proteins. In parallel experiments, a 30-amino acid peptide containing the amphipathic α-helix from Rab11-FIP1 (Fig. 2B) was included in the incubations with γ-35S-GTP-labeled Rab11a. The peptide markedly inhibited the binding of the Rab11 γ-35S-GTP overlays.
of Rab11-FIP proteins, we developed antibodies against Rab11-FIP1 and Rab11-FIP2. A mouse monoclonal antibody was raised against recombinant Rab11-FIP1 and a goat polyclonal antibody was raised against a Rab11-FIP2-specific peptide. The Western blot in Fig. 6A indicated that the monoclonal Rab11-FIP1 antibody is specific. Incubation of the antibody with recombinant Rab11-FIP1 for 1 h blocked in a concentration-dependent manner the antibody binding to an 89-kDa band in 100,000 × g microsomes isolated from gastric mucosa. Similar results were found with the polyclonal goat anti-Rab11-FIP2 antibody. The Rab11-FIP2 peptide used to immunize the goat blocked in a concentration-dependent manner antibody binding to a 68-kDa band in the same microsomal fraction in a concentration-dependent manner (Fig. 6B). Rab11-FIP1 antibodies did not recognize Rab11-FIP2 or Rab11-FIP3 and similarly anti-Rab11-FIP2 did not recognize Rab11-FIP1 or Rab11-FIP3 (data not shown).

Rab11-FIP1, Rab11-FIP2, GFP-Rab11-FIP3, and pp75/ R1P11 Colocalized with Rab11a in HeLa Cells—Since Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, and pp75/R1P11 interacted with Rab11a in the yeast two-hybrid binding assays, we sought to investigate the in situ localization of these proteins in cul-
tured cells. HeLa cells were double labeled with antibodies against Rab11a and antibodies against Rab11-FIP1, Rab11-FIP2, or pp75/Rip11, and then stained with anti-ZO1 antibodies along with antibodies against Rab11-FIP1, Rab11-FIP2, or pp75/Rip11 (Fig. 8A). Confocal immunofluorescence microscopy indicated the colocalization of Rab11-FIP1 and Rab11-FIP2 with Rab11a but in addition also demonstrated some staining in an annular perijunctional distribution.

Rab11-FIP1 and Rab11-FIP2 Translocate to the Secretory Canaliculus upon Stimulation with Histamine—Since Rab11-FIP1 was isolated from a parietal cell cDNA library, we investigated whether Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 were distributed with Rab11a within the parietal cell. We isolated and fractionated tubulovesicles from rabbit gastric mucosa. As in previous studies (13), Rab11a and H⁺-ATPase were enriched in the 100,000 g microsomes and in 20 and 27% sucrose gradient fractions derived from the 100,000 g membranes (Fig. 9). Western blots of parietal cell tubulovesicle preparations also indicated the coenrichment of Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 with Rab11a and H⁺-ATPase in the 100,000 g microsomes and the 20 and 27% sucrose gradient fractions (Fig. 9).

Rab11-FIP1 and Rab11-FIP2 Translocate to the Secretory Canaliculus upon Stimulation with Histamine—Previous work (18) has indicated that Rab11a translocates with H⁺-ATPase onto the secretory canaliculus following histamine stimulation. We studied whether Rab11-FIP1 and Rab11-FIP2 also translocated with Rab11a to the parietal cell secretory canaliculus upon stimulation with histamine. Cultured rabbit
gastric parietal cells were either maintained in their resting state or stimulated with histamine. Cells were then dual immunostained with antibodies to Rab11a or H⁺K⁺-ATPase and Rab11-FIP1 or Rab11-FIP2 along with fluorescently conjugated phalloidin to visualize F-actin (Fig. 10). Rab11-FIP1 colocalized with Rab11a in the resting parietal cell in a punctate vesicle pool that surrounded the F-actin-labeled canalicular membrane. Following stimulation with histamine, Rab11-FIP1 and Rab11a remained colocalized as they translocated onto the F-actin-containing canalicular membrane. Similarly, Rab11-
FIP2 colocalized with H⁺K⁻-ATPase in a punctate pericanalicular distribution in the resting parietal cell surrounding the F-actin labeled canaliculus (Fig. 10B). Upon stimulation with histamine, Rab11-FIP2 was translocated with H⁺K⁻-ATPase onto the canalicular membrane. These results suggest that both Rab11-FIP1 and Rab11-FIP2 are present on parietal cell tubulovesicles and translocate to the secretary canaliculus as the vesicles fuse with the canalicular membrane to deliver the H⁺K⁺-ATPase to the lumen.

**DISCUSSION**

The positioning of individual Rab proteins on discrete vesicle populations suggests their importance in regulating unique trafficking pathways. Previous studies have suggested that Rab proteins may organize multiprotein complexes associated with specific vesicle trafficking pathways. For example, early endosome fusion events mediated by Rab5 and the Rab5 effectors Early Endosomal Antigen-1 (EEA-1) and rabaptin-5 provided a model for SNARE tethering and vesicle fusion in early endosomes (38–41). Golgi complex organization and intra-Golgi transport may be directed by Rab6 and its microtubule-associating effector rabkinesin-6 (3, 42). Similarly, the association of Rab11a with myosin Vb coupled with the effects of the tail of myosin Vb in reducing transferrin and IgA trafficking indicated the importance of myosin Vb and possibly Rab11a in plasma membrane recycling systems (24). We sought to identify other proteins that interact with Rab11a to characterize further protein complexes that mediate the effects of Rab11a on vesicle trafficking through plasma membrane recycling systems. The results presented here indicate that interaction of Rab11a with a family of Rab11a-interacting proteins may account for complexities observed in the trafficking of cargo to plasma membranes.

The present studies demonstrate that Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, and pp75/Rip11 are members of a family of interacting proteins that bind Rab11a through an amphipathic α-helical motif not present in either myosin Vb or Rab11BP/ rabphilin-11. Amphipathic α-helices are known to participate in protein/protein interactions (32). In yeast two-hybrid assays and blot overlays, deletion of the carboxy-terminal α-helix abolished Rab11a binding. In addition, a peptide containing the carboxy-terminal α-helix could compete for Rab11a binding in blot overlays. The presence of a conserved Rab11a binding domain in these four structurally diverse proteins of likely disparate function indicates the complexity of steps involved in membrane recycling.

As reported previously (24) for myosin Vb, Rab11-FIP family members can interact with the GTP-bound forms of all three Rab11 family members Rab11a, Rab11b, and Rab25. The binding of Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, and pp75/Rip11 was specific for the Rab11 family, since yeast two-hybrid association assays indicate no binding with Rab2, Rab3a, Rab3b, Rab5, or Rab8a. In addition, γ₁⁵S-GTP-labeled Rab3a and γ₁³⁵S-GTP-labeled Rab5 overlay experiments also showed no binding to recombinant Rab11-FIP1, Rab11-FIP2, or Rab11-FIP3. Although all four interacting proteins associated with the dominant active form of Rab11a (Rab11aS20V), only Rab11-FIP2 interacted with the dominant negative Rab11a (Rab11aS25N). These latter results may indicate the existence of a new class of Rab11 chaperone molecules characterized by Rab11-FIP2 which function irrespective of Rab activation status.

As with pp75/Rip11 (25), Rab11-FIP1, Rab11-FIP2, and GFP-Rab11-FIP3 colocalized with Rab11a in HeLa cells. In MDCK cells, Rab11-FIP1 and pp75/Rip11 were colocalized on the apical recycling endosome system with GFP-Rab11a. Rab11-FIP2 was also sub-apically located with Rab11a, but colocalization was incomplete, and we observed Rab11-FIP2 staining in regions where Rab11a was not present. Interestingly, as observed previously with myosin Vb (24), Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 all dispersed upon treatment with nocodazole and moved to the apical corners upon treatment with taxol. Similarly in taxol-treated MDCK cells, while the majority of stained Rab11-FIP2 did colocalize with Rab11a in sub-apical corners, a significant subset of the immunoreactivity stained an annular region adjacent to the tight junctions where Rab11a immunoreactivity was not observed. This evidence supports a functional association between Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 with Rab11a based on their dynamic codistribution with Rab11a-containing vesicles. The somewhat different distribution of Rab11-FIP2 may indicate the presence of different functions for Rab11-FIP2 in polarized cells. This difference in distribution of Rab11-FIP2 could also reflect its potential association with different pools of nucleotide-bound Rab11 family members.

The results presented demonstrate that Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 are all enriched with H⁺K⁺-ATPase and Rab11a in gastric parietal cell tubulovesicle fractions. The gastric parietal cell represents the most highly developed example of an apical recycling system (43). The major function of the parietal cell, regulated acid secretion, utilizes the second messenger-dependent activation of intracellular tubulovesicle fusion with an intracellular canalicular target membrane to deliver H⁺K⁺-ATPase to the apical lumen of the stomach. We have demonstrated previously that parietal cell tubulovesicles are highly enriched in Rab11a and also contain Rab25 (13, 44). Duman et al. (20) reported that a dominant negative form of Rab11a (Rab11aN124D) inhibited H⁺K⁺-ATPase recruitment to the secretory canaliculus. We have also noted that when parietal cells are stimulated, Rab11a and H⁺K⁺-ATPase translocate to the expanded apical canalicular membrane (19). In the present studies, Rab11-FIP1 and Rab11-FIP2 also translocated to the secretory canaliculus with Rab11a and H⁺K⁺-ATPase, respectively. Maintenance of these Rab11a-associated proteins with the H⁺K⁺-ATPase-containing membranes after fusion suggests the existence of a functional recycling complex that is highly specialized for apical recycling in the parietal cells.

How can three different Rab proteins (Rab11a, Rab11b, and Rab25) within the same cell associate with at least 5 interacting proteins (Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, pp75/Rip11, and myosin Vb) and establish specific pathways for each member of the Rab11 family? Several possibilities exist. First, different pools of Rab11a, Rab11b, or Rab25 containing vesicles may be spatially segregated within a tubulovesicular compartment, or they may associate with other as yet unidentified interacting proteins performing more highly specialized functions. Second, we have observed that Rab11a has the ability to oligomerize.³ Oligomerized Rab11a could associate with multiple interacting partners performing various different functions. Thus Rab11a could be the nidus for the assembly of a multiprotein effector complex. A final explanation is that Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, and pp75/Rip11 all bind Rab11a, Rab11b, or Rab25, but their binding may be reserved for different functions oriented in time. Such interactions could then be regulated by either differences in in situ binding affinities or alterations in binding due to post-translational modifications, as postulated for pp75/Rip11 (25).

In summary, we have identified a family of four proteins that can associate with Rab11a, Rab11b, and Rab25. The interaction with Rab11a is dependent upon a carboxy-terminal amphipathic α-helix (Rab11 binding domain). Rab11-FIP1, Rab11-

³ J. R. Goldenring, unpublished results.
FIP2, and pp75/Rip11 colocalize on plasma membrane recycling system vesicles with Rab11a in both non-polarized HeLa cells and polarized MDCK cells, whereas GFP-Rab11-FIP3 colocalizes with Rab11a in HeLa cells. In addition, Rab11-FIP1, Rab11-FIP2, and pp75/Rip11 coenrich with Rab11a and H^+^-ATPase upon stimulating parietal cells with histamine. These results suggest that association of Rab11a with a number of protein regulators is required for the complex regulation of plasma membrane recycling systems.

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