Molecular Characterization of Rab11 Interactions with Members of the Family of Rab11-interacting Proteins*

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The Rab11 subfamily of GTPases plays an important role in vesicle trafficking from endosomes to the plasma membrane. At least six Rab11 effectors (family of Rab11-interacting proteins (FIPs)) have been shown to interact with Rab11 and are hypothesized to regulate various membrane trafficking pathways such as transferrin recycling, cytokinesis, and epidermal growth factor trafficking. In this study, we characterized interactions of FIPs with the Rab11 GTPase using isothermal titration calorimetric studies and mutational analysis. Our data suggest that FIPs cannot differentiate between GTP-bound Rab11a and Rab11b in vitro (50–100 nM affinity) and in vivo. We also show that, although FIPs interact with the GDP-bound form of Rab11 in vitro, the binding affinity (>1000 nM) is not sufficient for FIP and GDP-bound Rab11 interactions to occur in vivo. Mutational analysis revealed that both the conserved hydrophobic patch and Tyr^262 are important for the GTP-dependent binding of Rab11 to FIPs. The entropy and enthalpy analyses suggest that binding to Rab11a/b may induce conformational changes in FIPs.

Rab proteins constitute the largest family of small monomeric GTPases and play an important role in membrane trafficking pathways. More than 60 Rab proteins have been identified in the human genome (1, 2), and many of them have been implicated in the regulation of specific exocytic and endocytic transport steps. Several Rab proteins (Rab4, Rab5, Rab7, Rab9, Rab11, Rab15, Rab22, and Rab25) have been shown to localize to endosomes, consistent with the multiplicity of trafficking pathways mediated by these organelles (reviewed in Refs. 3 and 4). Rab7 and Rab9 associate with late endosomes and lysosomes, whereas the rest of the endosomal Rab proteins have been shown to localize to either early or recycling endosomes.

Rab11a, Rab11b, and Rab25 are three closely related Rab proteins that belong to the Rab11 subfamily (1, 2). Rab11 family proteins have been shown to play an essential role in protein recycling from endosomes to the plasma membrane (5), polarized transport in epithelial cells (6), transport from endosomes to the trans-Golgi network (7), insulin-dependent GLUT4 trafficking (8), and phagocytosis (9). Whereas Rab11a and Rab11b are ubiquitously expressed, Rab25 is present exclusively in epithelial cells (10, 11), hence its proposed role in regulating membrane trafficking pathways that are specific to epithelial cells. The different roles of the Rab11a and Rab11b isoforms remain unclear. Although several Rab11-binding proteins have so far been isolated (12–15), the differences in their binding to Rab11a and Rab11b isoforms have not been investigated.

Rab proteins cycle between inactive GDP- and active GTP-bound forms. In the GTP-bound form, they interact with effector proteins, and each Rab-effector complex is proposed to regulate a unique trafficking step/event such as vesicle docking, budding, transport, or fusion (3, 16). Recently, several Rab11 effector proteins, Rip11, FIP2, Rab-coupling protein (RCP), FIP3/eferin, and FIP4, were identified using biochemical and yeast two-hybrid methods (12–15). In addition to Rab11 binding, FIP3 and FIP4 also interact with ADP-ribosylation factor GTPases, thereby coupling these small GTPase families, allowing for potential crosstalk between two signaling pathways (17). All FIPs have a conserved C-terminal motif that is known as the Rab11/25-binding domain (RBD) (18). Based on the presence of additional structural domains, FIPs are classified into three groups: class I FIPs (Rip11, FIP2, and RCP) containing a C2 domain, class II FIPs (FIP3/eferin and FIP4) containing EF-hand motifs, and the class III FIP (FIP1) with no homology to known protein domains (reviewed in Ref. 19).

Despite the identification of the RBD that mediates the interaction of FIPs with Rab11, we are only beginning to understand the mechanisms of Rab11 and FIP interactions. It remains unclear whether different FIPs exhibit preferences for interaction with Rab11a or Rab11b isoforms. We still do not know whether these multiple effectors are capable of binding to Rab11a or Rab11b with affinities that are relevant to their physiological concentrations in cells. Interestingly, some recent work suggests that FIP2 and RCP bind to Rab11a in a GTP-independent manner (14, 20). In addition, although several effector proteins have been identified, our knowledge of the affinities and thermodynamic properties of Rab11-effector complex formation. Thus, to understand the properties of Rab11 and FIP binding, we have performed isothermal titration calorimetric (ITC) analysis on the interaction of class I FIPs with GDP- and Gpp(NH)p-bound forms of Rab11a and Rab11b. ITC analysis has been used previously to
study several protein–protein and protein–ligand interactions, as it directly measures enthalpy and the binding constant involved (22, 23). Our data indicate that all class I FIPs are capable of binding to the Gpp(NH)p-bound form of Rab11 with similar affinity. A weak interaction was also seen with the GDP-bound form of Rab11, although it was not sufficient to mediate GDP-bound Rab11 and FIP interactions in vitro. FIPs bound equally well to both isoforms of Rab11 in vitro and in vivo, suggesting that Rab11a and Rab11b mediate redundant and overlapping functions. The free energy (ΔG) for Rab11-FIP complex formation is contributed by enthalpic factors and exhibits a strong enthalpy-entropy compensation effect. Mutational analysis of the conserved amino acids in the RBD of Rip11 indicates that the hydrophobic patch is crucial for Rab11-FIP complex formation. In addition to these conserved hydrophobic residues, Rip11 Tyr628 also plays an important role in FIP association with Rab11. Finally, our data suggest that FIPs form a high affinity homodimer, which interacts with two Rab11 molecules to form a heterotetramer.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Glutathione S-transferase (GST) gene fusion constructs were prepared by cloning GST-Rip11, GST-FIP2, and GST-FIP3 into the baculovirus expression plasmid pcAGHLT-B (BD Biosciences) and Rab11a into pGEX-KG (Amer sham Biosciences). GST and green fluorescent protein (GFP) fusion constructs with the Rip11 C-terminal fragment consisting of amino acids 490–652 (referred to as Rip11-F1) were described previously (18). pGEX-FIP2-F1 (amino acids 378–511) and pGEX-RCP-F1 (amino acids 479–659) constructs were also prepared. Gpp(NH)p was purchased from Roche Applied Science. Miscellaneous chemicals were obtained from Sigma. Alexa 647-conjugated transferrin (TF) was purchased from Molecular Probes, Inc. (Eugene, OR). Rabbit anti-Rip11 polyclonal antibody was described previously (12). Rabbit anti-RCP polyclonal antibody was prepared by immunization with recombinant RCP. Cell culture reagents were obtained from Invitrogen unless otherwise specified.

Site-directed Mutagenesis and Expression and Purification of Proteins—Site-specific mutations were introduced into the pGEX-Rip11-F1 or pEGFP-Rip11-F1 construct using a Stratagene PCR-based mutagenesis kit. The GST-Rab11, GST-FIP2-F1, GST-RCP-F1, and GST-Rip11-F1 constructs were transformed into Escherichia coli BL21 Codon Plus (Stratagene). GST fusion proteins were expressed and purified from E. coli as described previously (24). The Gpp(NH)p- or GDP-bound forms of Rab11a and Rab11b proteins were prepared by processes through a series of nucleotide (Gpp(NH)p or GDP) exchange reactions in the presence of EDTA as described previously for Rab5 GTPase (25). Full-length FIPs were cloned into the baculovirus expression plasmid, and the GST fusion proteins were purified from insect cells using glutathione affinity column chromatography. Protein concentrations were determined by the Bradford assay.

In Vitro Binding Assays—Glutathione beads (50 μl) were coated with 5 μg of GST fusion protein and incubated with varying amounts of soluble protein in a final volume of 0.5 ml of reaction buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, 0.1% bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride) in the presence of either GTPγS or GDPγS (0.5 mM) as indicated. Samples were incubated at 4 °C for 1 h on a nutator with constant rotation. The samples were pelleted at 2000 × g for 3 min and washed three times with 1 ml of reaction buffer. Bound proteins were eluted with 1% SDS, analyzed by SDS-PAGE, and either stained with Coomassie Blue or immunoblotted.

Gel Filtration Analysis—The oligomeric status of Rab11, Rip11-F1, and Rip1-F1 mutants and the Rab11-Rip11-F1 complex was determined by analyzing the proteins on a Superdex 200 column that was connected to a refractometer. The native molecular mass of the protein was calculated from light scattering analysis using the software provided (Wyatt Technology Corp.).

ITC—ITC experiments were performed using a VP-ITC calorimeter (Microcal, LLC). All proteins used in the ITC studies were either in the thrombin-cleaved form (Rab11, Rip11-F1, and FIP2-F1) or in the GST fusion form (GST-RCP-F1). The presence of GST did not interfere with the binding studies.

Cell Culture, Transient Transfection, and Immunofluorescence—HeLa cells were cultured as described previously (18). The cells were transfected with wild-type and mutant GFP-Rip11-F1 constructs using LipofectAMINE 2000 (Invitrogen). At 18–24 h post-transfection, the cells were plated on collagen-coated glass coverslips, grown overnight, and fixed with 4% paraformaldehyde, followed by quenching with 0.1 M glycine. The cells were permeabilized with phosphate-buffered saline containing 0.4% saponin, 2% fetal bovine serum, and 1% bovine serum albumin for 30 min, followed by incubation with rabbit anti-Rip11 or anti-Rab11 polyclonal antibody (Zymed Laboratories Inc.). After extensive washing, cells were incubated with Alexa 594-conjugated secondary antibodies (Molecular Probes, Inc.) for 30 min, washed, and mounted with Vectashield (Vector Laboratories). Cells were imaged with a Zeiss Axiositter 200M deconvolution microscope.

FIG. 1. FIPs form homodimers in a Rab11-independent manner. Glutathione beads coated with GST alone or with various GST-FIPs were incubated with different FIPs in the absence (A) or presence (B) of recombinant Rab11a. The beads were washed, and the bound protein was eluted with 1% SDS. The homodimerization of FIPs was visualized by immunoblotting with anti-Rip11, anti-FIP2, or anti-FIP3 antibody. Thrombin-cleaved full-length FIPs were used as the source of protein in the binding experiments.

40–50 injections). The titrations were performed while samples were stirred at 300 rpm at 25 °C or at the indicated temperature. An interval of 4 min between each injection was allowed for the base line to stabilize. The blank ITC titration was performed against buffer by injecting the corresponding FIP that was used in Rab11 titration. The blank subtraction was done for all data used for analysis. The data were fitted via the one-set-of-sites model to calculate the binding constant (K) using Origin software (Microcal, LLC). All proteins used in the ITC studies were either in the thrombin-cleaved form (Rab11, Rip11-F1, and FIP2-F1) or in the GST fusion form (GST-RCP-F1). The presence of GST did not interfere with the binding studies.
RNA Interference Analysis—Rab11a and Rab11b isoforms were knocked down using small interfering RNAs (siRNAs) that were designed using human Rab11a and Rab11b sequences (Rab11a, 5'-agtgcagacgccagacgaaa-3'; and Rab11b, 5'-agaaactgactgtgaaac-3'). Rab11a siRNA, Rab11b siRNA, or a mixture of Rab11a and Rab11b siRNAs was cotransfected into HeLa cells using LipofectAMINE 2000. Transfected cells were incubated for 74 h and analyzed for Rab11a and Rab11b expression by Western blotting or used for subcellular fractionation. The remaining cells were plated on collagen-coated glass cover slips, fixed, and analyzed by immunofluorescence microscopy using a Zeiss Axiovert 200M inverted deconvolution microscope. Anti-Rab11 primary antibodies were labeled with Alexa 488 and anti-Rip11 primary antibodies with Alexa 594 using a Zenon labeling kit (Molecular Probes, Inc.) and were used for staining HeLa cells.

FACS-based Tf Uptake and Recycling Assays—HeLa cells (80–90% confluent, T-75 flask) were transiently transfected with GFP alone, GFP-Rip11-F1, GFP-Rip11-F1(Y628A), or GFP-Rip11-F1(I629E) using LipofectAMINE 2000. The cells were incubated with transfection mixture (60 μg of DNA and 120 μl of LipofectAMINE) for 3 h, and the medium was replaced. At 16 h post-transfection, the cells were trypsinized and resuspended first in complete medium and then subsequently in serum-free medium.

FACS-based Tf uptake and recycling assays were done as described previously (36). Briefly, for Tf uptake assays, the cells were incubated at 4 °C for 30 min with 20 μg/ml Alexa 647-conjugated Tf, followed by incubation at 37 °C for various time intervals in the continuous presence of Alexa 647-conjugated Tf. To measure plasma membrane-associated transferrin receptor (TfR), cells were incubated at 4 °C. In all cases, the cells were washed and terminated by pelleting and resuspending the cells in 3% paraformaldehyde. Cells associated Alexa 647-conjugated Tf were determined by FACS analysis. For recycling assays, cells were incubated at 4 °C for 30 min, followed by internalization for 20 min at 37 °C in the continuous presence of Alexa 647-conjugated Tf. Cells were then washed and incubated in complete medium supplemented with 50 μg/ml unlabeled Tf for various times prior to fixation. The experiment was terminated as described above and analyzed on a BD Calibur flow cytometer (BD Biosciences) equipped with 488- and 647-nm lasers, gating for transfected cells (10,000 GFP-positive cells), and the amount of Tf internalized was determined.
Rab11 Effector Proteins (FIPs) Form Homodimers—It has been previously suggested that FIPs can form homodimers and heterodimers, yet it remains unclear whether dimerization is dependent on Rab11 binding (26). To test this, we produced recombinant full-length GST-FIP fusion proteins in insect cells and truncated FIPs in E. coli and tested their ability to form homodimers or heterodimers with other FIP family member proteins in the presence or absence of Rab11a. As shown in Fig. 1, soluble full-length Rip11 bound to GST-Rip11. The binding was specific to Rip11 (Fig. 1A), as there was no binding observed with GST alone or with other FIP family member proteins, FIP2 (Fig. 1B) or FIP3 (Fig. 1A). Similarly, FIP2 and FIP3 interacted with only FIP2 and FIP3, respectively (Fig. 1, A and B), indicating that FIPs form only homodimers in vitro. Rab11a had no influence on FIP homodimerization, as FIPs formed homodimers in both its presence and absence (Fig. 1, A and B). Deletion of the N-terminal region (amino acids 1–490) of Rip11 had no effect on the Rip11-Rip11 interaction, whereas deletion of the C-terminal 28 amino acids completely abolished this interaction, indicating that the C-terminal region including the RBD (amino acids 630–652) is very important for homodimer formation (Fig. 1A). These results were further supported by cross-linking experiments showing that the Rip11-F1 protein formed a dimer in solution (data not shown).

To confirm that Rip11 forms a homodimer, we performed gel filtration followed by light scattering analysis on Rip11-F1 (amino acids 490–652) alone or complexed with Rab11 (Fig. 2). Consistent with cross-linking results, Rip11-F1 eluted as a dimer (36 kDa), whereas Rab11 eluted as monomer (24.3 kDa) and the Rab11-Rip11 complex as a heterotetramer of 85 kDa (Rip11-F1 dimer + two Rab11 molecules).

Class I FIPs Form Strong GTP-dependent Complexes with Rab11a and Rab11b—The binding affinity and thermodynamics of the interactions between Rab11 and FIP complexes were characterized using ITC analysis. Fig. 3 (A and B, upper panels) shows a typical calorimetric titration of Rip11-F1 with Rab11a at 25 °C in the presence of GDP and Gpp(NH)p, respectively. The size of the injection peaks decreased gradually as the injections progressed due to saturation of the binding sites. Fig. 3 (lower panels) shows the binding isotherm, in which the total heat per injection (kilocalories/mol of Rip11-F1 injected) has been plotted against the molar ratios of Rip11-F1 and Rab11a. Curve fitting the data using the identical-site model (Origin software) resulted in the following parameters: the binding constant ($K_B$), stoichiometry ($n$, enthalpy ($\Delta H$), and entropy ($\Delta S$). Both GDP- and Gpp(NH)p-dependent binding exhibited exothermic heat responses. The binding stoichiometry for the Rip11-F1 and Rab11a interaction was 0.5, indicating that one Rip11-F1 dimer bound to two Rab11a molecules. This agreed with the gel filtration analysis (Fig. 2). The affinity of Rip11-F1 for Gpp(NH)p-bound Rab11a (54 nM) was 17 times higher than that for GDP-bound Rab11a (950 nM). Similar results were obtained for the interaction of Rip11-F1 with Rab11b (Fig. 3, C and D). There was no exothermic heat response produced by the buffer control or with other Rab GTPases, indicating that the Rab11 and Rip11 interaction was specific and that ITC-based assays can be used to measure affinities between Rab proteins and their effector proteins (Fig. 3, E and F; and data not shown).

The ITC data suggest that Rip11 interacts with Rab11a and Rab11b in vitro with similar affinity. To examine these interactions in vivo, we used siRNAs designed against Rab11a and Rab11b isoforms. As shown in Fig. 4, knockdown of Rab11a or Rab11b alone had no effect on Rip11 subcellular distribution, indicating that Rab11b can compensate for Rab11a and vice versa. In contrast, knockdown of both Rab11 isoforms (Fig. 4, C and F) resulted in redistribution of Rip11 from endosomes to the cytosol. To gain additional insights into the interaction of Rab11 with FIP family effectors, we extended the ITC-based study to other Rab11 effector proteins, FIP2 and RCP, belonging to the Class I group of FIPs. The interactions of FIP2 and GST-RCP with Rab11a and Rab11b were studied under conditions similar to those for the Rab11-Rip11 interaction as described above. It is evident from Table I that a strong Gpp(NH)p-dependent interaction existed between FIP2 and Rab11b (40 nM), whereas the interaction with GDP-bound Rab11 was weak (>1000 nM), similar to the conditions for Rab11-Rip11 complex formation. Similar results were also obtained for the interaction of GST-RCP with Rab11, but with slightly decreased affinity (2–3 fold) compared with the Rab11-Rip11 interaction (Table I).

Rab11-FIP Interactions Are Thermodynamically Similar and Enthalpically Driven—The enthalpy changes associated with the formation of the Rab11-FIP complex were measured directly using ITC. The net $\Delta G$ and entropy ($\Delta S$) were calculated using the following equations: $\Delta G = -RT \ln K$ and $\Delta G = \Delta H - T \Delta S$, respectively. Negative enthalpy ($12.8$ kcal/mol for GDP and $-21.4$ kcal/mol for Gpp(NH)p) and entropy ($-4.6$ kcal/mol for GDP and $-11.6$ kcal/mol for Gpp(NH)p) values were obtained for GDP- as well as Gpp(NH)p-dependent Rab11-Rip11 complex formation; thus, this interaction must be driven by enthalpic rather than entropic factors, as the net favorable $\Delta G$ is contributed by enthalpic factors (Table I). The enthalpy for Gpp(NH)p-dependent Rab11-Rip11-F1 complex formation was almost twice higher, and the $\Delta G$ was about $-1.5$ to $-2.1$ kcal/mol larger than that with GDP-dependent Rab11-Rip11 complex formation at each temperature used in the study (Fig. 6A), indicating that Rab11 binds to Rip11 in the...
The Conserved Hydrophobic Patch and Tyr628 Are Important for Rab11-Rip11 Complex Formation—The thermodynamic analysis of Rip11 and Rab11 interactions suggested that hydrophobic amino acid residues may play an important role in Rab11-Rip11 complex formation. The amino acid sequence alignment of FIPs in the RBD indicates that the conserved hydrophobic residues (marked with asterisks) form a hydrophobic patch (Fig. 7A) (18). To test the importance of these hydrophobic residues, we introduced a conservative substitution of the central core isoleucine residue (Ile629) with either valine or alanine. Both substitutions decreased affinity, whereas the conservative substitution (I629V) had little effect on Rab11-Rip11 complex formation. The dependence of $K_D$ on temperature $T$ is shown with squares and circles, respectively. $K_D$ versus $T$ yields the magnitudes of the slope and intercept.

### Table I

Thermodynamic parameters for the association of Rab11 with Rab11 effector proteins

| Rab11-FIP-F1 complex | $K_D$ (nM) | $\Delta H$ (kcal/mol) | $\Delta S$ (kcal/mol K) | Free energy ($\Delta G$) (kcal/mol) |
|----------------------|-----------|----------------------|------------------------|-----------------------------------|
| Rab11a with GDP      | 948       | $-12.8$              | $-4.6$                 | $-8.2$                            |
| Rab11a with Gpp(NH)p | 54.4      | $-21.4$              | $-11.6$                | $-9.8$                            |
| Rab11b with GDP      | 797       | $-12.9$              | $-4.6$                 | $-8.3$                            |
| Rab11b with Gpp(NH)p | 45.9      | $-22.5$              | $-12.5$                | $-10$                             |
| FIP2 titration       |           |                      |                        |                                   |
| Rab11a with GDP      | 1317      | $-23.5$              | $15.5$                 | $-9.0$                            |
| Rab11a with Gpp(NH)p | 40.3      | $-27.4$              | $17.3$                 | $-10.1$                           |
| Rab11b with GDP      | 1106      | $-22.5$              | $14.4$                 | $-8.1$                            |
| Rab11b with Gpp(NH)p | 44.4      | $-27.7$              | $17.8$                 | $-10.1$                           |
| RCP titration        |           |                      |                        |                                   |
| Rab11a with GDP      | 1176      | $-16.7$              | $-8.6$                 | $-8.1$                            |
| Rab11a with Gpp(NH)p | 107.2     | $-25.7$              | $-16.2$                | $-9.5$                            |
| Rab11b with GDP      | 1287      | $-17.1$              | $-9.1$                 | $-8.0$                            |
| Rab11b with Gpp(NH)p | 173.3     | $-28.3$              | $-19.1$                | $-9.2$                            |

The presence of Gpp(NH)p with higher affinity. The enthalpy change upon binding showed a strong temperature dependence, which was essentially linear ($r = 0.99$) (Fig. 6A). GDP- and Gpp(NH)p-dependent Rab11-Rip11 complex formation both displayed a strong enthalpy-entropy compensation, resulting in a relatively constant $\Delta C_p$ at all temperatures tested (Fig. 6B). The change in heat capacity ($\Delta C_p$) values were calculated by performing linear regression analysis on a $\Delta H$ versus $T$ plot, yielding $-0.84$ and $-0.97$ kcal/mol/K, respectively, for both GDP- and Gpp(NH)p-dependent Rab11-Rip11 complex formation. The large negative $\Delta C_p$ values may be indicative of a hydrophobic component to the binding and may mean that a conformational change accompanies the association of Rip11 with Rab11.

Fig. 5. Western blot analysis of Rab11 siRNA-transfected cells. HeLa cells were transfected in the absence (Mock) or presence of Rab11a siRNA, Rab11b siRNA, or Rab11a and Rab11b siRNAs, and cells were then incubated for 74-h A, Triton X-100 extracts of these cells were separated by SDS-PAGE and immunoblotted with anti-Rab11, anti-Rip11, and anti-Rab4 antibodies. B, Rab11 siRNA-transfected cells were lysed and fractionated into cytosolic (Ctyo) and membrane (Mem) fractions. These fractions were then separated by SDS-PAGE and immunoblotted with anti-Rip11 antibody.

Fig. 6. Effect of temperature on the thermodynamic parameters for the Rab11-Rip11 complex. A, temperature dependence of enthalpy ($\Delta H$). The lines show the best fit of the data for $\Delta C_p$ values (slope) of $-0.84$ kcal/mol/K for Rab11-Rip11 complex formation in the presence of GDP (open circles) and $-0.97$ kcal/mol/K in the presence of Gpp(NH)p (closed circles). B, enthalpy-entropy compensation plot for GDP-dependent (open symbols) and Gpp(NH)p-dependent (closed symbols) Rab11-Rip11 complex formation. The dependence of $\Delta G$ and $\Delta H$ on $T \Delta S$ is shown with squares and circles, respectively. $\Delta H$ versus $T \Delta S$ yields the magnitudes of the slope and intercept.
had any effect on dimerization (as assessed by gel filtration analysis) (data not shown).

Inactive Rip11 Mutants Do Not Affect Transferrin Uptake or Recycling—The above-described ITC binding studies showed that the I629E mutation in the hydrophobic patch and an Ala mutation at the conserved Tyr628, a residue neighboring the hydrophobic patch, significantly reduced Rip11 interaction with endogenous Rab11. As shown in Fig. 8 (A–C), wild-type Rip11-F1 was localized to Rab11-positive compartments, which were tubulated and aggregated due to Rip11-F1 overexpression, consistent with previously published data (18). In contrast, the Rip11-F1 mutants were predominantly cytosolic and had no effect on the cellular distribution of Rab11 (Fig. 8, D–I), indicating that these mutants failed to interact with Rab11 in vitro, consistent with their interaction in vitro. Interestingly, co-overexpression of Rab11 caused cytosolic Y628A (Fig. 8, J–L), but not I629E (data not shown), to be recruited to a Rab11 compartment. Thus, the low affinity binding of Y628A to Rab11 can be compensated by the presence of excess Rab11.

We have previously reported that Rip11-F1 has a strong dominant-negative effect on Tf uptake and recycling since the overexpression of truncated Rip11 sequesters Rab11 and inhibits the formation of native Rab11-FIP complexes (18, 36). Here, we utilized a FACS-based assay to analyze the effects of overexpression of GFP-Rip11-F1 mutants on Tf uptake and recycling (see “Experimental Procedures”). Consistent with previous report (18, 36), Rip11-F1 had an inhibitory effect on Alexa 594-conjugated Tf uptake (Fig. 9A–D), indicating that these mutants failed to interact with Rab11 in vitro. Interestingly, co-overexpression of Rab11 caused cytosolic Y628A (Fig. 8, J–L), but not I629E (data not shown), to be recruited to a Rab11 compartment. Thus, the low affinity binding of Y628A to Rab11 can be compensated by the presence of excess Rab11.

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Table II
Thermodynamic parameters for the association of Rab11 with Rip11-F1 mutants

| Rab11-Rip11-F1 complex | \( K_d \) (nM) | \( \Delta H \) (kcal/mol) | \( \Delta S \) (kcal/mol/K) | \( \Delta G \) (kcal/mol) |
|------------------------|--------------|--------------------------|---------------------------|------------------------|
| Rab11b-Y628A           | 1900         | -7.6                     | 0.2                       | -7.8                   |
| Rab11b-Y628F           | 530          | -19.2                    | -10.8                     | -9.3                   |
| Rab11a-I629V           | 160          | -18.0                    | -8.7                      | -9.3                   |
| Rab11b-I629V           | 189          | -17.0                    | -7.7                      | -9.3                   |
| Rab11a-I629E           | 30,000       |                         |                           |                        |
| Rab11b-I629E           | 28,500       |                         |                           |                        |
| Rab11b-D630A           | 38           | -22.8                    | -12.7                     | -10.1                  |
| Rab11b-E638A           | 50           | -27.4                    | -17.4                     | -10.0                  |

Fig. 7. Mutational analysis of the conserved RBD of FIPs. A, amino acid sequence alignment of the RBD of FIPs. The residues marked with asterisks form the hydrophobic patch. Mutations were introduced at the conserved residues that are numbered here and characterized in this study. B, ITC analysis for Rip11-F1(Y628A) and Rip11-F1(I629V) (C). C, ITC analysis for Rip11-F1(Y628F) and Rip11-F1(I629E) (D). The upper panels in B and C correspond to the I629V and I629E mutants, respectively. ITC experiments were carried out as described in the legend to Fig. 3.

Fig. 8. Rip11-F1(1629E) and Rip11-F1(Y628A) are not capable of binding to Rab11 in vivo. A–I, HeLa cells were transiently transfected with GFP-Rip11-F1 (A–C), GFP-Rip11-F1(1629E) (D–F), or GFP-Rip11-F1(Y628A) (G–I). Rab11 was immunostained using anti-Rab11 polyclonal antibody, followed by Alexa 594-conjugated anti-rabbit secondary antibody. C, F, and I show merged images for A and B, D and E, and G and H, respectively. J–L, HeLa cells were cotransfected with GFP-Rip11-F1(Y628A) (green in J and L) and Myc-Rab11a (red in K and L). Yellow in L represents the overlap between Myc-Rab11a and GFP-Rip11-F1(Y628A) staining.
Rab11 GTPases play an important role in regulating membrane trafficking pathways by interacting with their effector proteins (FIPs). To date, six different FIPs have been isolated and shown to interact with Rab11a (reviewed in Ref. 19). Furthermore, different FIPs appear to play distinct roles in regulating membrane traffic (12–15, 17, 27). The presence of several Rab11 effectors and two Rab11 isoforms complicates our ability to understand the roles of Rab11 and FIPs. One possible mechanism is that various FIPs differentially bind to Rab11a or Rab11b, thus regulating distinct membrane transport steps or pathways. Alternatively, FIPs may bind to both Rab11 isoforms with similar affinity, thus competing with each other for this interaction. Our ITC analysis indicates that the affinities of both Rab11 isoforms for different FIPs are nearly identical. This was further supported by RNA interference analysis, where the absence of one isoform could compensate for the other, as the knockdown of either of the Rab11 isoforms had no effect on Rip11 localization, whereas the knockdown of both isoforms redistributed Rip11 into the cytosolic fraction. Thus, FIPs appear to play a role of scaffolding proteins that may regulate Rab11 function by colocalizing them with various regulatory proteins. Consistent with this idea, different FIPs are reported to interact with distinct sets of proteins that are known to regulate various endocytic pathways. For instance, FIP2 interacts with myosin Vb, whereas FIP3 and FIP4 bind to ADP-ribosylation factor GTPases, thereby generating putative Rab11-FIP2-myosin Vb or Rab11-FIP3-ADP-ribosylation factor complexes to regulate different transport steps and/or pathways (17, 27).

It has been previously reported that FIPs may form homodimers or even heterodimers (26). This raises an interesting possibility of cross-talk between different FIPs via heterodimerization. Our data did show that FIPs form homodimers in vitro. The homodimerization appears to be independent of Rab11 binding. Each of the dimer subunits can independently interact with Rab11, forming a final heterotetramer that contains two FIPs and two Rab11 molecules. Surprisingly, we saw very little evidence of heterodimerization between different FIPs. However, we cannot fully discount the possibility that FIPs heterodimerize in vivo. The functional meaning of FIP dimerization remains unclear. The dimerization of Rab11-FIP complexes on the opposing membranes may play a role in transport vesicle docking at its target membrane. Indeed, it has been suggested that dimerization of EEA1-Rab5 complexes mediates organelle docking during homotypic early endosome fusion (28–30).

Although FIPs interacted strongly with GTP-bound Rab11 (~50 nM), weaker (>1000 nM; $K_d$) GDP-dependent interactions were also observed, which raised the possibility that FIPs may also interact with GDP-bound Rab11 in vivo. We have determined the concentrations of Rab11, Rip11, and RCP in HeLa cells by Western blot analysis using available antibodies for these proteins. These analyses revealed that there is ~270 nM total Rab11, 200 nM Rip11, and 430 nM RCP present in HeLa cells. Therefore, in the cell, GDP-dependent interactions will not be favored due to weaker affinity, and the realistic concentrations of available GDP-bound Rab11 at any given time will be much lower compared with the estimated total concentration as described above. Indeed, the Rip11 mutant Y629A, which bound to GTP-bound Rab11 with an affinity comparable to that of wild-type Rip11 binding to GDP-bound Rab11, showed a predominant cytosolic distribution and was not capable of inhibiting TfR recycling. This ~20-fold difference in FIP binding affinity appears to be sufficient to ensure the specific interaction between FIPs and GDP-bound Rab11 in vivo. Even though we are able to determine the total Rab11 and FIP concentrations in the cell, they do not fully reflect the local effective concentrations of Rab11 and FIPs on endocytic membranes. Thus, it is possible that relatively low affinity interaction between GDP-bound Rab11...
and FIPs could be compensated by high concentrations of these proteins on the endosomes.

The thermodynamic properties of the formation of the Rab11-FIP complex described in this study showed invariably favorable enthalpies and unfavorable entropies, indicating that the FIP and Rab11 interactions are energetically driven by exothermic enthalpy. This is in contrast to most protein-protein interactions, which possess favorable entropy because of the similarity of many protein interfaces to the interiors of proteins (31). The favorable enthalpy may arise from the significant number of polar interactions such as formation of salt bridges and hydrogen bonds. On the other hand, the negative ΔC_p values for both GDP-dependent (−0.84 kcal/mol/K) and Gpp(NH)p-dependent (−0.97 kcal/mol/K) Rab11-Rip11 complex formation suggest the presence of significant hydrophobic interactions. This is further supported by the presence of a conserved hydrophobic patch among all FIPs. The substitution of the central core residue within this patch with a charged glutamic residue severely affected the interaction of Rab11 with Rip11. The large negative ΔC_p may also be the result of reduction in conformational entropy that occurs due to a structural change upon binding. Similarly, a large negative ΔC_p was also observed for the interaction between TCF4 and β-catenin (32). β-Catenin ligands were shown to undergo significant structural changes upon β-catenin binding, and the interfaces are mixture of polar and non-polar contacts (32–35). Interestingly, both GDP- and Gpp(NH)p-dependent Rab11-Rip11 interactions show similar negative ΔC_p values, and this is probably true for all FIPs, as FIP2 and RCP showed a similar trend (unfavorable entropy factor), indicating that FIP, but not Rab11, may undergo conformational changes upon binding. Our thermodynamic as well as mutational analyses suggest that the Rab11-FIP interaction is probably contributed by both polar as well as non-polar interactions.

The RBD in FIPs is a highly conserved domain among different species (reviewed in Ref. 19). It is predicted to exist in an α-helical conformation with hydrophobic and charged residues clustered on the opposite sides of the helix (18). Consistent with the involvement of hydrophobic residues in mediating Rab11 binding, mutations within the hydrophobic cluster inhibit Rab11 interaction with Rip11. In addition, mutational analysis of the highly conserved Tyr298 indicated that this residue may contribute to both hydrophobic and polar interactions. Surprisingly, mutations of the highly conserved Asp310 and Glu328 had no effect on Rab11 and Rip11 interactions or on homodimerization. Even though these residues do not directly participate in Rab11 binding, they still could be important for some other functions, such as in determination of the specificity of Rab11 binding or in the Rab11-independent interaction between FIPs and other binding proteins such as myosin Vb and/or the ADP-ribosylation factor GTPases (17, 27).

The data described in this study are the first glimpse into understanding the molecular properties of Rab11-FIP interactions, but many questions remain to be answered. Perhaps the most intriguing one is how cells regulate the timing and localization of Rab11 interactions with a specific FIP molecule. Since most of the cells express several FIPs, it is unlikely that they simply compete with each other for a limited number of Rab11 molecules. The presence of multiple phosphorylation and Ca2+-binding motifs suggests that additional cellular factors probably regulate Rab11 and FIP interactions in vivo. The basic characterization of Rab11 and FIP interactions allows us to start addressing these questions.

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