The Structural GDP/GTP Cycle of Rab11 Reveals a Novel Interface Involved in the Dynamics of Recycling Endosomes*

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The small GTP-binding protein Rab11 is an essential regulator of the dynamics of recycling endosomes. Here we report the crystallographic analysis of the GDP/GTP cycle of human Rab11a, and a structure-based mutagenesis study that identifies a novel mutant phenotype. The crystal structures show that the nucleotide-sensitive switch 1 and 2 regions differ from those of other Rab proteins. In Rab11-GDP, they contribute to a close packed symmetrical dimer, which may associate to membranes in the cell and allow Rab11 to undergo GDP/GTP cycles without recycling to the cytosol. The structure of active Rab11 delineates a three-dimensional site that includes switch 1 and is separate from the site defined by the Rab3/Rabphilin interface. It is proposed to form a novel interface for a Rab11 partner compatible with the simultaneous binding of another partner at the Rabphilin interface. Mutation of Ser25 to Phe in this epitope resulted in morphological modifications of the recycling compartment that are distinct from those induced by the classical dominant-negative and constitutively active Rab11 mutants. Recycling endosomes condensed in the perinuclear region where they retained recycling transferrin, and they clustered Rab11- and EEA1-positive membranes. Altogether, our study suggests that this mutation impairs a specific subset of Rab11 interactions, possibly those involved in cytoskeleton-based movements driving the slow recycling pathway.

Rab proteins form a large family of small GTP-binding proteins with a wide range of functions in the coordination of vesiculo-tubular traffic (1), which include directional tethering.

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†† The atomic coordinates and structure factors (codes 1OIV and 1OIW) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: GDI, guanine dissociation inhibitor; Tf, transferrin; TIR, transferrin receptor; Rab11wt, wild type human Rab11a; GFP, green fluorescent protein; MES, 4-morpholineethanesulfonic acid; GTPyS, guanosine 5’-O-(thiotriphosphate); GppNHp, guanyl-5’-imidodiphosphate.

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nelles at the cell periphery (20, 21). Rab11 also regulates a set of related endosomal functions such as the mobilization of endosomal membranes for phagocytosis in macrophages (22), the recycling of apical endosomes in Madin-Darby canine kidney cells (23), the targeting of secretory vesicles in gastric parietal cells (12), the modulation of cellular cholesterol transport and homeostasis (21), and the sorting of membrane proteins to melanosomes in melanocytes. In line with this diversity, Rab11 has various effectors that include Rabphilin-11, a protein with six WD-repeat domains (24), the FIP family characterized by a conserved Rab11 binding domain (25, 26), and the tail of the myosin Vb motor (27).

Structural and mutagenesis studies provide a means to gain insight into the unique structure-function relationships of Rab proteins. Recent structural studies of the GDP/GTP cycle of Rab proteins have shown that, despite their non-conventional cellular cycle, they do not depart from other small GTP-binding proteins and respond to the alternation of GDP and GTP by conformational changes and disorder-to-order transitions at the so-called switch 1 and switch 2 regions (28, 29). These studies of their GDP/GTP cycles, together with the structure of the complex of Rab3 with its effector Rabphilin3 (30), the structures of Rab proteins bound to either GDP (31) or GTP analogues (32–35), and genome-wide analysis of Rab sequences (5, 6), suggest that the structural specificity of Rab subfamilies resides in the combination of the nucleotide-sensitive switch regions with nucleotide-insensitive regions featuring subfamily specific sequences and/or conformations. These latter regions have been termed RabSF1–4 and include the N terminus/βI, a1/switch 1, a3–βS, and a5/C terminus, respectively (6). Yet, the implementation of the dual nucleotide/membrane cycle of Rab proteins, and how this cycle is distributed over various effectors, remain open questions.

In this study, we report the high resolution crystal structures of inactive GDP-bound and active GTPyS-bound human Rab11a. These structures yield a novel hypothesis as to the coupling between the GDP/GTP and membrane/cytosol cycles, which would involve a membrane-bound Rab11-GDP dimer, Rab11a. These structures yield a novel hypothesis as to the dynamics of inactive GDP-bound and active GTPyS-bound Rab11a. These structures yield a novel hypothesis as to the movement of Rab11a. These structures yield a novel hypothesis as to the movement of Rab11a. These structures yield a novel hypothesis as to the movement of Rab11a.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Protein Expression—Because initial trials to crystallize full-length human Rab11a failed, a truncated form (residues 1 to 173) lacking the hypervariable 43 C-terminal residues was used throughout the crystallographic study (referred to as Rab11 hereafter). In addition, a mutation impairing the spontaneous GTPase, Q70L, was introduced to grow crystals of the active form. Constructs of Rab11 and the Q70L mutant were generated by PCR using previously described pGEM-Rab11 constructs (20) as templates and the following oligonucleotides: R11-forward (5′-GGGATCCATCGTAGTATAATC4CAACTGTA-3′) and R11Del173-forward (5′-TCTGAGATCTGATATATGTTTCTGCTCAGG-3′) and R11Del173-reverse (5′-ATGAACTGAGTTTCTCTGTTTCTGCTCAGG-3′). The PCR products were then cloned as BamHI/PstI inserts in pQE32 vector (Qiagen). His-tagged Rab11 proteins were overexpressed in Escherichia coli, purified by nickel affinity and stored in 50 mM phosphate-buffered saline. After permeabilization markers were visualized by indirect immunofluorescence microscopy was performed using a Leica microscope (Leica Microsystems, Heidelberg, Germany). Confocal sections or slice projections were further processed using Adobe Photoshop.

The following antibodies were used: sheep anti-CN46 antibody (Serotec), goat anti-EEA1 (Santa Cruz Biotechnology), monoclonal anti-human TIR mouse antibody DF1513 (Sigma), fluorescein isothiocyanate, Cy3, and Cy5 donkey secondary antibodies (Jackson ImmunoResearch). Dulbecco’s modified Eagle’s medium, fetal calf serum, penicillin, streptomycin, sodium pyruvate, and glutamine were from Invitrogen. Human transferrin-Alexa 488 was from Molecular Probes. The antibody against Rab11 was purified from a rabbit antiserum raised against full-length recombinant Rab11 as described (20).

Transferrin Internalization and Recycling—For steady state internalization of transferrin, cells were incubated for 30 min at 37 °C with Alexa 633-labeled Tf to a final concentration of 5 μg/ml in internalization medium (Dulbecco’s modified Eagle’s medium (DMEM), 6.6–1.8 μM (NH₄)₂SO₄, 3–6% PEG 400, 3–10% 1,3-butanediol, 100 mM Tris-HCl, pH 8–8.5, and were optimized by microscopically. Prior to flash-freezing in liquid ethane, crystals were rapidly soaked in a stabilizing solution containing 33% glycerol as a cryoprotectant. Because no Mg⁡⁺⁺ could be located in the electron density map under these conditions, further crystallizations were done in the presence of MgCl₂ in stabilized Rab11(Q70L)-GTPyS (10 μg/ml) were grown in the presence of 5 mM GTPyS. Nucleotide exchange was allowed to proceed spontaneously at high Mg⁡⁺⁺ concentration, because EDTA rapidly precipitated the protein. Crystals appeared within 5–10 days in 1.1–1.3 M NaCl, 150–200 mM NaH₂PO₄, 150–200 mM KH₂PO₄, 100 mM Na-MES, pH 6.5, and were stabilized in the reservoir solution supplied with 22% xylitol prior to flash-freezing.

Data Collection, Structure Determination, and Analysis—All diffraction data sets were collected at 100 K at beamline ID14 at the ESRF synchrotron in Grenoble. Data were processed with DENZOSCALEPACK (36). Phases were obtained by molecular replacement with AMoRe (37). Refinements were performed with CNS (38) and model building was carried out with TURBO. Sec4-GDP (Protein Data Bank entry 1G16 (28)) with the switch regions removed was found to be the best molecular replacement search model for the Rab11-GDP structure. The asymmetric unit comprises two Rab11-GDP molecules related by a non-crystallographic 2-fold axis. Refined Rab11-GDP was then used as a model for Rab11(Q70L)-GTPyS. The refined structures of Rab11-GDP and Rab11-GTPyS have no Ramachandran outliers. Crystallographic statistics are summarized in Table I. Coordinates have been deposited with the Protein Data Bank with entry codes 1OIV (Rab11-GDP) and 1OJW (Rab11(Q70L)-GTPyS).

Multiple structure alignments were performed with TURBO using Rab11-GDP as a reference. A cutoff distance of 0.5 Å for including Ca atoms in the refinement of the superposition matrix was found to robustly superimpose conserved secondary structures without artificiually averaging significant structural differences. Figures were generated with Molscript (39) and rendered with Raster3D (40).

Cell Culture, Transfection, and Immunocytochemistry—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium as previously described (20). Transfections were performed by calcium phosphate precipitation and expression of GTP constructs was allowed for 24 h. For immunofluorescence, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline. After permeabilization markers were visualized by double labeling with primary antibodies followed by incubation with Cy3- and Cy5-conjugated anti-IgG (Molecular Probes). Coverslips were mounted in Mowiol (Hoechst AG). Confocal laser scanning microscopy was performed using a SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany). Confocal sections or slice projections were further processed using Adobe Photoshop.


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The Structures of Inactive and Active Rab11 Depart from Those of Other Rab Proteins—The GDP/GTP structural cycle of human Rab11a is reported here, including inactive Rab11-GDP at 2.3Å resolution and active Rab11-GTPγS-Mg²⁺ at 2.3Å resolution (Table I, Fig. 1). In both crystals forms, Rab11 has the overall fold common to other small GTP-binding proteins in including Rab proteins, with a conserved protein core and nucleotide-sensitive switch 1 and switch 2 regions (Fig. 1). The switch regions have well defined conformations in both the inactive and active forms, and undergo large conformational changes that span residues Glu39 to Val46 (switch 1) and Ala68 to Ala70 (switch 2). Other conformational changes are located at the interswitch β-turn (residues Ile53 to Ile60) and reflect its flexibility.

The unusually ordered conformation of the switch regions in Rab11-GDP is stabilized in a large symmetrical dimeric interface (Fig. 1A). The buried surface area is ~2000 Å², which is larger than average nonspecific interactions between crystal neighbors (42) but in the range (1600 ± 400 Å²) of biologically relevant protein-protein complexes (43). 75% of the buried area is contributed by switch 1 and 2 regions, which are therefore essentially inaccessible to protein-protein interactions in the dimer. Rab11-GDP lacks a bound Mg²⁺ ion at the nucleotide binding site, despite the high Mg²⁺ concentration in the crystallization conditions (Fig. 1B). The deficiency of stabilizing interactions resulting from the absence of the Mg²⁺ ion is made up by alternative interactions that maintain the nucleotide tightly bound, including hydrogen bonds of switch 1 with the α-phosphate and the ribose of GDP, and an alternative interaction of the invariant Mg⁴⁺-binding Ser from the P-loop (Ser45) with its α-phosphate (Fig. 1B). Furthermore, the invariant switch 1 Thr (Thr43 in Rab11a) that binds Mg²⁺ and the γ-phosphate of GTP in active Rab11, exchanges a symmetrical hydrogen bond with Gua⁴⁺ in strand β2 from the symmetrical Rab11 molecule (Fig. 1A). This interaction is strikingly reminiscent of the interaction of the equivalent Thr in small GTP-binding proteins of the Rho family with an aspartate from RhoGDI, which has been proposed to account for the inhibition of guanine nucleotide dissociation by RhoGDI (44).

In contrast, the GTPγS-bound form of Rab11 is monomeric in the crystal. The interactions of the guanine triphosphate nucleotide are similar to those found in other structures of Rab active forms, except that neither Ser²⁰ from the P-loop nor Ser²² from switch 1 interact with the most external γ-phosphate oxygen, which remains accessible to the solvent (Fig. 1C). Switch 2 of Rab11 is the region that diverges most from other GTP-bound Rabs, as it is not helical and it makes less interactions with switch 1 (Fig. 1D). Although an influence of crystal packing cannot be excluded, this unique conformation confirms the trend of Rab proteins in having structurally heterogeneous switch 2 regions in their active forms. The situation is more subtle at the switch 1 region, whose conformation in Rab11-GTP is closest to that in Rab3 and Sec4 despite their sequence divergence (Fig. 1D). Comparison of all structures of GTP-bound Rab proteins shows that switch 1 regions fall in two structural classes, which depend on the main chain conformation of a residue located at the beginning of switch 1 (Ser⁴⁰ in Rab11) (Fig. 2). This residue either points toward the nucleotide as in Rab11 (Fig. 2A) or is flipped by 180° (Fig. 2B), possibly as the result of a steric conflict with the facing residue from the P-loop (Ser²⁰ in Rab11) for binding the γ-phosphate (Fig. 2, B and C). The alternative conformations of switch 1 determine the shape of a three-dimensional site spanning residues 26–49 in Rab11, which combines switch 1 with an upstream subfamily specific region (α1 and α1-switch 1 loop) and a downstream Rab family motif (switch 1–β2 loop) (Fig. 2). This combination of nucleotide-dependent and Rab subfamily-specific characteristics suggested that this region may define a novel three-dimensional epitope for the interaction of Rab11 with specific partners. To test this hypothesis, Rab11 family-specific residues at the surface of this epitope were
changed into their counterparts in Rab3a and tested for their effects on the distribution of early endosomal markers and transferrin transport.

The S29F Mutation Unbalances the Distribution of Early Endosomal Markers in HeLa Cells—Residues Ser\(^{29}\) in Rab11 S25N, Arg\(^{33}\) in the switch 1 loop, and Ala\(^{49}\) in the switch 1 loop were mutated to Phe, Asp, and Lys, respectively. The mutants were fused to GFP to examine their cellular localization compared with wild type Rab11 and its classical mutants, and to markers of the endocytic pathway. All mutants strongly labeled membrane structures (Fig. 3, B, C, and F), as also observed for wild type Rab11 (Fig. 3D) or the Q70L mutant (Fig. 3E), but contrasting with the predominantly cytosolic staining of Rab11(S25N) (Fig. 3G) (19). Two of the mutants, A49K and R33D (Fig. 3, B and C), behaved essentially as Rab11wt or the constitutively active Q70L and S20V (not shown) mutants, whereas the S29F mutant (Fig. 3F) induced several unusual morphological effects that differed from those induced by the classical mutants. Cells overexpressing Rab11(A49K) and Rab11(R33D) showed TfR-positive tubulo-vesicular structures, located mostly at the cell periphery (Fig. 3, B and C, red staining) that were reminiscent of the membrane structures generated by the overexpression of Rab11wt (Fig. 3D) or the Q70L mutant (Fig. 3E, TfR) (20, 21). Depending on the level of overexpression, a relative depletion of the perinuclear staining for TfR was also observed compared with endogenous Rab11 (Fig. 3A). In striking contrast, overexpression of Rab11(S29F) led to the pericentriolar condensation of Rab11-positive membranes that were also positive for TfR proteins (Fig. 3, F and H).

None of the mutants induced significant modifications of the localization of trans-Golgi network (TGN), Golgi, and late endosomal markers when overexpressed in HeLa cells (TGN46, CTR433, and CD63, respectively, data not shown). In contrast, a remarkable effect was induced by the S29F mutant on the relative distributions of Rab11 and EEA1, a marker of the early endosomal-recycling network through its interaction with Rab5 that shows little, if any, intermixing with endogenous Rab11 (Fig. 4A) (1, 45–47). Segregation of Rab11 and EEA1 was maintained in HeLa cells overexpressing Rab11wt (not shown).
and the Q70L (Fig. 4B, Q70L), A49K (Fig. 4B, A49K), and R33D (not shown) mutants, whereas EEA1 barely colocalized with TfR-positive tubules induced by the overexpression of the cytosolic dominant-negative S25N mutant (Fig. 4B, S25N) (20, 21). Strikingly, in Rab11(S29F)-overexpressing cells the condensed Rab11-positive membranes were also strongly labeled for EEA1 (Fig. 4C). Merged images at higher magnification show a discrete apposition of the Rab11- and EEA1-positive membranes (Fig. 4C, zoomed merged area), which cluster without actual fusion of the corresponding membranes. This suggests that the Rab11(S29F) mutant has uncovered a novel transition step in the sequential processing of endosomal traffic.

**The S29F Mutation Defines a Novel Transferrin Transport Phenotype**—Disruption of the Rab11 function leads to downstream effects on recycling of Tf that have been documented in many cell types (18–20). We thus examined the trafficking of Tf within cells expressing the S29F mutant. Cells transfected with GFP-Rab11wt, the constitutively active Rab11(Q70L) or Rab11(S20V) mutants, the dominant-negative Rab11(S25N) mutant, and the new Rab11(S29F) mutant were allowed to internalize iron-loaded Tf for 30 min at 37 °C. Cells fixed at that point were considered as the initial time point for the chase experiment. After extracellular Tf was washed out at 4 °C, cells were observed at chased times of 5, 15, and 30 min at 37 °C. Effects on Tf recycling were apparent after 30 min chase, with intermediate chase times providing no further information (Fig. 5). In cells overexpressing Rab11wt as well as the Q70L and S20V mutants (not shown), Tf distributed to tubulo-vesicular structures that were also labeled for Rab11 (Fig. 5, A and B) and were similar to those labeled by TfR (Fig. 3D). Cells overexpressing the dominant-negative S25N mutant displayed a marked tubular staining for Tf emerging from the perinuclear region, whereas Rab11(S25N) was predominantly cytosolic (Fig. 5, I and J). In Rab11(S29F)-expressing cells, Tf concentrated in membranes in the perinuclear region where Rab11(S29F) was also strongly condensed.
endocytosis of Tf and its accessibility to Rab11-positive membranes are not abrogated by this new mutation.

A 30-min chase of internalized Tf led to almost complete depletion of Tf from the perinuclear endosomes in cells expressing Rab11wt (Fig. 5, C and D) and the Q70L and S20V mutants (not shown). In cells expressing Rab11(S29F), internalized Tf was still detected in the thin tubular structures after 30 min (Fig. 5, K and L). This is consistent with the reported effects of this mutant on slowing down the return of Tf-TfR complexes to the plasma membrane (18, 19). In contrast, even after 30 min of chase, Tf was still detected in the perinuclear region of cells expressing Rab11(S29F) (Fig. 5, G and H), with a level of retention that correlated with the level of Rab11(S29F) expression. Little Rab11(S29F)-negative structures remained stained with internalized Tf. This pool of retained Tf might thus correspond to the pool that escapes the fast recycling pathway that is not affected by this mutation and allows Tf-Tf complexes to recycle back to the cell membrane. We concluded that Rab11(S29F) associated to endocytic membranes promotes the dynamic retention of Tf within the endosomal recycling network. This was then confirmed by analyzing the rate of Tf recycling. As shown in Fig. 5M, expression of the Rab11(S29F) mutant promoted a strong retention of 125I-Tf previously internalized for 1 h at 37 °C. This effect was more pronounced than the inhibition of recycling induced by the Rab11(S25N) mutant. Remarkably, initial steps of recycling did not seem to be affected, whereas most of the effect appeared for later time points of the recycling kinetics. This emphasizes a specific effect of the new mutant of Rab11 on the late recycling pathway.

**DISCUSSION**

A Novel Interacting Region with Structural Properties Unique to Rab Proteins—The binding sites for the effectors and regulators of Rab proteins are expected to comprise regions whose conformations depend on the nucleotide status, together with amino acid sequences that identify individual Rab members and may be located outside switch regions. Our analysis of the structural GDP/GTP cycle of Rab11 suggests that switch 1,
Sequestration of Tf in cells expressing Rab11(S29F). HeLa cells were transfected with plasmids encoding for GFP-Rab11wt (A–D), -Rab11(S29F) (E–H), and -Rab11(S25N) (I–L). Overexpression was allowed for 24 h. Cells were then incubated with 5 μg/ml Alexa 633-coupled Tf for 30 min at 37 °C. A, B, E, F, I, and J, distribution of Tf after 30 min internalization. Merged images at high magnifications of the indicated area are shown for Rab11wt (B) and Rab11(S29F) (F) for improved visualization of structural details of the colocalization. An inset is included in J to better visualize the tubulation of Tf-positive membranes induced by overexpression of the Rab11(S25N) mutant. C, D, G, H, K, and L, distribution of Tf after 30-min chase time. Arrows indicate a cell expressing no or a very low level of the constructs. Arrowheads indicate a cell expressing a high level of Rab11(S29F) in which the condensation of sequestered Tf is clearer. Note that this phenotype is highly dependent on cellular expression. D, H, and L, pictures were taken with a SP2 confocal with identical excitation and detection parameters. Scale bars = 10 μm. M, HeLa cells were transfected with plasmids encoding for GFP-Rab11wt, Rab11(S29F), -Rab11(S25N), and -Rab11(Q70L). After 24 h cells were loaded with 125I-transferrin (200 ng/ml) for 1 h, washed at 4 °C, and then incubated at 37 °C. At the indicated times, media were collected and cells were harvested to assess the rate of radioactive transferrin recycling. Each time point was performed in triplicates. Data are representative of three separate experiments and are expressed as a % of total internalized transferrin as determined by counting intracellular and extracellular radioactivity. Mean ± S.D. of total counts/min in each cell population were: Rab11wt, 6056 ± 150; Rab11(S25N), 4786 ± 264; Rab11(Q70L), 6851 ± 174; Rab11(S29F), 4897 ± 223; and mock transfected cells (Ctl), 5874 ± 124.
which is a general target of protein-protein interactions in small GTP-binding proteins, combines with its upstream and downstream sequences to define a novel interaction site that meets these criteria in Rab11 and other Rab proteins. Notably, this site sorts into two structural classes in GTP-bound Rab structures, depending on whether switch 1 is flipped toward the center of the site or is involved in a non-conserved interaction with the γ-phosphate. The alternative conformations seem to result from the steric conflict of a residue located at the beginning of switch 1 with a facing residue in the P-loop for binding the γ-phosphate, and would thus depend solely on the sequence of this pair of residues, with large polar residues in switch 1 favoring the switched conformation (Fig. 2C). However, in other GTP-binding proteins, the residue equivalent to the flipped residue can have two different conformations, as exemplified by Tyr34 in RhoA, which binds the γ-phosphate of GTP (48) but flips outside to be replaced by the GTPase-activating protein arginine finger (49). We do not exclude, therefore, that both conformations could co-exist in an individual Rab protein, with the potential advantage of generating structural polymorphism for the binding of diverse effectors. The invariant Gly bulge of unknown function at the end of switch 1 (Gly45 in Rab11a) may provide the flexibility required for such a conformational change. The conformational subclasses do not, however, correlate with an endocytotic versus exocytotic classification.

**Mutations in the Novel Site Promote Morphological and Functional Alterations of the Early Endosomal Membranes—** Interactions of Rab proteins with their effectors have only been described so far for the Rab3-Rabphilin complex (30). The binding epitope defined by Rabphilin, which includes switch 2 and the Rab-SF1, -SF3, and -SF4 regions, is separate from our novel epitope. An attractive possibility would thus be that two effectors could be accommodated simultaneously by these two epitopes, or a combination of an effector and a regulator. If so, it is expected that mutants of this region should impair some, but not all aspects of Rab11 functions. As a first step toward addressing this issue, several Rab11 constructs carrying mutations located in this region were investigated in HeLa cells. Whereas the mutations located in switch 1-flanking loops behaved essentially as wild type or constitutively active Rab11, we identified a residue located in helix a1, Ser29, whose mutation to Phe yielded a unique cellular phenotype. This mutant was associated with membranes, indicating that its lipid modifications are correctly processed, and it did not affect the internalization of Tf to pericentriolar membranes. Its behavior in the cell differed from that of either the dominant negative or the constitutively active mutants of Rab11 in several respects, suggesting that it is not trapped in a GDP- or GTP-bound form and can undergo GDP/GTP cycles. On the other hand, its specific effects on the morphology and identity of early endosomal membranes, as well as on Tf recycling, suggests that the Rab11(S29F) mutant is likely to be impaired for a subset of its interactions, whereas other interactions take place normally. Various effectors have now been described for Rab11 (24–27), yet their specific functions are far from being deciphered, making it difficult to point to the implication of particular Rab11 partners at this stage. Yet, the morphological condensation of the endosomal membranes induced by Rab11(S29F) is reminiscent of the recently reported effect of the overexpression of two Rab11 interacting proteins, Rab11-FIP4 (26) or the tail of the myosin Vb motor (50). Notably, myosin Vb appears to be involved in a ternary complex with Rab11-FIP2 and Rab11, which would fit with our dual effector binding model. The tail of myosin Vb acts as a dominant negative on the exit of molecules such as Tf out of the recycling system, because it cannot link Rab11 functions to cytoskeleton-based movements. Notably, as found for Rab11(S29F), the tail of myosin Vb affects specifically the slow recycling pathway. Thus, the new interface affected by the mutation may be involved in the formation of this ternary complex.

Another interesting observation comes from the fact that Rab11(S29F) overexpression leads to the redistribution of a hallmark marker of the Rab5 compartment in the early endosomal pathway, EEA1, suggesting that this mutation also affects the molecular interactions in charge of the compartmental segregation of Rab5 and Rab11 membranes. However, a partition between Rab5 and Rab11 domains is maintained, suggesting that segregation and partition are controlled by different subsets of molecular interactions, of which only those involved in segregation are compromised by the S29F mutation. The structural and functional properties of the organelles involved in membrane trafficking are defined by molecular architectures that permit membranes to fuse with or exit from a given compartment. These architectures must therefore not be distributed randomly, which requires conflicting needs for communication and segregation. Maintenance of segregation between Rab-defined compartments and partition of Rab proteins into subdomains point to Rab proteins as key players in these processes (47, 51, 52), and form the basis for current models of Rab activities on membranes (1, 53). Divalent effectors that interact with two Rab proteins may act as the connectors between subdomains (51). Such effectors have been described for Rab4 and Rab11 (54), and more combinations may arise from the hetero-dimerization of certain effectors (55). The Rab11(S29F) compartment remains accessible to Tf and no fusion of the EEA1 and Rab11 membranes is observed, indicating that the communication mediated by connecting effectors is not fully impaired by this mutant. It is then tempting to speculate that both the spatial segregation of membranes, as defined by the cellular distribution of Rab5/EEA1 and Rab11 membranes, and the exit of molecules from the plasma membrane recycling system result from common mechanisms, whereas partition of membrane subdomains are not. In this respect, the role of myosin Vb in the spatial segregation of Rab5/EEA1 and Rab11 membranes remains to be explored.

**A Membrane-localized Rab11-GDP Dimer Pool Allowing GDP/GTP Cycling at the Membrane?**—Several studies, including ours, suggests that Rab11 may recognize several effectors in the course of its cellular function in endosomal traffic. The spatial and temporal coordination of these events is not known at the moment, and is compatible with various, although not exclusive, molecular models. For instance, Rab11-GTP could hop from one effector to the next, or Rab11 could couple effector changes to successive GDP/GTP cycles, or alternatively there could be pools of Rab11 distributed by RabGDI to different places where they fulfill different tasks. Together with the fading frontiers between effectors and regulators exemplified by the association of guanine nucleotide exchange factors and effectors in multiprotein complexes (8), this points to a challenging need for revisiting the implementation of the GDP/GTP cycle and its role in orchestrating the molecular interactions that mediate Rab functions. In this regard, the unique characteristics of the crystalline Rab11-GDP dimer address the question of whether it may represent a species biologically relevant to some aspects of Rab11 functions. The Rab11 dimer interface makes large fractions of switch 1 and switch 2 regions, which are universally involved in the interactions of small G proteins with their partners (56) including RabGDI (57, 58), unavailable for protein interactions. Thus the Rab11-GDP dimer, if it exists, cannot interact with and be solubilized by RabGDI, and should be located at the membrane rather than in the cytosol.
Accordingly, we were not able to detect the Rab11-GDP dimer in solution by gel filtration, whereas its formation in the crystal may result from the constrained crystalline environment that may mimic, to some extent, the diffusional restriction encountered by Rab11 upon membrane association. Furthermore, the dimer interface stabilizes the bound GDP nucleotide (see “Results”). Although speculative at the moment, dimerization could therefore provide an attractive mechanism, analogous to the action of RabGDIs in the cytosol, by which a pool of inactive membrane-bound Rab11-GDP could be maintained in a specific membrane subdomain where it could undergo multiple rounds of GDP/GTP exchange on the same membrane without recycling in the cytosol. We are now investigating further the homodimeric interaction of both soluble and membrane-bound wild type Rab11 and mutants modified in the dimer interface.

In conclusion, our combined structural and cellular analysis identified a novel binding site for a Rab11 partner, whose mutation leads to a neomorphic allele of Rab11 that unbalances the distribution of endosomal membranes and impairs trafficking through the plasma membrane recycling network. This site is proposed to specify a specific set of effectors that are necessary to maintain the identity of Rab11 membranes, possibly through cytoskeleton-based movements.

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