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Characterisation of the Rab binding properties of Rab coupling protein (RCP) by site-directed mutagenesis

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Abstract Rab coupling protein (RCP) is a member of the Rab11-family of interacting proteins (Rab11-FIPs). Family members are characterised by their ability to interact with Rab11. This property is mediated by a conserved Rab binding domain (RBD) located at their carboxy-termini. Several Rab11-FIPs can also interact with other small GTPases. RCP interacts with Rab4 in addition to Rab11. To dissect out the individual properties of the Rab4 and Rab11 interactions with RCP, conserved amino acids within the RBD of RCP were mutated by site-directed mutagenesis. The effect of these mutations on Rab4 and Rab11 binding, and the intracellular localisation of RCP, was examined. Our results indicate that Rab11, rather than Rab4, mediates the intracellular localisation of RCP, and that the class I Rab11-FIPs compete for binding to Rab11.

Keywords: Rab11-FIP; RCP; Endocytic recycling compartment

1. Introduction

Rab coupling protein (RCP) is a member of the recently identified Rab11-family of interacting proteins (Rab11-FIP) family, a family of proteins that are characterised by their ability to bind the small GTPase Rab11. The members of this family can be divided into two classes, the class I Rab11-FIPs possess homologous C2 domains near their amino-termini and the class II Rab11-FIPs possess an ERM-like domain and a proline rich domain. However, they all share a highly homologous Rab binding domain (RBD) at their carboxy-termini, through which they interact with Rab11 [1–4]. The class I Rab11-FIPs, to which RCP belongs, localise to the endocytic recycling compartment (ERC) where they function to regulate traffic from the ERC to the plasma membrane. A number of the Rab11-FIPs can also interact with other small GTPases. Rab11-FIP3 and Rab11-FIP4 (also known as arfophilin-1 and arfophilin-2, respectively) interact with Arf4, Arf5 and Arf6 [5–7]. RCP binds to the active conformation of Rab4, in addition to interacting with Rab11 [4]. The ability of RCP to bind to Rab4 is unique among the Rab11-FIPs and led us to propose that RCP serves as a 'molecular-link' between Rab4-regulated transport from the sorting endosome (SE) and Rab11-regulated transport from the ERC. Rabaptin-5 and Rabenosyn-5 have recently been described as divalent Rab effectors [8,9], in that they can both bind simultaneously to Rab5 and Rab4, thus serving as a link between Rab5- and Rab4-regulated membrane traffic. The Rab5 and Rab4 binding domains on these two effectors are located at different regions of the protein. In contrast, we have narrowed the Rab4 and Rab11 binding domains of RCP to the same 65 amino acids at the carboxy-terminus [4]. In order to help define the roles of the Rab4 and Rab11 interactions individually, we made a series of point mutations at key residues in its putative RBD. Using this method we have generated an RCP mutant that has the ability to bind Rab11 but not Rab4, and a mutant in which both Rab4 and Rab11 binding is abolished. We have transiently expressed these mutants in mammalian cells and show here that Rab11 recruits RCP to the recycling compartment. Furthermore, we demonstrate that the class I Rab11-FIPs compete with each other for binding to Rab11.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

The Rab4 and Rab11 yeast two-hybrid constructs, pEGFP-C3 RCP, pEGFP-C1 Rab4, and pEGFP-C1 Rab11-FIP3 constructs, have been described elsewhere [4,10–12]. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) according to the manufacturer's instructions. GFP-RCP or GFP-Rip11 was used as template and the following sense and antisense oligonucleotides were used (codon changes are indicated in bold).

RCP(Y620F) Sense	5′-GTCCGCGAGCTGGAAGAC**TTC**ATTG-ACAACCTGCTTGTC-3′
RCP(Y620F) Anti	5′-GACAAGCAGGTTGTCAAT**GAA**GTCT-TCAGCTCGCGGAC-3′
RCP(I621E) Sense	5′-CGCGAGCTGGAAGACTAC**GAG**GAC-AACCTGCTTGTCAGG-3′
RCP(I621E) Anti	5′-CCTGACAAGCAGGTTGTC**CTC**GTAG-TCTTCCAGCTCGCG-3′
RCP(D622N) Sense	5′-GCTGGAAGACTACATT**AAC**AACCTG-CTTGTCAGGG-3′
RCP(D622N) Anti	5′-CCCTGACAAGCAGGTT**GTT**AATGTA-GTCTTCCAGC-3′
Rip11(I630E) Sense	5′-CAGGAGCTGGAGAGCTAC**GAG**GAC-CGGCTGCTGGTGCGG-3′
Rip11(I630E) Anti	5′-CCGCACCAGCAGCCGGTC**CTC**GTA-GCTCTCCAGCTCCTG-3′

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Abbreviations: EGF, epidermal growth factor; ERC, endocytic recycling compartment; PMA, phorbol 12-myristate 13-acetate; Rab11-FIP, Rab11-family of interacting proteins; RCP, Rab coupling protein; SE, sorting endosome

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Incorporation of the mutations was confirmed by DNA sequencing. The cDNA for each of the RCP mutants was subcloned from the *Bam*HI site of pEGFP-C3 into the *Bam*HI site of the pGADGH yeast two-hybrid vector. To generate the bacterial expression vectors, the RCP mutants were subcloned into the *Bam*HI and *Eco*RI sites of pTrcHisB (Invitrogen) from pEGFP-C3.

2.2. Biochemical interactions

Escherichia coli lysates expressing RCP(WT) and mutants were separated by SDS–PAGE and transferred to nitrocellulose. The nitrocellulose was blocked overnight in Basic buffer (BB: 20 mM HEPES, pH 7.5; 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1% NP-40) plus 5% non-fat dried milk. Meanwhile, 10 μg of the GST-Rab fusion proteins was loaded with GTPγS by incubation in 20 mM HEPES, pH 7.5; 4 mM EDTA; and 100 μM GTPγS at 37 °C for 30 min. $MgCl_2$ was subsequently added to a final concentration of 10 mM. The fusion proteins were then overlayed onto the nitrocellulose in interaction buffer (BB plus 1% non-fat dried milk) and rocked at 4 °C for 4 h. The nitrocellulose was then washed with TBS–T (10 mM Tris, pH 7.5; 150 mM NaCl; and 0.1% Tween 20). Bound GST-Rab11 or Rab4 was revealed with an anti-GST antibody (Sigma).

2.3. Cell culture and immunofluorescence

HeLa and A431 cells were maintained in culture in DMEM (Bio-Whittaker) supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. For immunofluorescence, cells were seeded onto 10 mm glass coverslips. Transfections were performed using Effectene (Qiagen) according to the manufacturer's instructions. Approximately 16 h post-transfection the cells were fixed with 3% paraformaldehyde, quenched with 50 mM NH_4Cl, and permeabilised in 0.05% saponin/0.2% BSA. The fixed cells were either labelled with anti-RCP (1:150) [4] or anti-Rab11 (1:250) [11]. Texas red-labelled donkey anti-rabbit (Jackson Immunochemicals) was used as secondary antibody. Images were acquired on a Zeiss LSM 510 confocal microscope using a PlanApo 63X 1.4 NA oil immersion objective and processed using Image Examiner software (Carl Zeiss).

2.4. Miscellaneous protocols

The yeast two-hybrid experiments were performed as previously described [4]. To prepare membrane and cytosol fractions, HeLa cells expressing the GFP-RCP constructs were resuspended in fractionation buffer (FB: 50 mM Tris, pH 7.4, 250 mM sucrose, and 1 mM EDTA) and lysed by freeze-thawing and passage through a 26 gauge needle. A post-nuclear supernatant was generated by centrifugation at $5000 \times g$. The PNS was then spun at $200\,000 \times g$ for 30 min at 4 °C in an MLA-130 rotor (Beckman). The high-speed supernatant (cytosol) was removed and the high-speed pellet (membrane) was resuspended in an equal volume of FB. The same volumes of each fraction were separated on an SDS–PAGE gel, transferred to nitrocellulose and probed with anti-GFP (Abcam). The fractionation procedure was controlled by probing the blot with anti-TfnR (Zymed) and anti-β tubulin (Sigma).

3. Results

3.1. Generation of RCP Rab binding domain mutants

Multiple alignment of the carboxy-terminal region of each member of the Rab11-FIPs reveals a domain of approximately 20 residues that shows a high degree of sequence conservation (Fig. 1A) [3]. As this region has already been shown to mediate the interaction of Rip11 and Rab11-FIP3 with Rab11, we wished to investigate its importance in RCP binding to Rab4 and Rab11 [3]. Structural analysis of this 20 amino acid domain of RCP reveals that it has the potential to form an amphipathic helix (Fig. 1B). In order to establish if this region is responsible for binding to Rab11 and/or Rab4, the tyrosine, isoleucine, and aspartic acid residues at positions 620, 621, and 622, respectively, were individually mutated. We used the yeast two-hybrid system to test for the ability of these mutants to interact with the two Rab GTPases, and also for their ability to self-interact, since RCP has been shown to homodimerise [13] (Fig. 1C). The RCP(Y620F) mutant retains the ability to bind Rab11 and to dimerise, but displays weaker Rab4 binding. In contrast, an isoleucine to glutamic acid change at position 621, (RCP(I621E)), results in the complete abolition of Rab4 and Rab11 binding, and also the ability to dimerise. The aspartic acid to asparagine mutation at amino acid 622 generates a mutant that can bind efficiently to Rab11 and to itself, but not to Rab4. Western blotting of yeast lysates, that are expressing the RCP mutants, confirms that the wild-type and mutant proteins are expressed at similar levels and that the abolition of an interaction with Rab4 and Rab11 is not due to instability of the RCP(I621E) mutant (data not shown). To confirm the yeast two-hybrid data biochemically, a Far Western approach was adopted. Bacterial lysates expressing wild-type RCP and the three RCP mutants were transferred to nitrocellulose and overlayed with 10 μg of purified GST-Rab4 or GST-Rab11 that had been preloaded with GTPγS (see Section 2). Rab protein bound to RCP was detected with an anti-GST antibody (Fig. 1D). GST-Rab11 displayed a strong interaction with RCP(WT) and slightly weaker interactions with RCP(Y620F) and RCP(D622N), but no interaction with the I621E mutant. GST-Rab4 interacted with RCP(WT) and RCP(Y620F) but did not interact with either RCP(I621E) or RCP(D622N). Hence, the biochemical interactions fully support the two-hybrid data. It should be noted that the mobility of the RCP mutants on an SDS–PAGE gel is slightly altered, possibly due to an effect of the mutations on the RCP secondary structure.

Thus, the conserved 20 amino acids at the carboxy-terminus of RCP, which are likely to form an α-helix, mediate its interaction with both Rab4 and Rab11. This region is also responsible for the ability of RCP to dimerise. Since this binding motif is relatively small, and a single point mutation can abolish all three interactions, it is unlikely that both Rab4 and Rab11 can bind simultaneously to an individual RCP molecule. It is possible that the two small GTPases may compete for binding to RCP and this binding may depend on the activation state of RCP or on its intracellular localisation. Alternatively, the formation of an RCP dimer or higher order oligomer in vivo could provide a means whereby Rab4 and Rab11 could bind simultaneously to the same complex. Since the RCP(I621E) mutant does not dimerise, it is possible that dimerisation of RCP is an essential requirement for Rab binding.

3.2. Intracellular localisation of RCP and mutants

Wild-type RCP localises to the ERC, and we have recently demonstrated that treatment of cells with the epidermal growth factor (EGF), or phorbol 12-myristate 13-acetate, can induce its translocation from the ERC to the plasma membrane [11]. To determine the effect of the RBD mutations on the intracellular localisation of RCP, each mutant was fused to the C-terminus of the green fluorescent protein (GFP) and expressed in mammalian cells (Fig. 2A). As expected, wild-type GFP-RCP displayed a vesicular punctate pattern when expressed in HeLa cells. GFP-RCP(Y620F) and

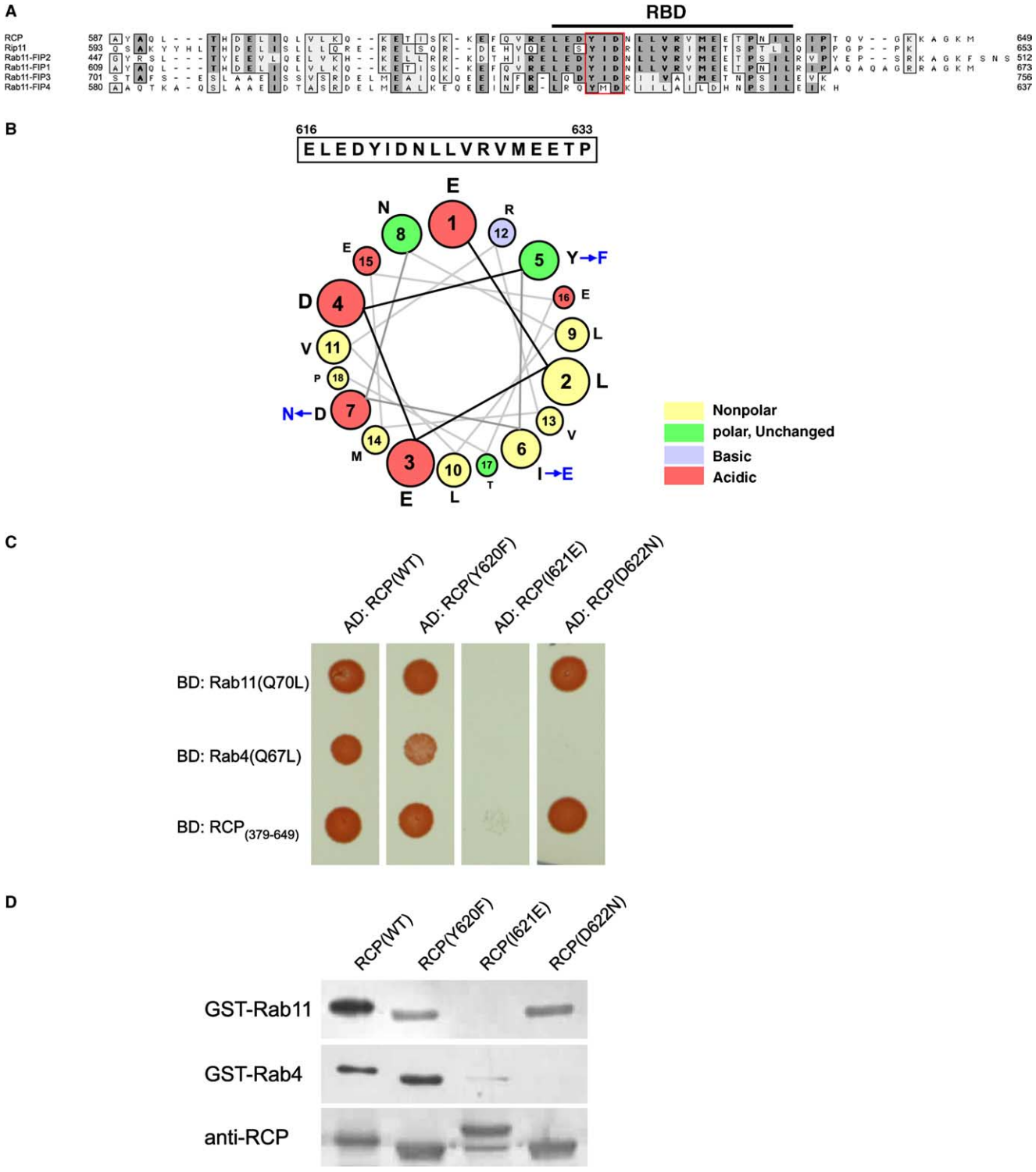

Fig. 1. Analysis of the Rab binding domain (RBD) of RCP. (A) ClustalW alignment of the carboxy-terminal regions of the Rab11-FIP family, with the RBD indicated [3]. The red box denotes the amino acids that were mutated. (B) Residues 616–633 of RCP have the potential to form an amphipathic α-helix (http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html). (C) Yeast two-hybrid analysis of the RCP mutants. Gal4 activation domain fusion constructs of RCP(WT), and mutants, were co-transformed into the L40 strain of *S. cerevisiae* with binding domain fusions of constitutively active Rab11 and Rab4, or with the carboxy-terminal region of RCP. Transformant colonies were spotted onto media lacking histidine (His−). An interaction is indicated by growth on the His− media. (D) Biochemical analysis of the Rab binding properties of wild-type RCP and mutants. *E. coli* lysates expressing His-RCP(WT) and mutants were separated by SDS–PAGE and transferred to nitrocellulose. The nitrocellulose was overlayed with GTPγS loaded GST-Rab11 or Rab4. Bound Rab protein was identified by blotting with anti-GST (top and middle panels). Loading of RCP fusion proteins was revealed by blotting with anti-RCP (bottom panel).

GFP-RCP(D622N) exhibited a pattern very similar to that of the wild-type protein. However, the GFP-RCP(I621E) fusion protein was completely cytosolic and did not localise to any vesicular structures. In some cases, with the I621E mutant some labelling of ruffles in the plasma membrane was observed. This localisation is likely to be mediated via the amino-terminal C2 domain [11]. To confirm the immunofluorescence results biochemically, HeLa cells expressing each of the GFP-fusion constructs were fractionated into a high-speed supernatant (cytosol) and high-speed pellet (membrane). Western blots of these fractions, normalised to cell volume, were then probed with an anti-GFP antibody (Fig. 2B). The GFP-RCP(WT), GFP-RCP(Y620F), and GFP-RCP(D622N) recombinant proteins were found in both the cytosol and membrane fractions. However, GFP-RCP(I621E) was found exclusively in the cytosolic fraction. These results verify our immunofluorescence data.

In summary, RCP(D622N) can bind Rab11 but not Rab4, whereas RCP(I621E) can bind neither Rab11 nor Rab4. Since RCP(D622N) can bind membranes and displays a vesicular pattern very similar to that of the wild-type protein, it can be concluded that Rab4 is not required for the correct intracellular localisation of RCP. Furthermore, the observation that RCP(I621E) is a cytosolic protein suggests that RCP localisation is mediated by Rab11. Thus, Rab11 is a likely candidate mediator of RCP recruitment onto the ERC membrane. This is consistent with our data indicating that there is very little RCP localised to the sorting endosome [4], the compartment where the majority of Rab4 resides, and fits with our model that RCP may serve as a recycling compartment target for Rab4-regulated transport vesicles originating from the SE.

3.3. Rab11 expression can alter RCP localisation

To further investigate if membrane-bound Rab11 recruits RCP to the ERC, we assessed the pattern of endogenous Rab11 or RCP in cells expressing various mutant constructs. Thus, GFP-RCP wild-type and mutant constructs were transfected into the A431 epidermal carcinoma cell line. Sixteen hours post-transfection, the cells were fixed and labelled for endogenous Rab11 (Fig. 3A). The Rab11 pattern did not differ significantly in cells expressing the different mutants, or from the pattern observed in non-transfected cells. The observation that Rab11 localisation was unaffected in cells expressing RCP(I621E), the Rab11-binding defective mutant is consistent with the hypothesis that Rab11 membrane binding is not dependent on RCP. In contrast, the expression of a dominant negative Rab11(S25N) mutant resulted in the redistribution of RCP to the cytoplasm (Fig. 3B). Rab11(S25N) is locked in the GDP-bound inactive conformation and is predominantly cytosolic [14]. Our yeast two-hybrid data suggest that RCP can bind to Rab11(S25N) indicating that, when expressed in cells, the mutant binds to RCP preventing it from interacting with endogenous Rab11 at the ERC. RCP displays a normal vesicular pattern in cells expressing wild-type Rab11 and constitutively active Rab11(Q70L). Rab4 or its mutants have no effect on endogenous RCP recycling compartment localisation (Fig. 3C).

3.4. The class I Rab11-FIPs compete for binding to Rab11

To investigate if the Rab11-FIPs compete for binding to Rab11, the localisation of endogenous RCP was examined in cells expressing GFP-fusions of the class I member Rip11, or the class II Rab11-FIP3 (Fig. 4). In cells expressing GFP-Rip11, the fusion protein has a normal vesicular pattern, whereas endogenous RCP displays a reduced membrane localisation when compared to non-transfected cells. Expression of GFP-Rab11 FIP3 results in the formation of an enlarged tubulo-vesicular structure to which RCP is found to colocalise with Rab11-FIP3. These results indicate that the class I Rab11-FIPs, but not the class II family members, compete for binding to Rab11. Overexpression of Rip11 results in the

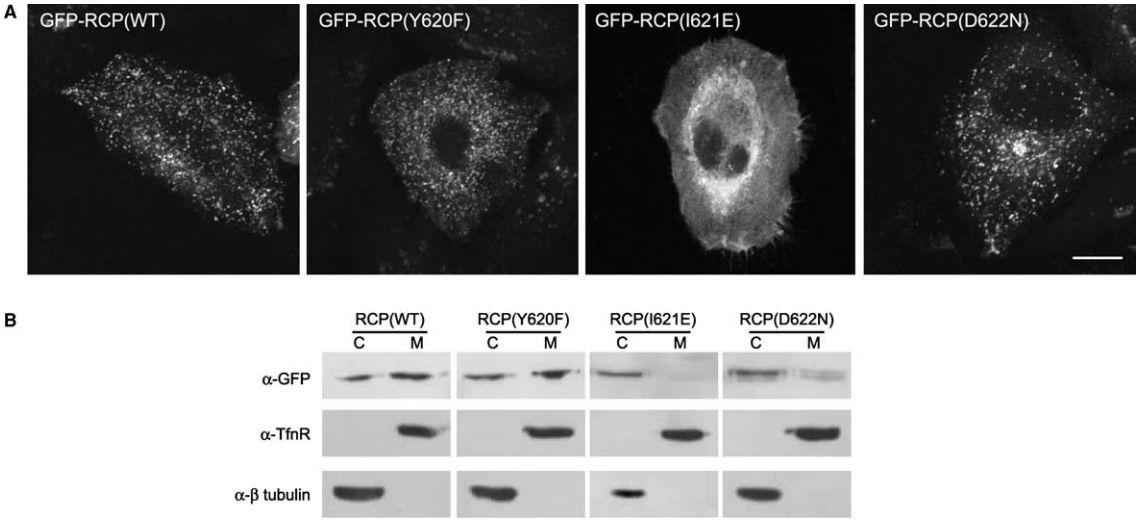

Fig. 2. The RCP/Rab11 interaction is required for RCP membrane association. (A) HeLa cells transfected with the indicated construct were fixed with paraformaldehyde and analysed by confocal microscopy. (B) HeLa cells expressing the indicated constructs were fractionated into a high-speed supernatant (C) and high-speed membrane pellet (M). Cytosol and membrane fractions, normalised to cell volume, were resolved by SDS–PAGE and transferred to nitrocellulose. Blots were probed with anti-GFP to detect the RCP fusion proteins. Anti-TfnR and anti-β tubulin were used as fractionation controls. Bar, 10 μm.

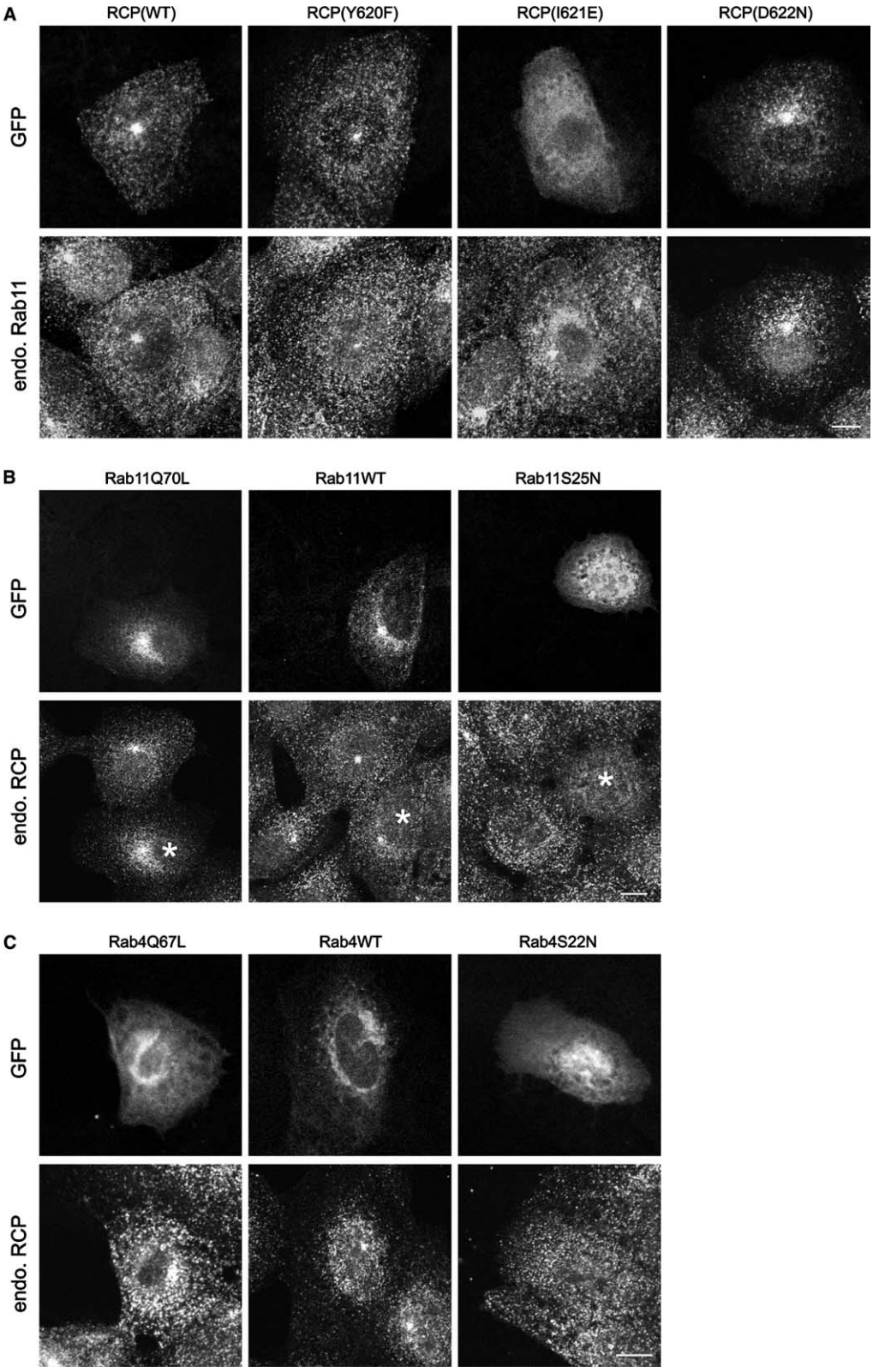

Fig. 3. Rab11 mediates RCP binding to the ERC. (A) A431 cells expressing the indicated GFP-fusion protein were fixed with paraformaldehyde, permeabilised with saponin, and labelled for endogenous Rab11. (B) The indicated Rab11 GFP-fusion constructs were transfected into A431 cells. 16 h post-transfection the cells were fixed, permeabilised, and labelled for endogenous RCP. (C) The indicated Rab4 GFP-fusion constructs were transfected into A431 cells. 16 h post-transfection the cells were fixed, permeabilised, and labelled for endogenous RCP. Transfected cells are indicated with an asterisk. Bar, 10 μm.

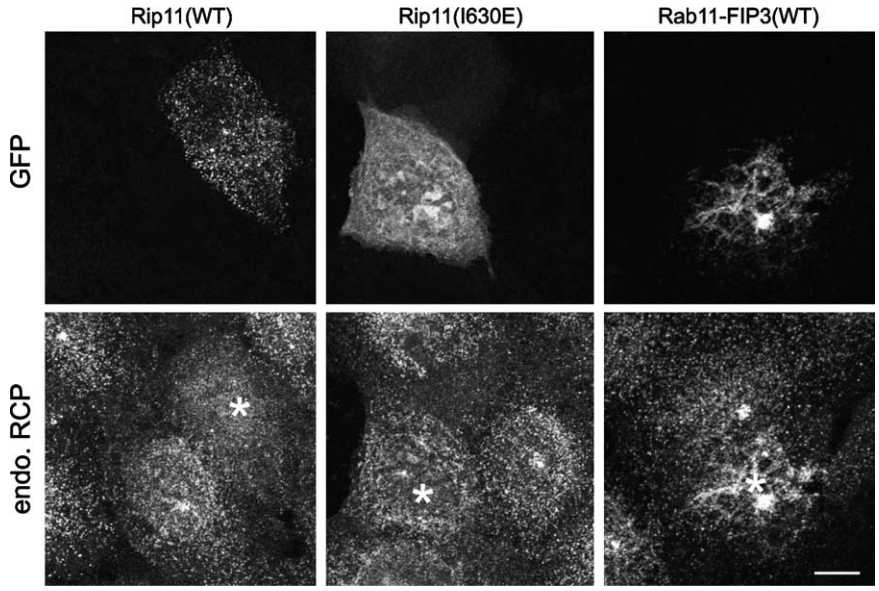

Fig. 4. Class I Rab11-FIP members compete in vivo for binding to Rab11. The indicated Rab11-FIP GFP-fusion constructs were transfected into A431 cells. 16 h post-transfection the cells were fixed, permeabilised, and labelled for endogenous RCP. Transfected cells are indicated with an asterisk. Bar, 10 μm.

sequestration of Rab11 from endogenous RCP, hence its increased cytoplasmic localisation. In contrast, expression of Rab11-FIP3 does not inhibit the membrane localisation of endogenous RCP, suggesting that the class II Rab11-FIPs employ another mechanism for their membrane localisation. This mechanism may involve the ARF GTPases to which they have been shown to bind [6,7], or may involve an as yet unidentified recruitment mechanism. To further confirm that the class I Rab11-FIPs compete for Rab11 binding, we made use of a Rip11 mutation, Rip11(I630E), which has been shown to abolish its Rab11 binding [15]. In cells expressing GFP-Rip11(I630E), the normal vesicular pattern of RCP is restored, demonstrating that the cytoplasmic localisation of RCP in GFP-Rip11 expressing cells is due to Rip11 sequestration of Rab11.

4. Discussion

We have used site-directed mutagenesis to assess the significance of the Rab4 and Rab11 binding properties of RCP. We investigated the importance of a conserved region at the carboxy-terminus of RCP that we believe mediates its Rab binding ability. By mutating individual amino acids in this region, we have generated an RCP mutant in which Rab GTPase binding is abolished and a mutant which can bind Rab11 but not Rab4. The mutant with aberrant Rab4, but normal Rab11 binding, displays a typical wild-type intracellular localisation when expressed as a GFP-fusion in mammalian cells. In contrast, the RCP(I621E) mutant which can bind neither Rab4 nor Rab11 is completely cytoplasmic. Fractionation studies of cells expressing each mutant yield similar results, in that the RCP(I621E) fusion protein partitions exclusively in the cytosolic fraction, whereas the wild-type protein and all mutants that maintain the ability to bind Rab11 are also detected in the membrane fraction.

RCP is localised to the ERC and the mutagenesis results suggest that Rab11, rather than Rab4, mediates this localisation. This hypothesis is further borne out by the observation that the expression of dominant-negative or dominant-active mutants of Rab4 does not perturb RCP intracellular localisation. However, expression of a dominant-negative mutant of Rab11, Rab11(S25N), results in the redistribution of endogenous RCP to the cytoplasm. Expression of the RCP mutants does not affect the localisation of Rab11. A perturbation of endogenous Rab11 localisation would be expected if RCP binding to the ERC preceded Rab11 binding. We also demonstrate that the class I Rab11-FIPs, but not the class II Rab11-FIPs, compete with each other for binding to Rab11. Overexpression of Rip11 results in the redistribution of RCP to the cytoplasm, consistent with a model in which the overexpressed protein out-competes RCP for binding to Rab11. In contrast, when Rab11-FIP3 is transiently overexpressed it forms a large tubulo-vesicular structure that is positive for RCP. Taken together, these data suggest that the class II Rab11-FIPs may have an alternative mechanism for binding vesicular membranes.

The data presented in this paper are consistent with a model in which Rab11 recruits cytosolic RCP to the ERC, where it can then act as a target for Rab4-regulated transport vesicles en route from the sorting endosome.

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