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Purification, crystallization and preliminary X-ray diffraction studies of Rab11 in complex with Rab11-FIP2

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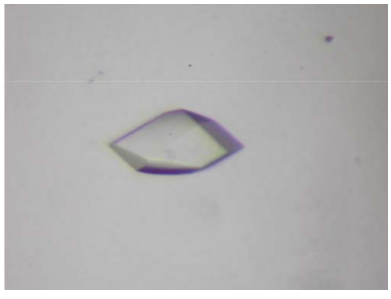
The small GTPase Rab11 regulates the recycling of endosomes back to the plasma membrane. In its active GTP-bound form, Rab11 binds a novel set of effectors termed the Rab11 family of interacting proteins (Rab11-FIPs) which contain a conserved C-terminal Rab-binding domain (RBD) of unknown structure. Here, a complex of Rab11 with the RBD of Rab11-FIP2 has been purified and crystallized in the trigonal space group $P3_121$, with unit-cell parameters $a = 64.99$, $b = 64.99$, $c = 112.59$ Å. Static light-scattering analyses of the molecular weight of the complex in solution are consistent with two copies of Rab11 and two copies of Rab11-FIP2 in the complex.

1. Introduction

The Ras superfamily of small GTPases regulate signalling pathways in living cells by oscillating between active GTP-bound forms and inactive GDP-bound states. The Rab family of proteins comprise the largest family in the Ras GTPase superfamily and play a critical role in maintaining the structure of intracellular compartments, vesicle trafficking and fusion (Bock *et al.*, 2001). All Rabs are anchored to the lipid bilayer *via* prenylation of C-terminal cysteine residues by geranylgeranyltransferase (GGTase) and in their active GTP-bound state Rabs localize to distinct subcellular compartments, where they carry out their specific function through the recruitment of effector proteins (Zerial & McBride, 2001).

The Rab11 subfamily comprises three isoforms: Rab11a, Rab11b and Rab25. Rab11a is a 218-residue (24.4 kDa) protein involved in the regulation of endocytic recycling pathways and has also been shown to localize to the trans-Golgi network (TGN) and regulate the traffic between endosomes and the TGN (Wilcke *et al.*, 2000). Recently, a novel set of effectors termed the Rab11 family of interacting proteins (Rab11-FIPs) have been identified that contain a highly conserved C-terminal Rab-binding domain (RBD) of unknown structure (Prekeris *et al.*, 2000; Hales *et al.*, 2001; Lindsay & McCaffrey, 2002). Rab11 effectors are classified into different classes based upon their sequence and domain organization. All Rab11-FIPs contain an RBD close to the C-terminal end and the family is subdivided depending on the presence of either C2 domains or EF-hand motifs at their N-termini. The class 1 Rab11-FIPs include Rip11 (Rab1-interacting protein), Rab11-FIP2 and RCP (Rab11-coupling protein) and are distinguished by having C2 phospholipid-binding domains at their N-termini (Lindsay & McCaffrey, 2004). Class 2 proteins include Rab11-FIP3/eferin and Rab11-FIP4 and these proteins contain EF-hand motifs for calcium sensing (Hales *et al.*, 2001; Wallace *et al.*, 2002). Rab11-FIP1 does not belong to either category and apart from the RBD lacks similarities to known proteins (Hales *et al.*, 2001; Prekeris, 2003). Previous biophysical studies of the RBD have revealed that it contains significant α-helical content and forms homodimers in solution (Junutula *et al.*, 2004). Modelling studies have predicted an amphipathic α-helical coiled coil that contains a Rab-binding hydrophobic patch along one face of the helix (Junutula *et al.*, 2004; Meyers & Prekeris, 2002; Lindsay *et al.*, 2002).

Rab11-FIP2 is a 512-residue protein that contains a C2 domain at the N-terminus (residues 1–129), a myosin Vb-binding region (129–



290) and an RBD at the C-terminus (477–496). Rab11-FIP2 protein has been found to be essential for the recycling of vesicles bearing the chemokine receptor CXCR2 back to the plasma membrane and the ternary complex Rab11–Rab11-FIP2–myosin Vb is proposed to link endosomes to the cytoskeleton and regulate delivery of vesicular cargo to the plasma membrane (Hales *et al.*, 2002; Lindsay & McCaffrey, 2002; Fan *et al.*, 2004). In order to understand the molecular basis for recognition by this novel Rab11 effector family, we have purified and crystallized a complex of Rab11 (residues 1–173, 19.4 kDa) with a C-terminal fragment of Rab11-FIP2 (residues 410–512, 11.9 kDa) containing the RBD. Although the conserved sequence within the Rab11-FIPs is restricted to a short 20-residue stretch, we chose a larger C-terminal segment of Rab11-FIP2 in order to encompass the entire globular region, thereby producing a soluble fragment for crystallization.

2. Materials and methods

2.1. Expression and purification

PTrcHisRab11-FIP2 and pTrcHisRab11aQ70L plasmids were used as template cDNA sources for the generation of the bacterial expression plasmids employed in this study. The construction of pTrcHisRab11-FIP2 has previously been described (Lindsay & McCaffrey, 2005). The cDNA encoding Rab11-FIP2 (amino-acid residues 410–512) was PCR-amplified from pTrcHisRab11-FIP2 and cloned into the vector pMAL-parallel 2 using the *Nco*I and *Eco*RI restriction sites. The expression vector is a modified pMAL-c2x plasmid with an rTEV protease-recognition site inserted between maltose-binding protein (MBP) and the protein of interest. Upstream and downstream primers were 5′-GCATACCATGGCAGCAAA-ATTCAGGGCTTCAAAT-3′ and 5′-ACCGGAATTCTTAACTGT-TAGAGAATTTGCCAGCTT-3′, respectively. Plasmid pTrcHis-Rab11aQ70L was generated by *Bam*HI/*Pst*I excision of pGEM1-Rab11Q70L (Wilcke *et al.*, 2000) and subcloning of the Rab11Q70L fragment into the *Bam*HI/*Pst*I sites of pTrcHisC.

Rab11-FIP2 (residues 410–512) was then amplified by PCR from pTrcHisRab11Q70L and cloned into the vector pMAL-parallel 2 using the *Nco*I and *Eco*RI restriction sites. Similarly, Rab11 (residues 1–173, Q70L mutant) was amplified by PCR with upstream and downstream primers 5′-AATGCCATGGGCACCCGCGACGAC-GAGTACGAC-3′ and 5′-ACCGGAATTCTTAGTATATCTCTGT-CAGAATTGTCT-3′, respectively. Thus, the construct of Rab11 used in our work dispensed with the last 45 amino acids (174–218) of the full-length protein. The PCR product was digested with *Nco*I and *Eco*RI and cloned into the corresponding sites in the pET-28b vector. The presence of the Q70L amino-acid substitution was confirmed by amino-acid sequencing. All ligations were carried out with the TaKaRa ligation kit.

The two resulting expression plasmids (pMAL-FIP2 and pET28b-Rab11) were co-transformed into BL21 (DE3) cells and cultured in EZMix 2×YT microbial medium (Sigma) supplemented with 100 µg ml^{-1} ampicillin and 30 µg ml^{-1} kanamycin at 310 K. At an OD_{600} of 0.6, protein expression was induced by addition of IPTG to a final concentration of 0.5 m*M*. Cells were grown for a further 3 h at 310 K, harvested by centrifugation and stored frozen at 253 K until use. Frozen pellets were resuspended in MBP extraction buffer (20 m*M* Tris–HCl, 200 m*M* NaCl, 5 m*M* $MgCl_2$ and 10 m*M* β-mercaptoethanol pH 7.8) and sonicated (2 × 1 min) on ice. Cell lysates were centrifuged at 20 000*g* to remove cell debris and the resulting supernatant was applied onto amylose resin (New England Biolabs). After extensive washing with MBP extraction buffer, bound protein was eluted with MBP elution buffer (extraction buffer supplemented with 10 m*M* maltose). Eluted protein was dialysed overnight against 10 m*M* Tris, 25 m*M* NaCl pH 8.0, having first added rTEV protease (10 µg per milligram of eluted protein). Cleaved protein was applied onto a MonoQ 5/50 GL column (GE Healthcare) equilibrated with dialyisis buffer and a gradient formed to 500 m*M* NaCl. The protein fractions corresponding to the Rab11–Rab11-FIP2 complex were pooled and further purified on a Superdex 200 16/60 column (GE Healthcare) equilibrated in column buffer (10 m*M* Tris–HCl, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT pH 8.0). The protein peak was prepared for crystallization on 10 kDa cutoff concentrators (Millipore) with a final concentration of 8.25 mg ml^{-1} as measured by Bradford dye assay.

The weight of the Rab11–Rab11-FIP2 complex was characterized by static light scattering using a miniDAWN instrument (Wyatt Technology Corp.). Following rTEV cleavage of the MBP effector in complex with Rab11, analytical amounts of the complex (500 µg in total) were run on a Superdex 200 10/300 GL column (GE Healthcare) coupled to the miniDAWN. The cleavage reaction liberated MBP from the mixture and the non-covalent Rab11–Rab11-FIP2 complex could be purified by Superdex 200 (GL 10/300) gel-filtration column chromatography.

2.2. Crystallization and X-ray data collection

Crystals of the Rab11–Rab11-FIP2 complex were grown by the hanging-drop vapour-diffusion method using 0.3 *M* ammonium

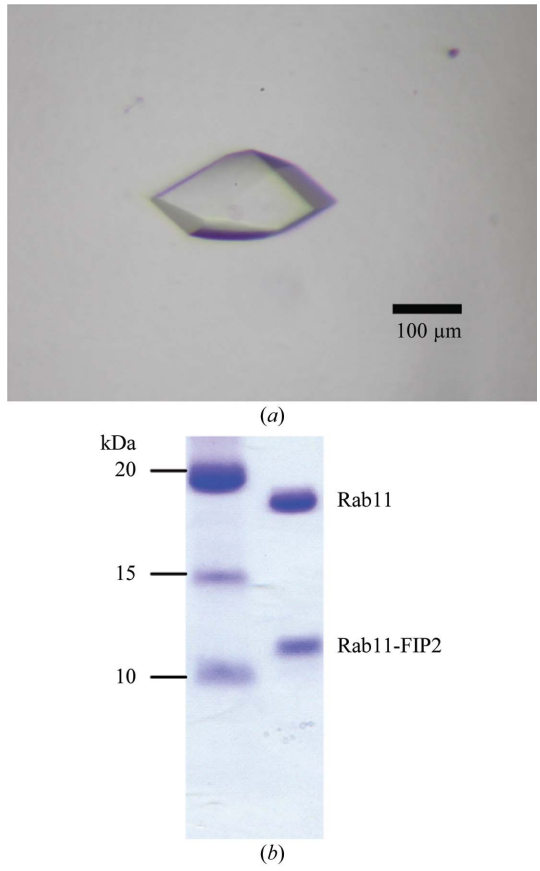


Figure 1
A typical native crystal of the Rab11–Rab11-FIP2 complex (*a*). Crystals were rigorously washed and analyzed by reducing SDS–PAGE (*b*) to confirm the presence of both proteins. The weight of the Rab11 construct (1–173) was 19.4 kDa, while the weight of Rab11-FIP2 (410–512) was 11.9 kDa.

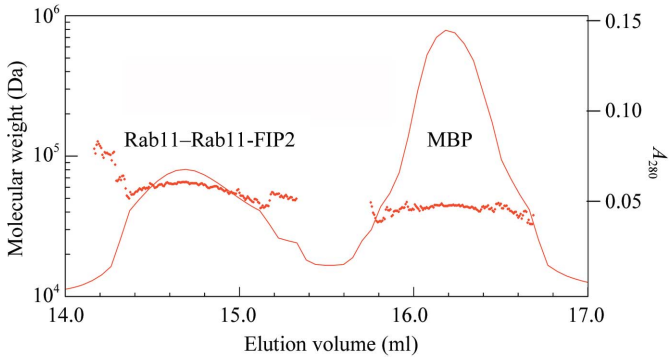


Figure 2
The molecular weight of the Rab11-FIP2 complex was determined by static light scattering (miniDAWN, Wyatt Technology Corp.) coupled to an AKTAbasic FPLC system. Affinity-purified complexes of Rab11 and Rab11-FIP2 (fused to the C-terminus of MBP) were subjected to cleavage by catalytic amounts of rTEV protease (see §2). The expected weight of MBP is 42 500 Da and the complex of Rab11–Rab11-FIP2 elutes at an earlier volume, with a molecular weight of 61 500 Da (±4%). The measured weight of the complex corresponds to the predicted weight (62.6 kDa) of two molecules of Rab11 (19.4 kDa) and two molecules of Rab11-FIP2 (11.9 kDa). Dotted red lines are weight measurements (left vertical scale); the solid thin line is UV absorbance (right vertical scale).

dihydrogen phosphate pH 4.8 as precipitant at 293 K (Fig. 1). 1 µl protein solution (above) was mixed with 1 µl precipitant and sealed over a 500 µl volume of precipitant. Typically, crystals appeared within 24 h and reached maximum dimensions within a week. Crystals were soaked in cryoprotectant containing 25%(*v*/*v*) glycerol, 0.3 *M* ammonium dihydrogen phosphate pH 4.8. Crystals were soaked for 2 min at room temperature and flash-cooled in liquid nitrogen. Data were collected on beamline BM30 at the European Synchrotron Radiation Facility (ESRF) from single (native) crystals of the complex and processed using *HKL*-2000 (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1.

3. Results and discussion

A stable complex of Rab11 with its effector Rab11-FIP2 was purified and crystallized using a co-expression strategy in *E. coli* cells (Fig. 1). Multi-angle light-scattering analyses of the complex in solution were consistent with a heterotetramer of two Rab11 molecules (19.4 kDa each) and two Rab11-FIP2 molecules (11.9 kDa each). The observed molecular weight was 61.5 kDa (±4%), which agrees well with the value of 62.6 kDa expected for the heterotetramer (Fig. 2). Crystals of the native protein diffract to 2.8 Å and the space group is trigonal $P3_121$, with unit-cell parameters a = 64.99, b = 64.99, c = 112.59 Å. Molecular replacement using the GTP form of Rab11 (PDB code 1oiw) resulted in a clear solution for Rab11 (not shown) and partial α-helical electron density for the effector Rab11-FIP2 (38% of the unknown weight in the crystals). We have also generated selenomethionine derivatives of the complex (crystals not shown) to permit structure determination by anomalous scattering methods, as a precaution in the event that the molecular-replacement solution fails to lead to refinement of the structure.

Table 1
Data-collection statistics.

Wavelength (Å)	0.977771
Resolution range (Å)	50–2.8
Measured reflections	61331
Unique reflections	7180
Completeness (%)	99.8
Mean $I/\sigma(I)$	10.3
$R_{\rm merge}$ (%)	6.8

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