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The activation cycle of Rab GTPase Ypt32 reveals structural determinants of effector recruitment and GDI binding

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ABSTRACT

Rab GTPases localize to distinct sub-cellular compartments and regulate vesicle trafficking in eukaryotic cells. Yeast Rabs Ypt31/32 and Sec4 have 68% homology and bind to common interactors, yet play distinct roles in the transport of exocytic vesicles. The structures of Ypt31/32 have not previously been reported in the uncomplexed state. We describe the crystal structures of GTP and GDP forms of Ypt32 to understand the molecular basis for Rab function. The structure of Ypt32(GTP) reveals that the switch II conformation is distinct from Sec4(GTP) in spite of a highly conserved amino acid sequence. Also, Ypt32(GDP) reveals a remarkable change in conformation of the switch II helix induced by binding to GDI, which has not been described previously.
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1. Introduction

Rab small GTPases are molecular switches that regulate vesicle trafficking in eukaryotic cells via interactions with effector proteins. Human Rabs comprise about 70 members, while *Saccharomyces cerevisiae* contains only 11 Rabs [1–5]. Rabs perform a variety of functions including vesicle formation, motility, tethering, fusion, and the various steps are mediated via recruitment of effector proteins [6]. Rab GTPases contain a flexible C-terminal tail that is post-translationally modified at Cys residues to enable attachment to lipid bilayers [3,7].

Rabs oscillate between an active (GTP) and inactive (GDP) state, regulated by GDP dissociation inhibitor (GDI), GTPase activating factors (GAPs), and GDP/GTP exchange factors [8]. Local conformational changes in switch I and switch II, adjacent to the γ-phosphate, distinguish these states. Active Rabs reside in distinct subcellular compartments and mediate their biological effects via recruitment of specific effector proteins. Some understanding of the molecular basis for effector recognition has emerged from the crystal structures of Rabs with the Rab-binding domains (RBDs) of effectors [3,9,10]. Generally, the GTP-sensitive switch regions,

switch I and II, as well as an invariant tryptophan residue in the interswitch region (between switch I and II) are important determinants of binding to α-helical motifs of RBDs. The exceptions are Early Endosomal Autoantigen 1 [EEA1 [10]] and the Lowe Syndrome protein OCRL1 [9], which have non-helical RBDs.

S. cerevisiae proteins Ypt31 and Ypt32 are homologs with 87% sequence identities (95% homology) in their globular Ras fold. They regulate vesicle exit from late Golgi compartments [11,12]. In the current model, Ypt31/32 together with phosphatidylinositol 4-phosphate recruit Sec2, which is also an exchange factor for Sec4. The nucleotide exchange action of Sec2 converts Sec4(GDP) to Sec4(GTP), and Sec2 also binds to Sec15, concomitantly releasing Ypt31/32 from the complex, thus facilitating the latter steps of vesicle delivery [13,14]. Both Sec4 and Ypt31/32 also bind to the globular tail domain of the actin-based class V myosin, Myo2 [15–17]. Sec15 is an effector of Sec4 and a component of the multi-protein exocyst, a docking complex that captures vesicles from the Golgi and promotes their fusion [18]. The equivalent interaction in mammalian cells is the Rab11/Sec15, which has been observed in photoreceptor cells of *Drosophila melanogaster* [19]. Thus, Ypt31/32 and Sec4 regulate consecutive steps in a complex cascade that involves Sec2, myosin V and the exocyst.

Here, we present the crystal structures of Ypt32 in the GTP and GDP bound states. The structure of active Ypt32 is compared with Sec4, which is required for a later step in the trafficking of

secretory vesicles [20,21]. Comparisons of their structures reveal significant differences in the conformation of switch II that are influenced by the underlying network of interactions with Rab sub-family specific (RabSF) regions. The structure of Ypt32(GDP) together with the previously determined structure of the Ypt31/GDI complex facilitates a complete description of the pathway leading from an active (membrane-bound) Ypt31/32 conformation to the GDI-bound (cytosolic) structure. Strikingly, the structures reveal that GDI induces a remodeling of the switch II helix prior to membrane extraction.

2. Materials and methods

2.1. Protein expression and purification

The Ypt32 construct of the globular domain in a constitutively active form (Q72L, residues 7-188) was generated as a fusion with maltose binding protein (MBP). The cDNA was cloned into the vector pMBP-parallel 1, which contains an rTEV protease cleavage sequence, at the NcoI (5′end) and SalI (3′end) restriction sites. Expression of the protein, cleavage of MBP, and subsequent purification was performed as described in previous work [22]. However, a second amylose affinity step was not performed, as significant amounts of free Ypt32 attached non-specifically to the resin. As an alternative, brief dialysis in low salt buffer (10 mM Tris–Cl, 10 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT, pH 8) was followed by ion-exchange chromatography using a MonoQ column (GE Healthcare) using a linear gradient to 500 mM NaCl. Ypt32 was further purified by size exclusion chromatography (Superdex 200 16/60 column, GE Healthcare) in gel filtration buffer (10 mM Tris–Cl, 150 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT, pH 7.5).

2.2. Crystallization and structure determination

Ypt32 (Q72L) at 10 mg/mL was incubated with 5 mM GTP (SIGMA) for about an hour on ice. Despite incubation of GTP with the mutant Ypt32(Q72L) at the beginning of crystallization trials, the crystals appeared with GDP, therefore it is likely that Ypt32 had hydrolyzed the γ-phosphate during the several weeks that were required for crystal growth. The crystallization condition was 21-22% PEG4000, 0.1 M Tris (pH 8.5) and 0.2 M $MgCl_2$. Single crystals of Ypt32(GDP) with maximum dimensions of $0.2 \times 0.1 \times 0.05$ mm were grown by micro-seeding. The crystallization procedure for active Ypt32 was identical, except that guanosine-5′-(βγ-imino)triphosphate (GppNHp), a non-hydrolyzable analog of GTP, was incubated with the protein at the beginning of trials. Crystals were optimized in 0.2M $MgCl_2$, 0.1M HEPES, 15%w/v PEG6000, pH 7. The hanging drop method in Linbro plates (291 K) was used for all crystallization experiments. Prior to data collection, crystals were soaked in cryoprotectant (25% xylitol) for approximately 30 seconds and flash cooled at 100 K in the cryostream. Data sets were collected at beamline BM14 at the European Synchrotron Research Facility (ESRF, Grenoble), and processed using the XDS package [23].

The crystal structure of Ypt32 in complex with GDP was determined by molecular replacement using MOLREP [24]. A solution with two molecules in the asymmetric unit was found using Ypt31 (PDB code 3cpj, 88% identical) as a search model. The initial electron density map for Ypt32(GppNHp) was obtained by molecular replacement using the refined Ypt32(GDP) structure. Refinement of the models was performed by Refmac [25] using the maximum likelihood method, individual *B*-factor refinement and TLS refinement, alternating with cycles of manual inspection using Coot [26]. At no point were non-crystallographic symmetry (NCS) restraints applied during refinement. The data collection and refinement statistics are summarized in Table 1.

Table 1
X-ray data collection and structure refinement.

	YPT32-GDP	YPT32-GppNHp
Wavelength (Å)	0.9794	0.9784
Resolution (Å)	40–1.7 (1.8–1.7)	40.0–2.0 (2.15–2.0)
Space group	C2	P212121
Asymmetric unit	2 molecules	1 molecule
Cell parameters		
a (Å)	130.0	47.09
b (Å)	45.2	49.89
c (Å)	73.4	90.66
β (°)	110.5	
R_{sym} (%)	5.2 (40.1)	6.9 (44.6)
$I/\sigma I$	19.5 (3.89)	23.57 (4.24)
Completeness (%)	99.4 (98.9)	99.6 (100)
Multiplicity	3.89 (3.9)	7.1 (7.2)
R_{work}/R_{free} (%)	16.7/20.3	19.4/24.0
R.M.S.D.		
Bonds (Å)	0.011	0.010
Angles (°)	1.33	2.01
Ramachandran plot		
Most favoured (%)	92.2	93.4
Disallowed	0	0

Values in parentheses are for highest resolution shell.

2.3. Structural alignments by least-squares methods

Pairwise alignments were performed using the secondary structure matching (SSM) algorithm implemented in COOT [26]. The switch regions, which undergo conformational transitions, were excluded from the calculations. The active conformations of Rab GTPases will be referred to as Rab(GTP), although in general, the uncomplexed proteins have been co-crystallized with GppNHp. The refined structure of Ypt32(GDP) was aligned with the previously determined structure of the complex Ypt31/GDI (PDB code 3cpj [27]). The two molecules in the asymmetric unit of Ypt32(GDP) are identical in conformation, with a root-mean-square (rms) deviation of 0.55 Å for 155 common C_α atoms, therefore molecule A was used for structural analyses. Alignment of Ypt32(GDP) with Ypt31 (as part of the GDI complex) resulted in an rms deviation of 0.87 Å for 149 common C_α atoms. Similarly, the structure of Ypt32(GTP) was aligned with Sec4(GTP). The two
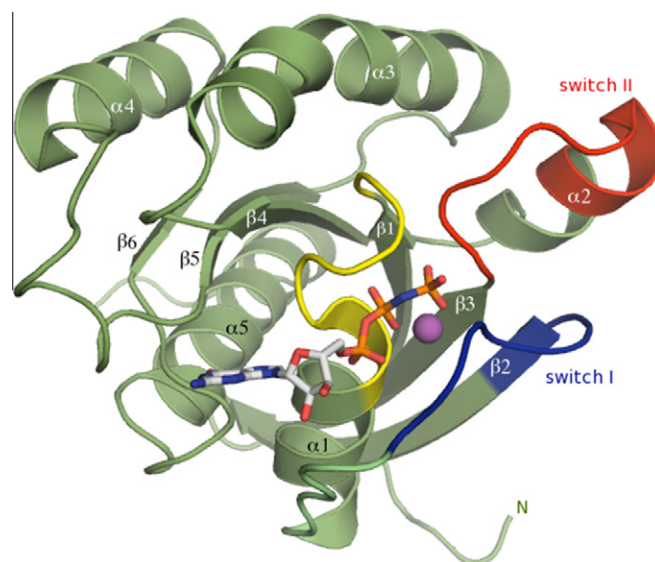

Fig. 1. Structure of Ypt32 small GTPase in the GTP-bound conformation. The nucleotide is shown as a stick model, the P-loop is yellow, switch I is blue, and switch II is red. The Mg^{2+} ion is a purple sphere.