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The structure and conformational switching of Rap1B

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ABSTRACT

Rap1B is a small GTPase involved in the regulation of numerous cellular processes including synaptic plasticity, one of the bases of memory. Like other members of the Ras family, the active GTP-bound form of Rap1B can bind to a large number of effector proteins and so transmit signals to downstream components of the signaling pathways. The structure of Rap1B bound only to a nucleotide has yet to be solved, but might help reveal an inactive conformation that can be stabilized by a small molecule drug. Unlike other Ras family proteins such as H-Ras and Rap2A, Rap1B crystallizes in an intermediate state when bound to a non-hydrolyzable GTP analog. Comparison with H-Ras and Rap2A reveals conservative mutations relative to Rap1B, distant from the bound nucleotide, which control how readily the protein may adopt the fully activated form in the presence of GTP. High resolution crystallographic structures of mutant proteins show how these changes may influence the hydrogen bonding patterns of the key switch residues.

1. Introduction

Ras-related protein 1 (Rap1) exists as two 95% identical isoforms A and B that play a number of crucial roles in regulating cell adhesion, cell junction formation, cell secretion, and cell polarity [1], but with quantifiable differences in localization and function [2]. Rap1 is a member of the small GTPase family that includes proteins such as H-Ras, K-Ras and M-Ras [3]. These proteins act as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound form [4,5]. They are involved in many cellular signal transduction pathways, controlling cell growth, migration and proliferation. Guanine nucleotide exchange factors (GEFs) catalyze the dissociation of GDP and binding of GTP, and GTPase activating proteins (GAPs) accelerate the GTP hydrolysis reaction, so that signaling is up- and down-regulated by these factors [6].

Previous analyses of H-Ras and M-Ras revealed that the majority of the structure is relatively invariant on replacing GDP with GTP, but two loop regions undergo substantial conformational changes [6–9]. These loop regions are called switch I and switch II, which are localized in-between a β strand and an α helix (either β2 or β3 and α1), close to the nucleotide binding site. The presence of a γ-phosphate group on the bound nucleotide and attendant magnesium ion is detected by the switch I and switch II regions forming bonds with them, creating a binding surface recognized by effector proteins. However, the GTP-bound GTPases may adopt two states, called state-1 and state-2, respectively. State-2 is defined by coordination of the magnesium ion by a conserved threonine side-chain in the switch I region (Thr-35 in Rap1B), and represents the activated form.

Different Ras family members show very different conformational preferences in the GppNHp bound form, with the state-2/state-1 ratio ranging from 0.072 for M-Ras to 16 for Rap2A [10,11], despite sharing an identical switch I sequence (YDPTIED). M-Ras shows the greatest tendency to adopt state-1 with GppNHp bound, leaving a spacious hydrophobic pocket near the nucleotide [12]. Small molecule Ras inhibitors have been developed by targeting this pocket, and there are hopes this may prove a generally useful strategy for inhibiting Ras signaling [13,14]. Since Rap1B is associated with several types of cancer, there is a strong interest in developing specific drugs that can down-regulate Rap1B signaling. Although the crystal structures are known of several complexes formed by Rap1B and effector proteins, no models are published of Rap1B in the presence of nucleotides alone. We have therefore

Abbreviations: GppNHp, guanosine 5′[β,γ-imido]triphosphate.

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determined high resolution crystal structures of rat Rap1B (wild-type and mutants) bound only to GDP or GppNHp, to clarify the mechanism of activation and assist drug design.

2. Materials and methods

2.1. Cloning

The rat Rap1B gene (Uniprot Q62636, residues 1–167) was amplified from a cDNA library of adult rat brain (GenoStaff) by PCR and cloned into the pET21b expression vector (Novagen), between the *Nde*I and *Not*I restriction sites. The gene was amplified by using the following pairs of primers:

5′-CGGGAATTCCATATGCGTGAATATAAGCTAGTCGTTC-3′ (*Nde*I site underlined)
5′-TTTTCCTTTTGCGGCCGCTTATCTGTTAATTTGCCGCACTAGG-3′ (*Not*I site underlined)

2.2. Expression and purification

The pET21b/Rap1B (residues 1–167) plasmid was transformed into *Escherichia coli* BL21 (DE3) cells including a pGro7 chaperone vector (Takara). Cells were grown at 37 °C in LB medium (containing 50 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 2 mg/ml L-Arabinose) up to OD_{600} of 0.8. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and shaking overnight at 22 °C. Rap1B was purified using Q-Fast flow (GE Healthcare) and HiLoad 16/600 Superdex 200 columns (GE Healthcare).

2.3. Nucleotide exchange

Rap1B-GDP was prepared by incubating purified Rap1B in the presence of rat SynGAP C2-GAP domain (Uniprot ID Q9QUH6, residues 237–714) and 1 mM GDP (Nacalai tesque) overnight at 4 °C in 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. Phosphate and SynGAP C2-GAP domain were removed by gel filtration. Rap1B-GppNHp (wild-type and mutants) were prepared as described [15].

2.4. Crystallization of Rap1B-GppNHp and Rap1B-GDP

Rap1B-GppNHp (10 mg/ml) and Rap1B-GDP (10 mg/ml) were dialyzed against 20 mM HEPES pH 7.4, 50 mM NaCl, and 5 mM $MgCl_2$. The wild-type and L9V mutant Rap1B-GppNHp crystals were obtained by hanging drop vapor diffusion method using a reservoir solution containing 0.1 M HEPES pH 7.5, 12% (w/v) PEG3350, and 5 mM $CdCl_2$ at 20 °C. Crystals of wild-type Rap1B-GDP were grown by same method using the reservoir solution containing 0.1 M Li_2SO_4, 0.1 M Tris–HCl pH 8.5, 30% (w/v) PEG4000, and 1 mM $CdCl_2$. The Rap1B(T65A)-GppNHp crystals were obtained by hanging drop vapor diffusion using a reservoir solution containing 0.2 M Li_2SO_4, 0.1 M bis-Tris pH 5.5, and 25% (w/v) PEG3350 at 20 °C.

2.5. Data collection and structure determination

All X-ray diffraction data were collected at beam-line 17A of the Photon Factory (Tsukuba, Japan) using an ADSC Quantum 270 CCD detector or DECTRIS PILATUS-3S 6M detector. Diffraction data were processed with HKL2000 [16] or iMOSFLM [17]. General data handling was carried out with the CCP4 suite [18]. The crystal structure of Rap1B-GppNHp was determined by molecular replacement with the program MOLREP [19] using a Ras-GppNHp structure (PDB entry 5P21). Further models were solved by bootstrapping from this Rap1B model. The models were manipulated with COOT [20]. Refinement was carried out with PHENIX [21]. The final structures were validated with MolProbity [22]. Data collection and refinement statistics are shown in Table 1.

2.6. Accession numbers

The models have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) with accession codes 3X1W (Rap1B-GDP), 3X1X (Rap1B-GppNHp), 3X1Y (Rap1B(L9V)-GppNHp) and 3X1Z (Rap1B(T65A)-GppNHp).

3. Results

Rat Rap1B has an N-terminal catalytic domain of 167 residues, identical to the human protein, except that Cys 139 (human) is replaced by serine (rat); this surface residue is over 20 Å from the bound nucleotide and has no appreciable effect on the structure. The catalytic domain of rat Rap1B was chosen for analysis because it contains fewer cysteine residues that may accidentally form disulfide bonds. Within the N-terminal domain, human Rap1A and Rap1B differ at only three positions, Cys 48→Ala, Glu 107→Asp, and Cys 139→Asn. A sequence alignment of Rap1B and H-Ras is given in Fig. 1A, and the structure of the catalytic domain is shown in Fig. 1B.

3.1. Comparison of the native GDP and GppNHp complexes

Very high resolution X-ray diffraction data were collected for both Rap1B complexes, and a summary of the refinement statistics is provided in Table 1. The catalytic domain of the Ras family consists of a central β-sheet (β1-β6) with five α-helices (α1-α5) and ten loops, (L1-L10) [23]. Comparing Rap1B with other Ras family catalytic domains, the rmsd values of the Cα atoms are in the region of 1.2 Å. L1 is known as the phosphate binding loop (P loop), and consists of residues 10-17 in Rap1. The P loop interacts with the bound nucleotide but its conformation is the same in both the GDP and GTP bound forms. Ser 17 coordinates the Mg^{2+} ion. The GDP and GppNHp complexes were both crystallized in space-group $P2_12_12_1$ with very similar cell parameters and a single molecule in the asymmetric unit. Switch I, residues 32-38, is a loop found between α1 and β2. Switch II, residues 60-70, forms half of L5 and half of α2. Representative electron density maps covering the ligands for the GDP and GppNHp complexes are shown in Fig. 2A and B respectively.

In the GppNHp complex, the switch I and II regions form a mutual crystallographic contact, with the main-chain nitrogen atoms of Thr 35 and Ile 36 of one molecule making hydrogen bonds to the side-chain oxygen atom of *Asn 74* of a neighbor. (Residue names of neighboring molecules are shown in italics.) The side-chain of Thr 35 points away from the nucleotide, and lies close to the side-chain of *Lys 5*. Tyr 32 also contacts *Tyr 71*. The electron density map is very clear in this region and shows both switch I and switch II to be well ordered, but separated from the GppNHp by a number of water molecules hydrating the phosphate groups (Fig. 2B). The crystal contact formed by the switch regions is significantly smaller in the GDP bound form; Thr 35 no longer makes any contact with the neighboring molecule, and *Asn 74* makes a hydrogen bond to the carbonyl oxygen of Pro 34. In both structures the Mg^{2+} ion is coordinated by a water molecule in place of Thr 35 (Fig. 2A and B). Although the crystal contacts may influence the conformations of switch I and switch II in the structures described here, differences between the two crystal forms show that the phosphate groups of the nucleotide ligand still exercise