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Crystal structure of Rab6A′(Q72L) mutant reveals unexpected GDP/Mg^{2+} binding with opened GTP-binding domain

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ABSTRACT

The Ras small G protein-superfamily is a family of GTP hydrolases whose activity is regulated by GTP/GDP binding states. Rab6A, a member of the Ras superfamily, is involved in the regulation of vesicle trafficking, which is critical for endocytosis, biosynthesis, secretion, cell differentiation and cell growth. Rab6A exists in two isoforms, termed RabA and Rab6A′. Substitution of Gln72 to Leu72 (Q72L) at Rab6 family blocks GTP hydrolysis activity and this mutation usually causes the Rab6 protein to be constitutively in an active form. Here, we report the crystal structure of the human Rab6A′(Q72L) mutant form at 1.9 Å resolution. Unexpectedly, we found that Rab6A′(Q72L) possesses GDP/Mg^{2+} in the GTP binding pockets, which is formed by a flexible switch I and switch II. Large conformational changes were also detected in the switch I and switch II regions. Our structure revealed that the non-hydrolysable, constitutively active form of Rab6A′ can accommodate GDP/Mg^{2+} in the open conformation.

1. Introduction

The Ras-superfamily of small G proteins is a family of GTP hydrolases that includes more than 100 members in eukaryotes from yeast to human [1]. The activity of this family is regulated by the GTP binding state: GDP-bound inactive and GTP-bound active forms [2]. Most of this family of proteins are ubiquitously present inside cells and are key components to various cellular processes, including cytoskeletal organization, mitogenesis, vesicle trafficking, and nuclear transport [3].

The Rab GTPase family is one of the Ras-superfamily of small G proteins and plays an important role in vesicle trafficking, which is essential for endocytosis, biosynthesis, secretion, cell differentiation and growth [4]. The active form of the Rab proteins, which contains GTP, can recruit specific binding partners, such as sorting adaptors, tethering factors, kinases, phosphatases and motor proteins, and affects vesicle formation, transport, and tethering [5]. Many Rab GTPase family proteins share the same interacting partners and perform unique roles in specific locations [6–13]. Because a functional loss of the Rab pathways has been implicated in a variety of diseases, the Rab GTPase family has been extensively studied [14].

Rab6 family is a representative Rab GTPase family. They are particularly involved in membrane trafficking in the Golgi by residing at the membrane of the Golgi apparatus and the trans-Golgi network (TGN) [15,16]. Three different types of Rab6 has been identified in mammals (Rab6A–C) and Rab6A has two isoforms, termed Rab6A and Rab6A′ [17–19]. Rab6A′ is produced by alternative splicing of the duplicated exon within Rab6A [17]. The sequence of human Rab6A′ differs from that of Rab6A in only three amino acid residues (Val62 → Ile, Thr87 → Ala, and Val88 → Ala) [17]. Both Rab6A and Rab6A′ were similarly detected in the Golgi apparatus, are ubiquitously expressed in various tissues in human and show similar GTP-binding activity [17,20]. The active forms of these proteins inhibit the secretory pathway in vesicle transport [17]. Although they appear similar, several studies have reported that the two isoforms are functionally different. For example, it is well known that Rab6A interacts with Rabkinesin-6 but Rab6A′ does not [17,21]. Although conflicting results have been reported regarding the roles of Rab6A′, it is believed that this protein plays unique roles in several cellular processes [4,17,22–25].

Mg^{2+} is a critical cofactor for the activity of the Ras-superfamily of small G proteins and is coordinated with GTP or GDP in the GTP binding pocket of the protein [26]. GTP/Mg^{2+} binds more strongly

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to the Rab GTPase family than GDP/Mg^{2+} [27]. The structure of Ras-superfamily of small G proteins, including the Rab GTPase family, was shown to be changed in the absence or presence of GTP, GDP and Mg^{2+} and these conformational changes can affect the activity of the Ras-superfamily of small G proteins [27–29].

Although several structures of Rab6A have been reported [30], no structures of Rab6A′(Q72L) are available. In the present study, we report the crystal structure of the mutant form of human Rab6A′(Q72L), which has been shown to be in a GTP lock form, at 1.9 Å resolution to better understand the functional uniqueness of Rab6A′ and determine the molecular mechanism by which GTP and GDP control activity. Although the Q72L mutant has been shown to be in the GTP-lock form, we found that Rab6A′(Q72L) possesses GDP/Mg^{2+} in the GTP binding pocket, which is formed by a flexible switch I and switch II. Large conformational changes were also detected in switch I and switch II regions. Our structure revealed that non-hydrolysable, constitutively active, GTP-lock form of Rab6A′ can accommodate GDP/Mg^{2+} in the open conformation.

2. Materials and methods

2.1. Protein expression and purification

The methods used for expression and purification methods in this study have been described elsewhere in detail. Briefly, human Rab6A′(Q72L), which corresponds to amino acid residues 5–178, was expressed in BL21 (RIPL) *Escherichia coli* competent cells under overnight induction at 293 K. Rab6A′(Q72L) contained a N-terminal His-tag and was purified by nickel affinity chromatography followed by gel-filtration chromatography over a Superdex 200 gel-filtration column (GE Healthcare) that had been pre-equilibrated with a solution of 20 mM Tris pH 8.0 and 150 mM NaCl. The target protein was concentrated to 10–12 mg ml^{-1}.

2.2. Crystallization and data collection

The crystallization conditions were initially screened at 293 K by the hanging-drop vapor-diffusion method using various screening kits. Initial crystals were grown on plates by equilibrating a mixture containing 1 μl protein solution (4.53 mg ml^{-1} protein in 20 mM Tris pH 8.0, 150 mM NaCl) and 1 μl of reservoir solution containing 18% PEG 8000, 0.2 M calcium acetate and 0.1 M sodium cacodylate pH 6.5 against 0.4 ml of a reservoir solution. Crystals appeared within 3 days and grew to maximum dimensions of 0.2 × 0.2 × 0.1 mm in the presence of 20% PEG 8000, 0.3 M calcium acetate and 0.1 M sodium cacodylate pH 6.7. A 1.9 Å native diffraction data set was collected from a single crystal at the beamline BL-4A at the Pohang Accelerator Laboratory (PAL), South Korea. The data sets were indexed and processed using HKL2000 [31].

2.3. Structure determination and analysis

The structure was determined by molecular replacement phasing method using *Phaser*. The previously solved structure of GTP-bound Rab6A (PDB code: 2GIL) [30] was used as a search model. Model building and refinement were performed by COOT [32] and Refmac5 [33], respectively. Water molecules were added using the ARP/wARP function in Refmac5. The final model contained residues 13–174 for the A chain and residues 12–175 for the B chain. One GDP/Mg^{2+} was placed at each chain. The geometry was inspected using PROCHECK and was found to be reasonable. A total of 93.98% of the amino acids were located in the most favorable region and 6.02% were in the allowed regions of the Ramachandran plot. The data collection and refinement statistics are summarized in Table 1. All the molecular figures were generated using the program Pymol (The pymol Molecular Graphics System (2002), DeLano Scientific, San Carlos, USA).

Table 1
Crystallographic statistics.

Data collection	Native
Space group	$P22_12_1$
Cell dimensions	
a, *b*, *c*	36.84 Å, 96.78 Å, 109.99 Å
Resolution	50–1.9 Å
R_{sym}[a]	8.9% (48.7%)
Mean $I/\sigma(I)$[a]	29.1 (3.6)
Completeness[a]	99.8% (99.9%)
Redundancy[a]	6.7 (6.7)
Refinement	
Resolution	50–1.9 Å
No. of reflections used	30283
R_{work}/R_{free}	19.6%/25.0%
No. of atoms	
Protein	2484
Water and other small molecule	305
Average *B*-factors	25.0 $Å^2$
R.m.s deviations	
Bond lengths	0.028 Å
Bond angles	3.175°
Ramachandran plot	
Most favored regions	93.98%
Additional allowed regions	6.02%

[a] Highest resolution shell is shown in parenthesis.

2.4. Protein Data Bank accession code

The coordinate and structure factor have been deposited in the Protein Data Bank (PDB) with Accession code 4DKX.

3. Results and discussion

3.1. Structure of Rab6A′(Q72L) complex with GDP/Mg^{2+}

The exact mechanism by which GTP and GDP mediate the activity of the Rab GTPase family is still not clear. To obtain a better understanding of this process, we determined the crystal structure of Rab6A′(Q72L), which is a well-known GTP lock and constitutively active mutant form, at a resolution of 1.9 Å using the coordinates of the previously solved isoform of Rab6A (PDB ID: 2GIL) as the search model for molecular replacement. The asymmetric unit contained two molecules, Chains A and B. The final model contained residues 13–174 for Chain A and residues 12–175 for Chain B (Fig. 1) and one GDP/Mg^{2+} was placed at each chain. The structure was refined to an R_{work} = 19.6% and R_{free} = 25.0%. The overall structure of Rab6A′(Q72L) showed the typical Ras-like GTPase fold, which was comprised of five α-helices surrounding six β-strands [34] (Fig. 1).

3.2. Unexpectedly located GDP/Mg^{2+}

The Rab6 family has the lowest GTPase activity among the Rab family and Q72L mutant of Rab6A was previously shown to lack GTP hydrolysis activity [30,35]. Since we expected that they used endogenous GTP and Mg^{2+}, we did not supply any GTP or Mg^{2+} during determination of the structure and expected GTP/Mg^{2+} or only GTP in the crystal structure of Rab6A′(Q72L) mutant. However, we found that Rab6A′(Q72L) possesses GDP/Mg^{2+} in the GTP binding pockets, even though it does not possess GTP hydrolysis activity

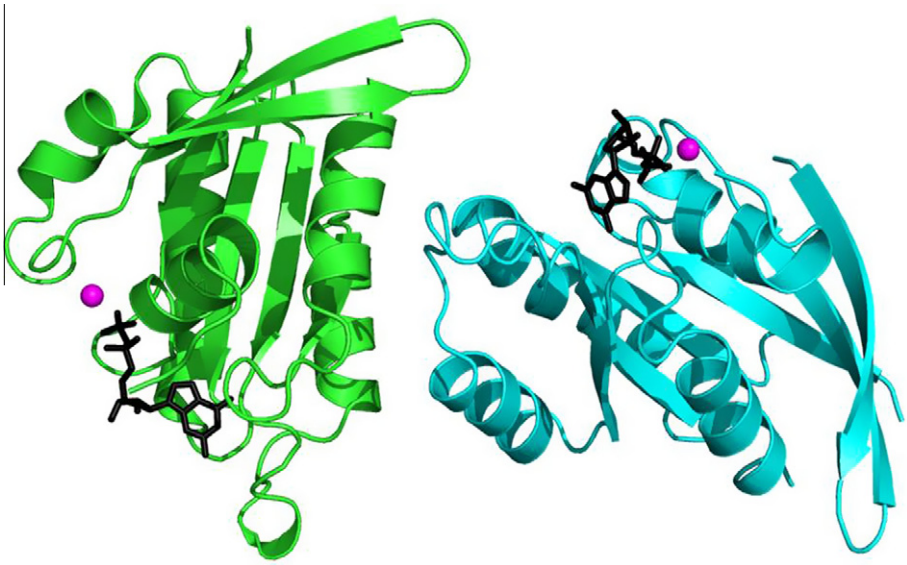

Fig. 1. Crystal structure of Rab6A′(Q72L)-GDP/Mg^{2+}. Ribbon diagram of Rab6A′(Q72L)-GDP/Mg^{2+}. Chain A (Green color) and Chain B (Cyan color) are shown separately. GDP is shown by the black stick. Ball colored in magenta indicates Mg^{2+}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

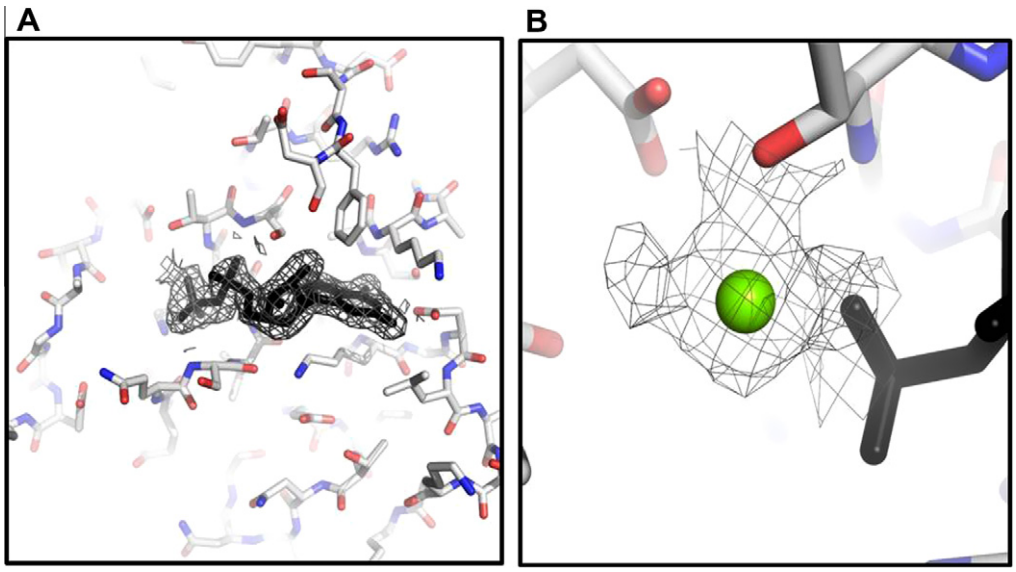

Fig. 2. Environment of GDP and Mg^{2+} binding sites. (A) An omit density map contoured at the 1-σ level around GDP. GDP is shown by the black colored stick and the density map is shown in the gray color. (B) An omit density map contoured at the 1-σ level around Mg^{2+}. Green ball indicates Mg^{2+}. GDP is shown by the black colored stick and the density map is shown in the gray color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2A and B). Mg^{2+} in the Rab6A′(Q72L) mutant was more closely located to the site where γ-phosphate of GTP was placed in the structure of Rab6A–GTP/Mg^{2+} (Fig. 3). The structure of the GDP/Mg^{2+} bound form of Rab6A′(Q72L) (hereafter referred to as the Rab6A′(Q72L)-GDP/Mg^{2+}) might have formed due to several possible reasons. First of all, endogenous GDP in the cell can bind to the protein. Because the concentration of GTP in normal cells is much higher than that of GDP, this might be an unusual case. Secondly, GTP bound to the protein may have been hydrolyzed by an unknown factor during purification and crystallization of the protein. Finally, it is possible that GTP can be hydrolyzed without the active site Q72. Rab6A′(Q72L) might have a weak activity compared with that of wildtype.

3.3. Structural comparison with Rab6A–GTP/Mg^{2+} complex

The human Rab6A–GTP/Mg^{2+} structure was solved in a previous study [30]. The α-carbon atoms of Rab6A′(Q72L)-GDP/Mg^{2+} was superimposed with those of Rab6A–GTP/Mg^{2+} and the root-mean-square deviation (r.m.s.d.) was 1.5. Pair-wise structural alignments between Rab6A′(Q72L)-GDP/Mg^{2+} and Rab6A–GTP/Mg^{2+} showed that most parts of the structure were similar except switches I and II, which are critical for controlling the activity of small G protein [28]. The most significantly different region was the orientation of loop in switch II (Fig. 3). One of the interesting features of switch II was the presence of Gln72, which is critical for the hydrolytic activity of Rab6A and Rab6A′. Our structure

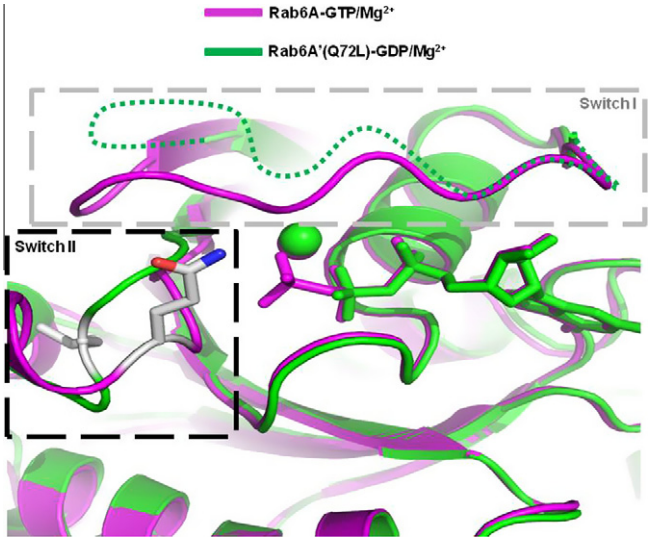

Fig. 3. Structural comparison with Rab6A–GTP/Mg^{2+} at the GTP binding domain. The structures of Rab6A–GTP/Mg^{2+} (PDB ID: 2GIL) and Rab6A′(Q72L)-GDP/Mg^{2+} (PDB ID: 4KDX) are shown in the magenta and green color, respectively. Gray dot-box and black dot-box indicate switches I and II, respectively. Q72L mutant is shown at the switch II region using a stick representation. Unclear switch I of Rab6A′(Q72L)-GDP/Mg^{2+}, which is shown with a green dot (model was built with unclear density). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

showed that the side chain of Leu72 was located on the opposite side of Gln72, which caused the loop in switch II to be located in the opposite position (Fig. 3). This indicates that the loss of hydrolytic activity of the Q72L mutant form of Rab6A was likely due to the location of Gln72 (Leu72), which is a critical residue for GTP hydrolysis at small G proteins.

In our structure, it was hard to build a model of the switch I region because of the poor quality of the map due to its high flexibility. Based on the disconnected and poor electron density, a potential model was built and is shown in the Fig. 3 (gray colored dot-box). The structure of switch I might be more close to the open form that was detected in the structure of Mg^{2+}-free and GDP bound form of RhoA (PDB ID: 1DPF), which is also a Ras superfamily of small G protein [28].

A structural homology search using the DALI server [36] showed that the Rab6A′(Q72L) mutant has more structural similarity with several different classes of the Ras superfamily of small G protein. The top seven matches, with *Z*-scores from 27.5 to 25.6, were Rab6 from *Plasmodium falciparum* [37], Rab6B [38], Rab6A [30], Rab5C [39], SEC4 [40], Rab21 [39], and Rab8A [41] (Table 2). The structural similarity based on the high *Z*-scores indicates that our structure was more similar with Rab6 from *P. falciparum* and Rab6B among the same Rab6 family and Rab21 among the same Rab family.

Table 2
Structural similarity search using DALI [36].

Proteins and Accession Nos.	*Z*-score	R.m.s.d (Å)	Identity (%)	References
Rab6 (1D5C)	27.5	0.8	69	[37]
Rab6B (2FFQ)	27.0	1.3	91	[38]
Rab6A (2GIL)	26.9	1.5	97	[30]
Rab5C (1Z0D)	26.8	1.0	46	[39]
SEC4 (1G16)	25.9	1.2	35	[40]
Rab21 (1Z08)	25.5	1.3	39	[39]
Rab8A (3QBT)	25.4	1.3	44	[41]

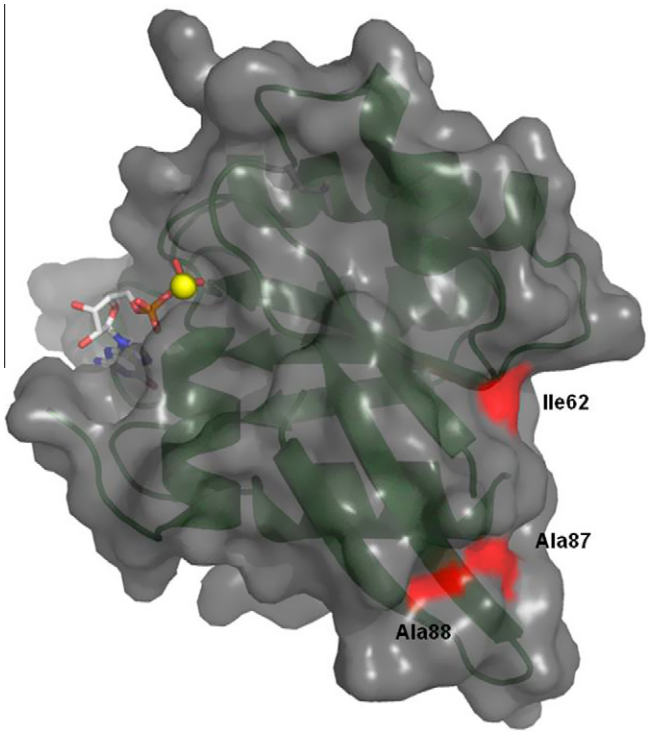

Fig. 4. Mapping of different amino acids between Rab6A and Rab6A′. Molecular surface is shown in the gray color and different amino acids are marked in the red color. GDP and Mg^{2+} are shown as a stick and sphere, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Mapping of different amino acids between Rab6A and Rab6A′

The sequence of human Rab6A′ differs from that of Rab6A in only three amino acid residues (Val62 → Ile, Thr87 → Ala, and Val88 → Ala) [17]. It has been shown that the two isoforms are functionally different even though they have almost the same amino acids sequence. This functional difference may be caused by their interaction partner. For example, it is well known that Rab6A interacts with Rabkinesin-6 but Rab6A′ does not [17,21].

Mapping of the three amino acid residues (Val62 → Ile, Thr87 → Ala, and Val88 → Ala) that were different between the two isoform onto the Rab6A′(Q72L)-GDP/Mg^{2+} surface showed that all three residues were located on the side opposite of the GTP/Mg^{2+} binding site (Fig. 4). Because Rab6A and not Rab6A′ is known to interact with Rabkinesin-6, it is possible that this mapped region of the Rab6A′(Q72L)-GDP/Mg^{2+} surface is critical for its interaction with Rabkinesin-6.

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