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Crystal structure of GTPase-activating domain from human MgcRacGAP

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ABSTRACT

Cytokinesis in animal cells relies on a centralspindlin complex consisting of male germ cell RacGap (MgcRacGAP) and mitotic kinesin-like protein 1 (MKLP1). Rho GTPases act as molecular switches to regulate the actin cytoskeleton for cytokinesis, of which Rac1 is regulated by MgcRacGAP. In this study, we determined the crystal structure of the GTPase-activating protein (GAP) domain of MgcRacGAP at a resolution of 1.9 Å. The conformation of Arg385, which is a key residue for GAP activity, was found to be different from that of previously reported GAP proteins, and MgcRacGAP (residues 348–546) was found to exist as a monomer in solution, according to Stokes radii. We also measured the GAP activity of MgcRacGAP mutants for Rac1.

1. Introduction

The final step of cell division is cytokinesis, wherein 2 daughter cells are produced from a single cell. The assembly of the central spindle during anaphase in animal cells directs contractile ring constriction and cleavage furrow formation through the Rho family of GTPases and its regulators [1]. The Rho family of GTPases and its effectors mediate spindle signaling during anaphase [2]. GTPase-activating proteins (GAPs) inactivate GTPases by stimulating intrinsic GTPase activity, while guanine nucleotide-exchange factors (GEFs) activate GTPases by promoting the exchange of GDP for GTP [3]. GTPase activity is maintained by a balance of local GTPase inactivation by GAPs and local GTPase activation by GEFs [3].

Rho GTPases act as molecular switches to regulate the actin cytoskeleton; 3 common members of the Rho family are RhoA, Rac1, and Cdc42 [3]. Centralspindlin, which consists of mitotic kinesin-like protein 1 (MKLP1), which is a Rho GTPase activating protein (Rho-GAP), and male germ cell RacGap (MgcRacGAP), plays an essential role in the assembly of the central spindle in cytokinesis [4,5]. The N-terminus of MgcRacGAP interacts with MKLP1 by forming a coiled coil, and the C-terminus of MgcRacGAP contains GAP activity for RhoA-family GTPases [6]. MgcRacGAP localizes precisely to the central spindle and functions as an important regulator to recruit other cytokinetic regulators [7]. MgcRacGAP stimulates the intrinsic GTPase activities of Rac1 and Cdc42, but not that of RhoA [8]. However, MgcRacGAP is functionally changed into a RhoA GAP by Ser387 phosphorylation mediated by Aurora B kinase [9]. Moreover, the GAP activity of MgcRacGAP is down-regulated by PRC1 binding, and it contributes to Cdc42 activation and mitotic spindle formation [10]. CYK-4 (the MgcRacGAP homolog from *Caenorhabditis elegans*) stimulates GTP hydrolysis of Cdc42 and the Rac1 homolog (Rac), but it has little effect on RhoA [11]. In parallel with the activation of RhoA by Ect-2 (RhoGEF), inactivation of Rac by CYK-4 functions to drive cytokinesis [12].

The crystal structures of several small GTPases, GAPs, and their complexes have been previously reported. In the crystal structure of p50rhoGAP, Arg85 and Asn194 are important in binding to RhoA (or Cdc42 GTPase) and enhancing GTPase activity [13–15]. The crystal structure of human Cdc42 (GMPPNP, the non-hydrolysable GTP analog) in complex with the GAP domain of p50rhoGAP showed that Cdc42 makes contact with a shallow pocket on the GAP domain through its switch I and II regions [14]. A conformational change (rotation of 20° between the Rho and GAP domains) was observed in the crystal structure of the RhoA-p50rhoGAP complex (GDP and AlF_4, the transition-state analog) when the complex structure was compared with the Cdc42 (GMPPNP bound)-p50rhoGAP complex [15]. The crystal structures of the Cdc42 (GDP and AlF4)-p50rhoGAP and Cdc42 (GDP and AlF_4)-p50rhoGAP (R305A mutant) complexes showed that an arginine residue on the A–A′ loop is important to stabilize the transition state of the GTPase reaction [16]. Position 95 of GTPase (Glu95 of Cdc42, Glu97 of RhoA, and Ala95 of Rac1) is an important determinant of binding specificity between GTPases and GrafGAP on the basis of structural and mutational studies of the GAP domain of the *Gallus gallus*

Abbreviations: MgcRacGAP, male germ cell RacGap; MKLP1, mitotic kinesin-like protein 1; GAPs, GTPase-activating proteins; GEFs, guanine nucleotide exchange factors; GST, glutathione S-transferase; TEV, tobacco etch virus; IPTG, isopropyl-β-D-1-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

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GTPase regulator associated with focal adhesion kinase (Graf) [17]. The structure-based model of the Rac-specific GAP domain of β2-chimaerin suggested that Phe315 and Glu317 in the A′ helix interact with Ala88 and Ala95 of Rac1, which are key determinants of contact with Rac1 [18]. In spite of the number of structural studies conducted so far, the structure of human MgcRacGAP remains uncharacterized. To provide further insight into the structure of MgcRacGAP, we report the crystal structure of the GAP domain of human MgcRacGAP. The GAP activity assay and mutational studies on MgcRacGAP provide molecular insight into how MgcRacGAP activates Rac1 to regulate cytokinesis.

2. Materials and methods

2.1. Protein preparation

Human cDNA clones encoding MgcRacGAP (clone ID: hMU005205) and Rac1 (clone ID: KU000510) were purchased from the 21C Frontier Human Gene Bank (http://genbank.kribb.re.kr). The GAP domain of human MgcRacGAP (amino acids 348–546, MgcRacGAP$_{348–546}$) was cloned into the parallel vector pGST2 [19] and expressed in *Escherichia coli* strain BL21(DE3) cells as a fusion protein with an N-terminal GST (glutathione S-transferase) tag followed by a TEV protease cleavage site. The MgcRacGAP$_{348–546}$ was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and expressed at 25 °C for 16 h. Cells were lysed by passing them through a French press in a lysis buffer containing 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5% (w/v) glycerol. Protein was purified using an affinity column on Glutathione Sepharose 4B (GE Healthcare) and cleaved with TEV protease. The MgcRacGAP$_{348–546}$ was further purified by size exclusion chromatography (HiLoad 16/600 Superdex 200 prep grade, GE Healthcare) and concentrated to 10 mg/ml by ultrafiltration. The R385A and N495A mutations of MgcRacGAP$_{348–546}$ were introduced by site-directed mutagenesis by using a Quikchange™ kit (Stratagene). The MgcRacGAP$_{348–546}$ mutants (R385A and N495A) were expressed and purified in the same manner as the wild-type MgcRacGAP$_{348–546}$. Full-length human Rac1 (amino acids 1–192) was cloned and expressed in the same way as human MgcRacGAP$_{348–546}$. Cells were lysed by passing them through a French press in 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 5% (w/v) glycerol. Protein was purified using a Glutathione Sepharose 4B affinity column and by size exclusion chromatography (HiLoad 16/600 Superdex 200) without removing the GST-tag.

2.2. Crystallization, structure determination, and refinement

Crystals of human MgcRacGAP$_{348–546}$ were grown at 298 K using the sitting drop method by mixing 1 μl of a 10 mg/ml solution of MgcRacGAP$_{348–546}$ in 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, and 1 mM dithiothreitol with 1 μl of reservoir solution consisting of 100 mM sodium cacodylate buffer (pH 5.7) and 13% polyethylene glycol 6000. The crystals were transferred to a solution containing the reservoir solution and 25% glycerol for cryoprotection. Data was collected at 100 K in 1° oscillations at the 7A beamline of the Pohang Light Source (Pohang Accelerator Laboratory).

Crystals of MgcRacGAP$_{348–546}$ diffracted to a resolution of 1.9 Å, and the diffraction data were processed and scaled using the *HKL2000* software package [20]. The crystal belonged to space group $P2_12_12_1$, with unit cell parameters of $a = 41.47$ Å, $b = 63.16$ Å, and $c = 74.08$ Å. The structure was solved using the molecular replacement method using human p50rhoGAP model (PDB ID: 1RGP) as a probe. A cross-rotational search followed by a translational search was performed using the *PHASER* program [21]. Subsequent manual model building was performed using the *COOT* program [22] and restrained refinement was carried out using the *REFMAC5* program [23]. Several rounds of model building, simulated annealing, positional refinement, and individual *B*-factor refinement were performed using the *COOT* and *REFMAC5* programs. Table 1 lists the refinement statistics. The atomic coordinates and structure factors were deposited in the Protein Data Bank (accession codes 3W6R).

2.3. Analytical gel filtration

Purified MgcRacGAP$_{348–546}$ was subjected to analytical gel filtration chromatography on a Superdex 200 (10/300 GL) column with a running buffer (20 mM Tris–HCl pH 8.0 and 200 mM NaCl) at a constant flow rate of 0.5 ml/min. The standard curve was obtained using molecular weight markers (Sigma). The Stokes radii of β-amylase, alcohol dehydrogenase, carbonic anhydrase, and cytochrome C were calculated from the crystal structures of each protein (PDB codes: 1FA2, 2HCY, 1V9E, and 1HRC, respectively) by using the *HYDROPRO* program [24].

2.4. GAP activity assay

Purified GST-Rac1 and MgcRacGAP$_{348–546}$ (or its mutants) were mixed to obtain a concentration of 10 μM each in 20 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl, 10 μM $MgCl_2$, and 1 mM GTP (guanosine 5-triphosphate, Sigma) and incubated for 30 min at 22 °C. As a control, the other reaction components were run without introducing MgcRacGAP$_{348–546}$ into the reaction tube. GTP hydrolysis was monitored by measuring the production of inorganic phosphate at 660 nm using an acidic ammonium molybdate solution (ammonium molybdate tetrahydrate, Sigma) with malachite green oxalate (Sigma) [25]. Standard curves produced using known amounts of phosphate (KH_2PO_4, Sigma) were linear and highly consistent.

Table 1
Statistics for data collection and refinement.

Data set	MgcRacGAP348-546
A. Data collection statistics	
X-ray source	BL-7A (Pohang Light Source)
X-ray wavelength (Å)	0.97928
Space group	P212121
a, *b*, *c* (Å)	41.47, 63.16, 74.08
Resolution range (Å)	50–1.9
Total/unique reflections	93,141/15,787
Completeness (%)	99.2 (98.3)[a]
Average $I/\sigma(I)$	51.6 (5.2)[a]
R_{merge}[b] (%)	8.5 (48.5)[a]
B. Model refinement statistics	
Resolution range (Å)	25.3–1.9
R_{work}/R_{free}[c] (%)	22.1/25.9
Number/average B-factor (Å^2)	
Protein nonhydrogen atoms	1584/22.4
Water oxygen atoms	133/26.6
R.m.s. deviations from ideal	
Bond lengths (Å)	0.005
Bond angles (°)	0.94
Protein-geometry analysis	
Ramachandran favored (%)	96.46
Ramachandran allowed (%)	3.54
Ramachandran outliers (%)	0

[a] Values in parentheses refer to the highest resolution shell (1.93–1.90 Å).

[b] $R_{merge} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)_i$, where $I(hkl)$ is the intensity of reflection *hkl*, $\sum_{hkl}$ is the sum over all reflections, and $\sum_i$ is the sum over *i* measurements of reflection *hkl*.

[c] $R = \sum_{hkl}||F_{obs}| - |F_{calc}||/\sum_{hkl}|F_{obs}|$, where R_{free} was calculated for a randomly chosen 5% of reflections, which were not used for structure refinement and R_{work} was calculated for the remaining.