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Expression, purification, crystallization and preliminary crystallographic analysis of human Rad GTPase

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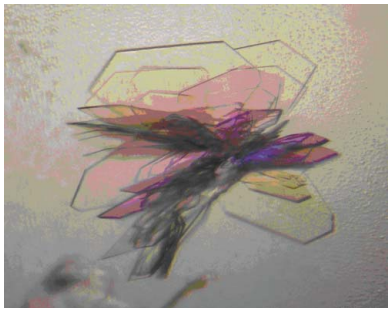

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Human Rad is a new member of the Ras GTPase superfamily and is overexpressed in human skeletal muscle of individuals with type II diabetes. The GTPase core domain was overexpressed in *Escherichia coli* and purified for crystallization. Crystals were obtained at 293 K by vapour diffusion using a crystallization robot. The crystals were found to belong to space group $P2_1$, with unit-cell parameters a = 52.2, b = 58.6, c = 53.4 Å, β = 97.9°, and contained two Rad molecules in the crystallographic asymmetric unit. A diffraction data set was collected to a resolution of 1.8 Å using synchrotron radiation at SPring-8.

1. Introduction

Small GTPases regulate a wide variety of cellular processes, including intracellular signalling, vesicular transport, cytoskeletal rearrangement and nuclear transport (Herrmann, 2003). These enzymes possess two conformational states in the form of a binary switch that cycles between the active GTP-binding and the inactive GDP-binding forms. The active form recognizes target proteins or effectors and triggers a response until the switch is turned off by GTP hydrolysis.

Rad is a new member of the Ras GTPase superfamily and is overexpressed in human skeletal muscle of individuals with type II diabetes (Reynet & Kahn, 1993). Within the Ras superfamily of small GTPases, Rad has been included in a novel subfamily referred to as RGK, together with Gem, Kir, Rem and Rem2 (Olson, 2002). The human Rad gene was localized to chromosome 16q22 (Doria *et al.*, 1995) and encodes a protein that possesses 60% amino-acid sequence identity to Gem (Maguire *et al.*, 1994) and Kir (Cohen *et al.*, 1994). The GTPase core domain of Rad exhibits ∼35% identity with those of other Ras-superfamily members and contains five conserved amino-acid motifs G1–G5 that are highly related in all members of the superfamily.

Rad possesses a distinct structural feature among the Ras GTPase superfamily that might affect GTP binding or GTPase activity. Interestingly, Rad contains nonconserved residues within regions G1, G2 and G3 of the GTPase core domain. These regions are known to be involved in GTP binding and GTP hydrolysis (Zhu *et al.*, 1995). Rad has NH_2- and COOH-terminal extensions consisting of 88 and 31 amino acids, respectively (Moyers *et al.*, 1997). Although Rad possesses 100% identity to Gem/Kir over the 11 amino acids at the COOH terminus (Moyers *et al.*, 1997), it lacks the typical CAA*X* prenylation motifs needed for post-translational modifications that have been described for other Ras GTPase proteins (Takai *et al.*, 2001).

In humans, Rad is most highly expressed in heart, lung and skeletal muscle (Moyers *et al.*, 1996). Rad exhibits interactions with skeletal muscle β-tropomyosin and cytoskeleton in a guanine nucleotide-dependent manner (Zhu *et al.*, 1996). Rad has been shown to inhibit insulin-stimulated glucose uptake in myocyte and adipocyte cell lines (Moyers *et al.*, 1996). Rad GTPases possess unique characteristics in terms of binding calmodulin and calmodulin-dependent protein kinase II, suggesting a role such as Rad-like GTPase activation of serine/threonine kinase cascades (Moyers *et al.*, 1997). Recently, Gem and Rad have been shown to bind Rho kinases and subsequently inhibit their protein kinase activity (Ward *et al.*, 2002). In this paper, we report the expression, purification, crystallization and preliminary

X-ray analysis of the GTPase core domain of Rad. This is the first crystallization of an RGK-family GTPase.

2. Expression and purification

The gene of the GTPase core domain of human Rad encoding residues 87–258 was cloned into the glutathione-*S*-transferase (GST) fusion modified vector pPRO-EX (Invitrogen). The plasmid was transformed into and expressed in *Escherichia coli* strain BL21 Star (Invitrogen). *E. coli* cells were grown in LB media supplemented with 100 µg ml^{-1} ampicillin at 310 K to an OD_{660} of 0.5. Expression of Rad was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to 0.5 m*M*. Cells were cultured for 3 h following induction at 310 K and then harvested by centrifugation at 6000 rev min^{-1} for 15 min. Cells were washed with 0.9% NaCl, pelleted by centrifugation at 7000 rev min^{-1} for 30 min and then resuspended in buffer *A* (300 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* DTT, 1% Triton X-100, 50 m*M* Tris–HCl pH 8.0) containing protease inhibitor. Cells were disrupted by sonication in an ice bath and the resultant cell suspension was clarified by ultracentrifugation at 37 000 rev min^{-1} for 30 min.

Purification of the protein was carried out by affinity chromatography using Glutathione Sepharose 4B (GSH-4B; Amersham Bioscience). The lysate supernatant was loaded onto a GSH-4B column equilibrated with buffer *A*. The resin was washed with 25 column volumes of buffer *B* (buffer *A* without Triton X-100). GST-fusion Rad bound to the resin was digested on the column with 0.24 mg ml^{-1} His-TEV-protease for 3 h at 293 K. The column was eluted with six volumes of buffer *C* (150 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* DTT, 50 m*M* Tris–HCl pH 8.0) and the effluent was passed through an Ni–NTA column (Qiagen) to remove the His-TEV-protease. The GST-free Rad was then concentrated by centrifugration using an Amicon Ultra-15 10 000 MWCO (Amicon). The buffer was then changed to buffer *D* (50 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* DTT, 50 m*M* Tris–HCl pH 8.0) using HiPrep Desalting 26/10 column (Pharmacia). SDS–PAGE (12.5%) analysis of the protein showed one major band corresponding to 18 kDa. The sample was then concentrated to 22 mg ml^{-1} using an Amicon Ultra-4 (Amicon) with 100 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* DTT, 10 m*M* Tris–HCl pH 8.0.

3. Crystallization

Crystallization screening was carried out by the sitting-drop method using Nextal Biotechnologies screening kits. The Rad protein complex solution was mixed in a 1:1 ratio with the reservoir solution and drops were set up with 200 nl sitting-drop volume and 100 µl reservoir solution using a Hydra II crystallization robot (Matrix Technologies Corporation). Initial crystallization conditions at 293 K using Sparse Matrix 3 (Nextal Biotechnologies) containing 100 m*M* MOPS pH 7.0, 20% PEG 3350, 100 m*M* $MgCl_2$ produced a small stack of needle-shaped crystals. Optimization was carried out using the hanging-drop method by changing the pH, the PEG molecular weight and concentration and the magnesium concentration and by additionally using Additive Screen and Detergent Screen (Hampton Research, USA). Optimal crystal growth was achieved at 293 K in a 4 µl hanging drop containing 100 m*M* MOPS pH 7.0, 25% PEG 3350, 25 m*M* $MgCl_2$, 10 m*M* DTT as a precipitant in a 1:1 volume ratio with 11 mg ml^{-1} protein solution and 500 µl 100 m*M* MOPS pH 7.0, 21% PEG 3350, 25 m*M* $MgCl_2$, 10 m*M* DTT as reservoir solution (Fig. 1).

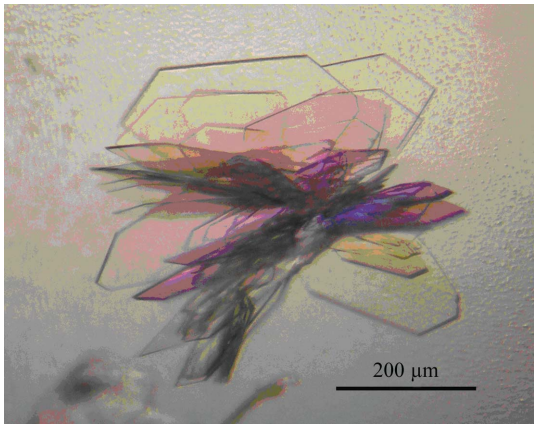

Figure 1
Crystal of human Rad in 100 m*M* MOPS pH 7.0, 25% PEG 3350, 25 m*M* $MgCl_2$ and 10 m*M* DTT.

Table 1
X-ray diffraction data of human Rad.

Statistics for the outer resolution shell are given in parentheses.

Beamline	SPring-8/BL41XU
Detector	ADSC Quantum 315 CCD
Wavelength (Å)	1.00
Temperature (K)	100
Oscillation range (°)	180 (1.5° × 120 images)
Exposure time (s)	30
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 52.2, b = 58.6, c = 53.4, β = 97.9
Resolution (Å)	50–1.80 (1.86–1.80)
Total reflections	97986
Unique reflections	28220
Completeness (%)	94.6 (72.2)
Mosaicity	0.3–0.5
$\langle I/\sigma(I)\rangle$	12.4 (2.2)
R_{merge}† (%)	6.6 (44.4)

† $R_{merge} = \sum|I_i - \langle I_i\rangle|/\sum\langle I_i\rangle$, where I_i is the observed intensity and $\langle I_i\rangle$ is the average intensity over symmetry-equivalent measurements.

4. X-ray data collection

Crystals were transferred into cryoprotectant solution containing 20%(*v*/*v*) glycerol in crystallization solution, mounted on a nylon loop and flash-frozen in liquid nitrogen. An X-ray diffraction data set for the Rad crystal was collected at SPring-8, Japan on beamline BL41XU using an ADSC Quantum 315 CCD detector system and the *HKL*2000 program suite (Otwinowski & Minor, 1997).

5. Results and discussion

Fig. 1 shows crystals of the human Rad GTPase core domain. The crystallographic data and intensity data-processing statistics are summarized in Table 1. The crystals were found to belong to space group $P2_1$, with unit-cell parameters a = 52.2, b = 58.6, c = 53.4 Å, β = 97.9°. A Matthews coefficient (Matthews, 1968) of 2.1 Å^3 Da^{-1} was calculated assuming the presence of two Rad molecules in the asymmetric unit, which corresponds to 42% solvent content by volume. Calculation of the self-rotation map did not yield a peak in the κ = 180° section, which suggests that the dimer is not associated with a local twofold symmetry or is associated with a non-crystallographic twofold axis that is parallel to the crystallographic 2_1 axis. Efforts are currently being directed towards solving the structure of Rad GTPase using the molecular-replacement method.

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