Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary structural characterization of human Rap1GAP

Oliver Daumke, Alfred Wittinghofer* and Michael Weyand

Max-Planck-Institute für Molekulare Physiologie, Abteilung Strukturbiologie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany

Correspondence e-mail: alfred.wittinghofer@mpi-dortmund.mpg.de

Received 20 January 2004
Accepted 29 January 2004

Human Rap1GAP, the GTPase-activating protein (GAP) for the small GTPase Rap1, was recombinantly expressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. Crystals were obtained using PEG 3350 as a precipitating agent and belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 170.7, b = 224.5, c = 48.7 Å. A complete data set was collected to 2.9 Å resolution at 100 K using synchrotron radiation. The structure may reveal features of the unique reaction mechanism of Rap1GAP.

1. Introduction

Rap1 belongs to the Ras subfamily of small GTPases and with 50% amino-acid identity is the closest relative of the founding member Ras (for a review, see Bos *et al.*, 2001). Rap1 acts as a molecular switch, cycling between a GTP-bound and a GDP-bound state, in which the GTP-bound form is specifically able to interact with effector molecules and modify their activity or localization. Rap1 is thought to regulate cell adhesion and proliferation (Stork, 2003). Its best characterized role is the regulation of integrins in T-lymphocytes (Abraham, 2003), but it has also been reported to be involved in the processes of learning and memory (Morozov *et al.*, 2003) and in the development of certain types of leukaemia (Ishida *et al.*, 2003).

The nucleotide-loading state of Rap1 is tightly regulated by guanine nucleotide exchange factors (GEFs) that catalyse the dissociation of bound nucleotide and GTPase-activating proteins (GAPs), which accelerate the hydrolysis of bound GTP. Rap1GAP, a 663-amino-acid protein of molecular weight 73 kDa, was the first protein for which a specific GAP activity towards Rap1 was identified (Rubinfeld *et al.*, 1991). A core domain comprising amino acids 75–415 was shown to be necessary and sufficient for this activity. This domain (hereafter referred to as Rap1GAP) shows no sequence homology to known GAPs of other small GTP-binding proteins. However, two human proteins Spa1 and E6TP1 were found to have sequence homology to Rap1GAP, whilst tuberin only shows sequence homology in the C-terminal 160 amino acids (Maheshwar *et al.*, 1997).

In nearly all small GTP-binding proteins, a catalytic glutamine is crucial for the GTP hydrolysis reaction since this amino acid positions the attacking water molecule (Krengel *et al.*, 1990). A mutation of this residue to any other amino acid leads to a dramatic reduction of GTPase activity, *e.g.* 10^5-fold for Q61*X* in Ras. Rap1, however, contains a threonine at this position, pointing to a different mechanism of GTP hydrolysis.

All GAPs characterized so far, with the exception of RanGAP, introduce an arginine residue, the so-called arginine finger, into the active site of their corresponding G protein (Scheffzek *et al.*, 1998). This arginine provides a positive charge, which is thought to stabilize the transition state of GTP hydrolysis. However, in previous experiments we were not able to identify a conserved arginine that when mutated to alanine markedly reduced the activity of Rap1GAP (Brinkmann *et al.*, 2002). We concluded that Rap1GAP does not employ catalytic arginine. However, the mutation of two conserved lysines (Lys194 and Lys285) to alanine dramatically reduced the activity of Rap1GAP (by 25-fold and 100-fold, respectively).

In order to clarify the roles of these conserved lysines and to identify the catalytic residue(s) of Rap1GAP, we decided to solve the structure of the catalytic domain of Rap1GAP by X-ray crystallography. The large-scale purification and crystallization of wild-type and mutant Rap1GAP are described in this article.

2. Overexpression and purification

Rap1GAP (amino acids 75–415) was purified as a GST-fusion protein according to Brinkmann *et al.* (2002). A pGEX4T1 (Amersham Biosciences) expression construct was transformed in *Escherichia coli* Bl21DE3. A 10 l bacterial culture was grown at 310 K in TB medium. At an A_{600} of 0.2, 50 µ*M* IPTG was added and the culture was grown overnight at 291 K. The bacterial pellet was resuspended in phosphate-buffered saline (PBS) containing

5 m*M* $MgCl_2$, 5 m*M* dithioerythritol (DTE), 1 m*M* ATP and 100 µ*M* phenylmethylsulfonyl fluoride pH 7.5. A soluble cell extract was prepared and applied onto a glutathione Sepharose column. The column was washed extensively, first with PBS containing 5 m*M* $MgCl_2$, 5 m*M* DTE and 0.1 m*M* ATP and then with PBS pH 7.5 and 5 m*M* DTE. GST was cleaved overnight by the addition of 300 units of thrombin (Serva) under continuous circulation at 277 K. After elution with PBS pH 7.5 and 5 m*M* DTE, the protein (90% purity as judged by SDS–PAGE; data not shown) was concentrated by ultrafiltration. Rap1GAP was further purified on a Sephadex 200 gel-filtration column using 20 m*M* HEPES pH 7.5, 100 m*M* NaCl and 5 m*M* DTE as buffer. The protein eluted in two peaks, the first containing bound chaperone and eluting in the exclusion volume and the second eluting as an apparent trimer and containing pure Rap1GAP. Proteins from the second peak were pooled, concentrated and washed twice with 20 m*M* HEPES pH 7.5 and 5 m*M* DTE using Amicon concentrators (10 kDa cutoff) to remove NaCl. The protein was finally concentrated to 60 mg ml^{-1} and flash-frozen in liquid nitrogen. A typical protein purification yielded 3 mg of protein per litre of culture.

The Rap1GAP(Q204A) mutant was generated in a previous experiment when looking for conserved amino acids important for catalysis (Brinkmann *et al.*, 2002). This mutant had wild-type activity in a Rap1–GTP-hydrolysis assay, but showed a threefold higher expression of soluble protein than the wild type under the same expression conditions. The same purification protocol was applied as for the wild-type protein.

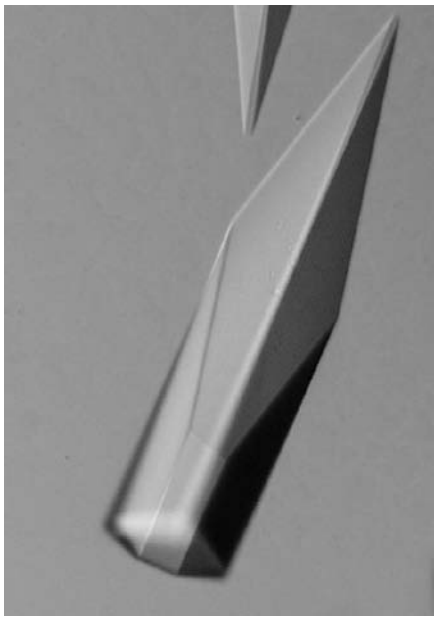

Figure 1
Photograph of a Rap1GAP(Q204A) crystal (~0.5 × 0.2 × 0.1 mm).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.934
Resolution (Å)	15.0–2.9 (3.0–2.9)
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 170.7, b = 224.5, c = 48.7
V_M ($Å^3$ Da^{-1})	3.09
Total measurements	208333
Unique reflections	42290
Average redundancy	4.9 (4.8)
$I/\sigma(I)$	17.2 (3.9)
Completeness (%)	98.9 (99.8)
Wilson B ($Å^2$)	71
R_{sym}†	6.0 (34.5)

† $R_{sym} = \sum |I(h)_j - \langle I(h)\rangle| / \sum I(h)_j$, where $I(h)_j$ is the scaled observed intensity of the jth symmetry-related observation of reflection h and $\langle I(h)_j\rangle$ is the mean value.

3. Crystallization

Since Rap1GAP is very susceptible to protease impurities, all crystallization trials were carried out at 277 and 285 K. For initial trials, Rap1GAP was thawed rapidly and diluted to 20 mg ml^{-1} using 20 m*M* HEPES, pH 7.5 and 5 m*M* DTE. The hanging-drop method was used in all experiments.

An initial condition resulting in spherulites was found at 285 K using a Hampton Research PEG/Ion screen. It was found that the Q204A mutant protein yielded larger and better diffracting crystals than the wild type. Consequently, the Q204A mutant was used for all further crystallization experiments. The optimized conditions for these crystals (400 × 100 × 50 µm), which appeared after 4 d, were 45 mg ml^{-1} Rap1GAP(Q204A), 9%(*w*/*v*) PEG 2000 MME, 230 m*M* lithium acetate, 7%(*v*/*v*) MPD and a drop size of at least 6 µl.

These crystals diffracted to only 3.5 Å and had a very large orthorhombic unit cell (~300 × 140 × 100 Å) with between eight and 12 protein molecules predicted per asymmetric unit. Furthermore, the crystals were poorly reproducible. Switching to 277 K and modifying the reservoir solution led to a different and better diffracting crystal form with a smaller unit cell. The final optimized crystallization conditions used in all diffraction experiments and resulting in crystals of dimensions 400 × 100 × 100 µm after 4 d were 20 mg ml^{-1} Rap1GAP(Q204A), 10–12%(*w*/*v*) PEG 3350, 3–5%(*v*/*v*) MPD, 100 m*M* HEPES pH 7.0 and 100 m*M* $MgSO_4$ (see Fig. 1).

To reduce radiation damage, crystals were transferred into a cryosolution and flash-frozen in liquid nitrogen. Since Rap1GAP crystals are sensitive to osmotic changes, the cryoprotectant concentration was slowly increased while keeping the buffer (100 m*M* HEPES pH 7.0) and salt concentration (100 m*M* $MgSO_4$) constant. Crystals were first incubated in 10% PEG 3350 and 6% MPD. In two further incubation steps, the cryoprotectant concentration was increased to 15% PEG 3350 and 7% MPD and to a final concentration of 20%(*w*/*v*) PEG 3350 and 8%(*v*/*v*) MPD. All incubation steps took less than 2 min.

3.1. Data collection

A data set was collected from a Rap1GAP(Q204A) crystal at 100 K at ESRF beamline ID 14-EH1 at a wavelength of 0.934 Å using an ADSC Q105 CCD detector. The crystal-to-detector distance was 270 mm, the oscillation range was 0.6° and 207 frames were collected.

Data were indexed, integrated and scaled with the *XDS* package (Kabsch, 1993). The crystals diffracted to better than 3.0 Å resolution (Table 1) and systematic absences revealed that the crystals belong to space group $P2_12_12$, with four monomers per asymmetric unit, corresponding to a V_M value of 3.09 $Å^3$ Da^{-1} (Matthews, 1968) and a solvent content of 60%. Currently, we are trying to crystallize the selenomethionine-substituted Rap1GAP protein in order to solve the phase problem.

The authors are thankful to the staff at the ESRF, Grenoble, France and Ilme Schlichting and Wulf Blankenfeldt for data collection. OD is supported by the Boehringer Ingelheim Fonds.

References

Abraham, R. T. (2003). *Nature Immunol.* **4**, 725–727.
Bos, J. L., de Rooij, J. & Reedquist, K. A. (2001). *Nature Rev. Mol. Cell Biol.* **2**, 369–377.
Brinkmann, T., Daumke, O., Herbrand, U., Kuhlmann, D., Stege, P., Ahmadian, M. R. & Wittinghofer, A. (2002). *J. Biol. Chem.* **277**, 12525–12531.
Ishida, D., Kometani, K., Yang, H., Kakugawa, K., Masuda, K., Iwai, K., Suzuki, M., Itohara, S., Nakahata, T., Hiai, H., Kawamoto, H., Hattori, M. & Minato, N. (2003). *Cancer Cell*, **4**, 55–65.
Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.
Krengel, U., Schlichting, L., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E. F. & Wittinghofer, A. (1990). *Cell*, **62**, 539–548.
Maheshwar, M. M., Cheadle, J. P., Jones, A. C., Myring, J., Fryer, A. E., Harris, P. C. &

Sampson, J. R. (1997). *Hum. Mol. Genet.* **6**, 1991–1996.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Morozov, A., Muzzio, I. A., Bourtchouladze, R., Van Strien, N., Lapidus, K., Yin, D., Winder, D. G., Adams, J. P., Sweatt, J. D. & Kandel, E. R. (2003). *Neuron*, **39**, 309–325.
Rubinfeld, B., Crosier, W. J., Albert, I., Conroy, L., Clark, R., McCormick, F. & Polakis, P. (1992). *Mol. Cell. Biol.* **12**, 4634–4642.
Rubinfeld, B., Munemitsu, S., Clark, R., Conroy, L., Watt, K., Crosier, W. J., McCormick, F. & Polakis, P. (1991). *Cell*, **65**, 1033–1042.
Scheffzek, K., Ahmadian, M. R. & Wittinghofer, A. (1998). *Trends Biochem. Sci.* **23**, 257–262.
Stork, P. J. S. (2003). *Trends Biochem. Sci.* **28**, 267–275.