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Crystallization and preliminary crystallographic analysis of the Ras binding domain of RalGDS, a guanine nucleotide dissociation stimulator of the Ral protein

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Abstract

The RalGDS is a guanine nucleotide dissociation stimulator which activates the Ral protein, a Ras-like small GTPase. The C-terminal domain of the RalGDS (C-RalGDS) binds tightly to the effector loop of Ras suggesting that the RalGDS may be a crossing point of two signal tranduction pathways associated with the Ras and Ral proteins. C-RalGDS has been purified and crystallized in space group $C2$, with unit-cell dimensions $a = 108.8$, $b = 30.7$, $c = 51.3$ Å, $\beta = 91.7°$ at 277 K and $a = 103.8$, $b = 30.55$, $c = 51.4$ Å, $\beta = 94.9°$ for data collected at 100 K. The crystals diffract to 1.8 Å at a synchrotron radiation source. To use the multiple-wavelength anomalous diffraction method for phasing, a selenomethionine derivative of the protein has also been crystallized.

1. Introduction

The Ras protein functions as a crucial mediator of many biological responses and is mutationally activated in a wide variety of tumors (Barbacid, 1987; Bollag & McCormick, 1991). Ras is the prototype of a large family of small GTP-binding proteins (Lacal & McCormick, 1993). Each of these proteins functions as a molecular switch, transmitting a signal in the active GTP-bound state, and reverting to an inactive state when the bound GTP is hydrolyzed to GDP by an intrinsic GTPase activity (Bourne, Sanders & McCormick, 1991). The function of the small GTPases is positively regulated by guanine nucleotide exchange factors or dissociation stimulators (GDS proteins), which catalyze the exchange of GDP by GTP (Boguski & McCormick, 1993; Lowy & Willumsen, 1993). The Ras protein contains a stretch of nine amino acids in the amino-terminal half, termed the effector loop (Polakis & McCormick, 1993), which undergoes a major conformational shift when Ras binds GTP (Kim, Privé & Milburn, 1993).

The Ras effector loop is known to interact with a number of different effector molecules (White *et al.*, 1994). Recent evidence suggests that Ras can signal to Ral, another member of the Ras subfamily of small GTPases (Feig & Emkey, 1993). The Ras effector loop interacts with the RalGDS (Albright, Giddings, Liu, Vito & Weinberg, 1993), a guanine nucleotide exchange factor specific for Ral (Hofer, Fields, Schneider & Martin, 1994; Spaargaren & Bischoff, 1994; Kikuchi, Demo, Ye, Chen & Williams, 1994). The interaction is mediated by a segment within the non-catalytic, C-terminal domain of the RalGDS (C-RalGDS), approximately 100 amino acids in length (residues 767–864 in rat RalGDSb). The interaction occurs both *in vivo* and *in vitro*; it is specific, dependent on activation of Ras by GTP, and blocked by mutations that affect Ras effector function. The RalGDS therefore appears to mediate signaling from Ras to Ral, which in turn may signal to other small GTPases (Cantor, Urano & Feig, 1995; Julien-Flores *et al.*, 1995). Thus, C-RalGDS appears to be at a crossroads of two signal transduction pathways, one associated with Ras and the other with the Ral protein. Structural studies of the RalGDS C-terminal domain will, therefore, provide insight into the functioning of the Ras effector loop and the mechanism of signaling between small GTPases. Here, we describe the purification and crystallization of the C-terminal domain of the RalGDS and preliminary crystallographic analysis. Recently the crystal structure of the complex between the Ras-related protein Rap1A and the Ras-binding domain of Raf has been solved (Nassar *et al.*, 1995). Determination of the structure of the RalGDS C-terminal domain will allow a comparison of the different Ras-interacting domains, the analysis of common structural features and the identification of possible differences between these molecules. This information will be necessary for the future design of specific inhibitors of Ras function.

2. Materials, methods and results

2.1. *Bacterial expression and protein purification*

A full-length cDNA clone of the RalGDS was provided by R. A. Weinberg (Albright *et al.*, 1993). The C-terminal domain of the RalGDS (amino acids 767–864 of rat RalGDSb) was amplified by the polymerase chain reaction and cloned into the pGEX-2T vector (Pharmacia) to generate the expression plasmid pGEX98. C-RalGDS was expressed as a glutathione S-transferase fusion protein (Hofer *et al.*, 1994), which was purified by a modification of the procedure of Smith & Johnson (1988). 2 l cultures of *E. coli* transformed with pGEX98 were grown for 4 h at 310 K in the presence of 0.1 m*M* isopropyl-β-D-thiogalactoside (IPTG, Clontech, Palo Alto, CA). The cells were harvested, resuspended in 20 ml phosphate-buffered saline (PBS) supplemented with protease inhibitors and fractured in a french press. Triton X-100 (Sigma) was added to a concentration of 1% and the extract clarified by centrifugation. The supernatant of the fusion protein from 2 l of LB culture was loaded onto a 15 ml glutathione sepharose 4B (Pharmacia Biotech, Alameda, CA) affinity column, and allowed to circulate overnight. The column was washed: (1) twice with 50 ml of PBS, 1% Triton X-100, 1 m*M* phenylmethylsufonyl fluoride (PMSF, Sigma), and 1 m*M* EDTA (Sigma); (2) once with 50 ml of wash buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl); and (3) once with 50 ml of cleavage buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, and 2.5 m*M* $CaCl_2$). Thrombin [0.5%(*w*/*w*) of the fusion protein] was diluted in 15 ml of cleavage buffer and loaded onto the affinity column. After incubation at room temperature for 1.5 h, C-RalGDS was eluted with wash buffer. The protein was concentrated and dialyzed into 20 m*M* Bis-Tris propane, pH 6.8 (Sigma), 1 m*M* PMSF

(Sigma), 1 m*M* EDTA (Sigma), and 1 m*M* DTT (Boehringer-Mannheim). It was then applied onto a Mono Q 5/5 column (Pharmacia) equilibrated with the same Bis-Tris propane buffer. Protein was eluted with a linear gradient from 0 to 1 *M* NaCl at 1 ml min^{-1}. Finally the fractions containing C-RalGDS were pooled, concentrated, and applied to a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 50 m*M* Hepes, pH 7.5 (Sigma), 1 m*M* PMSF, 1 m*M* EDTA, and 1 m*M* DTT. C-RalGDS protein was eluted with the same Hepes buffer at 0.5 ml min^{-1}. On a small scale (2 l culture) this protocol yields 2 mg of purified protein per liter of bacterial culture; on a large scale (12 l of fermentor culture) the yield is 8 mg l^{-1}. The homogeneity and authenticity of the purified protein were examined by SDS polyacrylamide gel electrophoresis followed by silver staining, and mass spectrometry. The ability of the purified C-RalGDS to bind to Ras was confirmed by incubating the protein with active Ras and monitoring complex formation by non-denaturing gel electrophoresis.

2.2. *Crystallization of native C-RalGDS*

The initial crystallization conditions were determined by the sparse-matrix screening method (Jancarik & Kim, 1991). Crystals were grown by the vapor-diffusion method from a solution containing 6 mg ml^{-1} C-RalGDS, 0.5 m*M* DTT, 0.5 m*M* PMSF, 0.5 m*M* EDTA, 0.1 *M* calcium acetate, 0.05 *M* Tris–HCl pH 8.5, and 11.5% polyethylene glycol (PEG) 8K equlibrated with a reservoir solution containing 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 8.5, and 23% PEG 8K. The crystal size was $0.6 \times 0.05 \times 0.05$ mm after 2 weeks at 277 K. Preliminary X-ray diffraction data were collected at the Stanford Synchrotron Radiation Laboratory (Fig. 1). Diffraction was observed to a resolution of 1.8 Å. Subsequently, the crystal size has been improved to $0.8 \times 0.12 \times 0.1$ mm (Fig. 2) using the following conditions: 5 mg ml^{-1} protein, 0.5 m*M* DTT, 0.5 m*M* PMSF, 0.5 m*M* EDTA, 0.1 *M* calcium acetate, 0.05 *M* Tris–HCl pH 8.5, and 10% PEG 8K in the drop equilibrated against 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 8.5, and 20% PEG 8K in the reservoir. The crystallization drop was microseeded after a week of equilibration. C-RalGDS crystals were sealed in glass capillaries. Data collection was carried out at 277 K. Alternatively, the crystals were flash-frozen in the presence of 40% PEG 8000 using an Oxford Cryostream liquid-nitrogen cooling system (Stoe Diffraction Systems) and data were collected at 100 K.

2.3. *Expression, purification, and crystallization of SeMet C-RalGDS*

To apply the multiple-wavelength anormalous diffraction method (Hendrickson, 1990) we have also made a selenomethionine (SeMet) derivative of the protein by substituting three methionine residues in the protein by the amino acid SeMet. The pGEX98 plasmid was transformed into the methionine auxotrophic *E. coli* strain JB (DE3), pACYC177. The cells were then grown in minimal medium in the presence of SeMet. The SeMet C-RalGDS protein was purified using the same protocol as for the native protein, with the addition of 10 m*M* DTT and 2 m*M* EDTA in all buffers. The SeMet incorporation efficiency was 99%, as determined by mass spectrometry. The SeMet protein was crystallized under similar conditions to those described above and the crystals are of similar morphology. The protein derivative crystals diffract to 2.3 Å at beamline X-4 of Brookhaven National Laboratory.

3. Results

We have purified and crystallized the C-terminal domain of RalGDS. Wild-type and derivative protein crystals exhibit same mophology and grow as long needles. The space group of the crystals is *C*2 with unit-cell dimensions $a = 108.8$, $b = 30.7$, $c = 51.3$ Å, $\beta = 91.7°$ for crystals grown at 277 K. The unit-cell dimensions of the flash-frozen crystal in the presence of 40% PEG 8K are $a = 103.8$, $b = 30.55$, $c = 51.4$ Å, $\beta = 94.9°$. The molecular weight of the protein is 11 337 Da. If there is one molecule per asymmetric unit, or four proteins per unit cell, $V_M = 3.6$ Å^3 Da^{-1}; if there are two molecules per asymmetric

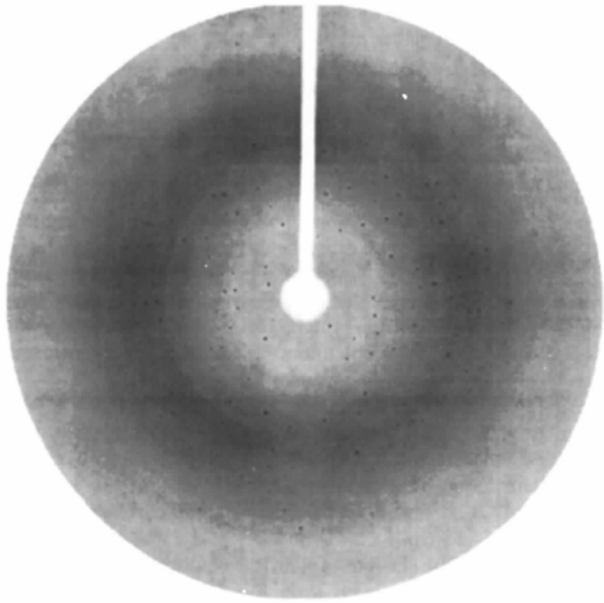

Fig. 1. A 3° rotation diffraction pattern from a native crystal of C-RalGDS ($0.6 \times 0.05 \times 0.05$ mm) at 277 K. The image was taken at SSRL 7-1 beamline.

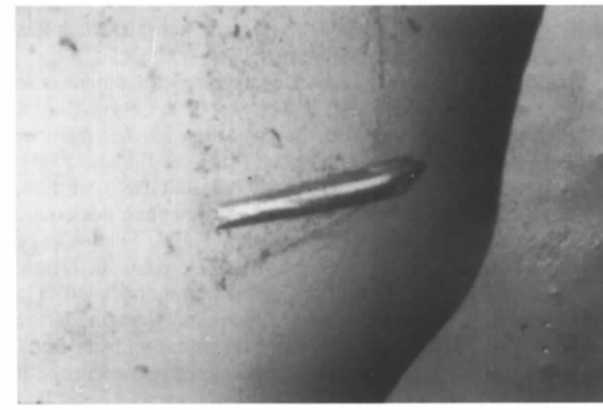

Fig. 2. A single native crystal of C-RalGDS grown from 20% PEG 8K, $CaCl_2$ and Tris buffer at pH 8.5 with dimensions $0.8 \times 0.12 \times 0.1$ mm. The space group is monoclinic *C*2.

unit, or eight proteins per unit cell, $V_M = 1.8$ Å^3 Da^{-1}. Thus, the unit-cell dimensions are consistent with either one or two molecules per asymmetric unit. However, given the fact that the crystals diffract to 1.8 Å, it is likely that the molecules form a close contact, and thus two molecules per asymmetric unit with the solvent content of about 30% in the unit cell.

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