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Preliminary crystallographic analysis of the complex of the human GTPase RhoA with the DH/PH tandem of PDZ-RhoGEF

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PDZ-containing RhoGEF (PDZ-RhoGEF) is a multidomain protein composed of 1522 amino acids that belongs to the guanine nucleotide exchange factors family (GEF) active on Rho GTPases. It is highly specific for RhoA and is thought to transduce signals from $G\alpha_{12/13}$-coupled receptors to the RhoA-dependent regulatory cascades. The protein shows high sequence homology to LARG, p115-RhoGEF and *Drosophila* DRhoGEF2. The exchange reaction is catalyzed by a DH domain, which is directly downstream of a PH domain in all known Rho-specific GEFs. The DH/PH tandem of PDZ-RhoGEF and C-terminally truncated RhoA were overexpressed in *Escherichia coli* as TEV protease-cleavable fusion proteins containing GST and a hexahistidine tag at the N-termini, respectively. The nucleotide-free DH/PH–RhoA complex was purified by gel filtration and crystallized. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 88.6$, $b = 119.0$, $c = 91.5$ Å, $\beta = 114.7^\circ$.

1. Introduction

The activation of small Ras-like cytosolic GTPases of the Rho (Ras-homology) family is mediated by numerous guanine nucleotide exchange factors (GEFs) which catalyze the exchange of GDP for GTP (Whitehead *et al.*, 1997; Schmidt & Hall, 2002). The signals initiating these events are typically transmitted by the tyrosine kinase family of receptors, but recently a unique family of RhoGEFs that are activated by G-protein-coupled receptors was identified. These GEFs include the mammalian proteins PDZ-RhoGEF (Fukuhara *et al.*, 1999), also known as GTRAP48 (Jackson *et al.*, 2001), p115RhoGEF (Kozasa *et al.*, 1998) and the leukemia-associated RhoGEF LARG (Fukuhara *et al.*, 2000), as well as orthologs found in *Drosophila melanogaster* (Halsell *et al.*, 2000) and *Caenorhabditis elegans* (Yau *et al.*, 2003). All contain an RGSL (RGS-like) domain capable of binding the α-chain of $G_{12/13}$ (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Fukuhara *et al.*, 1999, 2000; Suzuki *et al.*, 2003).

Like all RhoGEFs, the large multidomain members of this family catalyze GDP/GTP exchange using a tandem of DH (Dbl-homology) and PH (pleckstrin-homology) domains. The RGSL-containing GEFs are unique in their strict preference for RhoA as a substrate and are not active on Cdc42 or Rac, the other major RhoGTPases. The principal features of the molecular mechanisms by which the DH domains stabilize nucleotide-free RhoGTPases were proposed on the basis of several crystal structures of complexes, *i.e.* those of the Tiam1 DH/PH tandem with Rac1 (Worthylake *et al.*, 2000), the ITSN DH/PH with Cdc42 (Snyder *et al.*, 2002), Dbs (DH/PH)–RhoA (Suzuki *et al.*, 2003) and Dbs (DH/PH)–Cdc42 (Rossman *et al.*, 2002). These studies have shown that while all the residues essential for the nucleotide-exchange process are found in the DH domain, the PH domain, at least in some cases, substantially contributes to the selectivity and specificity of interaction. It was shown that the PH domain is in direct contact with a GTPase in the structures of Dbs–Cdc42 (Rossman *et al.*, 2002) and Dbs–RhoA (Snyder *et al.*, 2002), but that the residues involved in these interactions are not conserved in the $G_{12/13}$-activated GEFs.

To better understand the structure–function relationships and the molecular basis of the intermolecular interactions involving the RhoA-specific exchange factor PDZ-RhoGEF, we have undertaken crystallographic studies of this protein. We have previously determined the crystal structure of the RGSL domain of PDZ-RhoGEF (Longenecker *et al.*, 2001). In the present paper, we report the successful preparation of X-ray quality crystals of the complex of the human nucleotide-free RhoA and the DH/PH tandem of PDZ-RhoGEF.

2. Materials and methods

2.1. Protein expression and purification

The DH/PH tandem of PDZ-RhoGEF and RhoA were obtained separately as recombinant proteins expressed in *Escherichia coli*. Using the Gateway cloning system, a fragment encoding the DH/PH domains (residues 712–

1081) was inserted into the pDEST15 expression vector (Invitrogen) to create a GST-fusion protein. An rTEV-specific protease-recognition site was introduced during the amplification step in order to cleave and separate the GST protein from the DH/PH tandem. The verified fusion construct was then transformed into the BL21(DE3)-RIL *E. coli* strain (Stratagene) and the bacterial cultures were grown in Luria–Bertani medium supplemented with 100 µg ml^{-1} ampicillin. Protein expression was induced at OD_{600} = 0.8–1.0 with 0.5 m*M* isopropylthiogalactopyranoside (IPTG) and the cultures were incubated overnight at 293 K. Cells were harvested by centrifugation at 6000 rev min^{-1} for 20 min and the pellet was either immediately used for protein purification or frozen at 253 K. All subsequent purification steps were carried out at 277 K. The thawed cells were resuspended in lysis buffer (10 m*M* Tris–HCl, 150 m*M* NaCl, 5 m*M* EDTA pH 7.5) containing 1 m*M* PMSF and disrupted by sonication. The resulting cell lysate was centrifuged at 15 000 rev min^{-1} for 1 h and the supernatant containing the soluble fraction was applied to a glutathione-Sepharose 4B column (Pharmacia) pre-equilibrated with chilled lysis buffer. Binding to the matrix was achieved by gently rocking the column for 1 h. After this time, the flowthrough fraction was collected and the matrix was washed extensively with 2 l washing buffer (10 m*M* Tris–HCl, 500 m*M* NaCl, 5 m*M* EDTA pH 7.5). The GST-DH/PH fusion was released with elution buffer (50 m*M* Tris–HCl, 50 m*M* NaCl, 20 m*M* glutathione pH 7.5). The purified protein was digested with rTEV protease (Life Technologies) in the same buffer for 48 h and then dialyzed against the lysis buffer to remove glutathione. The protein solution was then passed twice over a glutathione-Sepharose 4B column to remove GST and the uncut fusion protein, while simultaneously collecting the DH/PH tandem as a flowthrough fraction. Concentrated samples of the DH/PH domains were next injected onto a Superdex-75 size-exclusion column (Pharmacia) equilibrated with 20 m*M* Tris–HCl, 150 m*M* NaCl and 1 m*M* DTT pH 7.1 and fractions containing the DH/PH domains were pooled, concentrated and analyzed by SDS–PAGE. Pure DH/PH appeared on the gel as a single band corresponding to a molecular weight of approximately 45 kDa, in agreement with the calculated value of 43.2 kDa.

A human RhoA-encoding sequence with an F25N mutation, which stabilizes the bacterially expressed protein (Self & Hall, 1995), and with the C-terminus truncated after residue 181 in a pETUni vector (Sheffield *et al.*, 1999) was used for expression of RhoA as an N-terminal fusion with a His-tag sequence in BL21(DE3) *E. coli*. The bacterial cells were grown in Luria–Bertani broth containing ampicillin (100 µg ml^{-1}) and were induced (at $OD_{600} \simeq 1.0$) by addition of IPTG to a final concentration of 0.5 m*M*. After an additional 3.5 h of growth at 310 K, the cells were pelleted at 6000 rev min^{-1} for 20 min and stored at 253 K. Cell pellets were resuspended in homogenization buffer (50 m*M* Tris–HCl, 400 m*M* NaCl, 1 m*M* $MgCl_2$, 50 µ*M* GDP, 5 m*M* imidazole pH 8.0) and lysed by sonication. The lysate was then clarified by centrifugation at 15 000 rev min^{-1} for 1 h. An Ni–NTA agarose column equilibrated with the same buffer was used for affinity binding of the soluble His-tag–RhoA fusion protein from a soluble fraction. After 1 h, the matrix was washed with 200 ml homogenization buffer and the fusion proteins were eluted with elution buffer (50 m*M* Tris–HCl, 250 m*M* NaCl, 5 m*M* $MgCl_2$, 50 µ*M* GDP, 150 m*M* imidazole pH 8.0). To remove the His tag, rTEV protease (Life Technologies) was added and, after removal of imidazole, the protein sample was reloaded onto the Ni–NTA agarose column to separate RhoA from the His tag and uncut fusion protein. The sample was then loaded onto a Superdex-75 size-exclusion column (Pharmacia) equilibrated with 25 m*M* Tris–HCl, 200 m*M* NaCl, 5 m*M* $MgCl_2$ and 1 m*M* DTT pH 8.0. Fractions containing purified RhoA were pooled, concentrated and stored on ice.

2.2. Guanine nucleotide exchange fluorescence assay

To test that the DH/PH tandem of PDZ-RhoGEF is functional and capable of stimulating nucleotide exchange on RhoA, a spectroscopic fluorescence analysis using the *N*-methylanthraniloyl-GTP (mant-GTP) was performed with a Jasco FP-750 spectrofluorimeter. The exchange reaction was carried out at 293 K in a quartz cuvette containing 1 µ*M* RhoA and 2 µ*M* mant-GTP in 20 m*M* Tris–HCl, 150 m*M* NaCl, 5 m*M* $MgCl_2$ and 1 m*M* DTT pH 7.5, which was continuously stirred at 700 rev min^{-1}. After ~200 s equilibration time, the DH/PH tandem was added at 100 n*M* and the rate of the exchange reaction was monitored by the increase in mant-GTP fluorescence intensity (λ_{ex} = 356, λ_{em} = 445 nm) as a result of its incorporation into the GTPase. An equivalent volume of reaction buffer instead of the DH/PH tandem was added in a control experiment. The initial rates of intrinsic RhoA nucleotide exchange and that stimulated by the DH/PH tandem were determined by fitting the data using the program *GraFit* (Erithacus Software Ltd). Addition of the DH/PH tandem results in a high rate of exchange: 2.25 s^{-1} × 10^{-2}, in contrast to the intrinsic exchange rate of RhoA, which is 3.27 s^{-1} × 10^{-4}. The exchange reaction is stimulated 68.8-fold. The result is shown in Fig. 1.

Although direct comparison with results published for other GEFs is not possible because of differences in the experimental conditions, our data are consistent with other reports found in the literature, showing that Rho-specific GEFs substantially accelerate the nucleotide-exchange activity of RhoGTPases *in vitro*. For instance, a 57-fold stimulation for Cdc42 and a 26-fold stimulation for RhoA has been reported in the case of the Dbs (Dbl's big sister) exchange factor (Rossman *et al.*, 2002). Similarly, 46.8-fold stimulation of Cdc42 was reported for intersectin (ITSN; Snyder *et al.*, 2002).

2.3. Preparation of the complex

The DH/PH–RhoA complex was formed by mixing the two proteins in a 1:1.5 molar ratio. The resulting sample was dialyzed against 20 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* DTT and 5 m*M* EDTA pH 7.1 overnight at 277 K in order to remove Mg^{2+} and consequently the guanine nucleotide from RhoA to obtain a high-affinity complex between DH/PH tandem and GTPase. The sample was then injected onto a Superdex-75 size-exclusion column equilibrated with 20 m*M* Tris–HCl and 150 m*M* NaCl pH 7.2

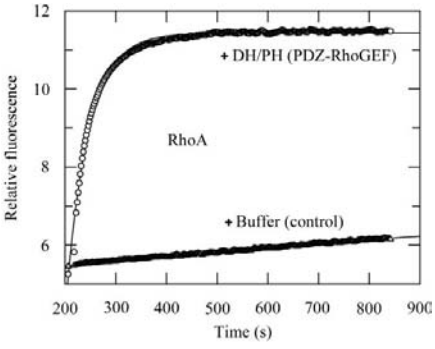

Figure 1
The DH/PH tandem of PDZ-RhoGEF stimulates guanine-nucleotide exchange on RhoA *in vitro*. The rate of the exchange reaction was monitored by the increase in the mant-GTP fluorescence intensity as a result of its incorporation to the Rho GTPase (open circles). The intrinsic exchange rate of RhoA is represented by the control experiment (open triangles). Details are given in §2.

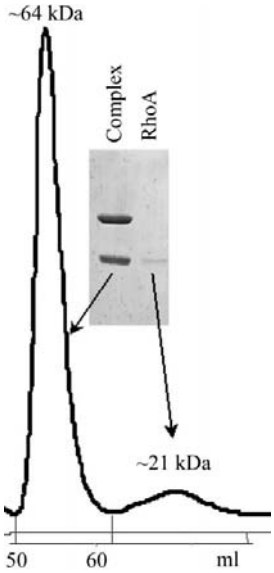

Figure 2
Size-exclusion analysis of the DH/PH complex with RhoA (Superdex-75 prep-grade column). The average molecular weights were estimated on the basis of retention times and the peak fractions were analyzed by SDS–PAGE (inset).

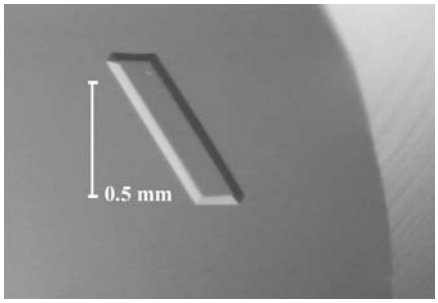

Figure 3
A single crystal of the complex of the DH/PH tandem from PDZ-RhoGEF with RhoA.

and, as a result, two peaks appeared on the chromatogram. As judged by SDS–PAGE, the first dominant peak corresponded to 64.1 kDa heterodimer DH/PH (PDZ-RhoGEF)–RhoA and the second (very small) peak contained free RhoA (Fig. 2). Finally, the fractions containing the complex were pooled, concentrated to $\sim$10 mg ml^{-1} and used for crystallization trials.

2.4. Crystallization

The initial search for crystals was performed with an original compilation of 96 crystallization conditions consisting of commercially available and home-made formulations (to be published elsewhere). The screening was carried out using a cloverleaf plate (Emerald Biostructures) and the sitting-drop vapor-diffusion method by mixing 1 µl 10 mg ml^{-1} protein solution and 1 µl mother liquor. The best crystals appeared in a solution containing 20% PEG 3350 and 0.2 *M* tripotassium citrate monohydrate pH 8.3. Perfectly shaped prisms grew overnight and reached maximum dimensions of 0.7 × 0.2 × 0.15 mm within days (Fig. 3). Preliminary X-ray characterization showed that crystals tended to crack when transferred to cryoprotectant solutions including agents such as glycerol, sucrose and MPD. Because of the low stability of the crystals, we decided to introduce cryoprotectant additives directly into the crystallization solution. The final conditions found were 20% PEG 3350 and 0.2 *M* tripotassium citrate monohydrate pH 8.3 with the addition of 10–12.5% glycerol.

Table 1
Crystallographic data statistics.

Values in parentheses refer to the highest resolution shell.

Wavelength (Å)	1.0
Resolution (Å)	25.0–2.7 (2.8–2.7)
Total reflections	155176
Unique reflections	46522 (4247)
Completeness (%)	98.7 (91.2)
R_{sym}† (%)	7.8 (29.6)
Average $I/\sigma(I)$	18.2 (2.6)

† $R_{sym} = \sum |I - I_{avg}| / \sum I$.

2.5. Preliminary diffraction analysis.

Crystals were frozen by immersion in liquid nitrogen. X-ray data were collected at the Advanced Photon Source, CSB beamline ID19, with the use of an ADSC CCD detector. The space group was identified as $P2_1$, with unit-cell parameters a = 88.6, b = 119.0, c = 91.5 Å, β = 114.7°. Data were processed and reduced with *HKL*2000 (Otwinowski & Minor, 1997) and the statistics are shown in Table 1. Assuming the molecular weight of the complex to be 64.1 kDa, the Matthews coefficient corresponding to two molecules per asymmetric unit is 3.42 Å^3 Da^{-1}. This value is consistent with a pronounced twofold crystallographic symmetry observed in the crystals. The non-crystallographic twofold axis is perpendicular to the b axis and is very close to the diagonal between a^* and c^* (ω = 120.6, φ = 0, κ = 180°), leading to pseudo-$C222_1$ symmetry, although the extent of this phenomenon appears to vary among the crystals, which exhibit significant non-isomorphism.

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