Biochemical and Biophysical Research Communications 420 (2012) 188–192

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure of the Rasputin NTF2-like domain from *Drosophila melanogaster*

Tina Vognsen, Ole Kristensen *

Biostructural Research, Department of Drug Design and Pharmacology, Faculty of Health Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 21 February 2012
Available online 3 March 2012

Keywords:
Rin
Rasputin
G3BP
RasGAP
NTF2
Crystal structure
Ras signaling

ABSTRACT

The crystal structure of the NTF2-like domain of the *Drosophila* homolog of Ras GTPase SH3 Binding Protein (G3BP), Rasputin, was determined at 2.7 Å resolution. The overall structure is highly similar to nuclear transport factor 2: It is a homodimer comprised of a β-sheet and three α-helices forming a cone-like shape. However, known binding sites for RanGDP and FxFG containing peptides show electrostatic and steric differences compared to nuclear transport factor 2. A HEPES molecule bound in the structure suggests a new, and possibly physiologically relevant, ligand binding site.

1. Introduction

The Ras signaling pathway is a complex network of cell signaling events important for cell proliferation and differentiation. Ras proteins cycle between an active GTP-binding form and an inactive GDP-binding form. This is done with help from guanine nucleotide exchange factors (GEFs) and Ras GTPase Activating Protein (RasGAP). In response to signals from tyrosine kinase receptors, GEFs like Son of Sevenless (SOS) activate Ras by exchanging GDP with GTP, while RasGAP is a negative regulator that promotes GTP hydrolysis. In its active state, Ras stimulates a cascade of downstream effectors such as RAF and MAPK leading to cell proliferation (reviewed in [1]).

One of many proteins suggested being involved in regulation of Ras activity is the Ras GTPase Activating Protein SH3 Domain Binding Protein (G3BP). As the name implies, G3BP binds to the SH3 domain of RasGAP and can hereby impinge on Ras signaling [2]. In humans, G3BP exists in three isoforms: G3BP1, G3BP2a and G3BP2b. The main difference between these three variants is the number of PxxP motifs found in the central region of the protein [3]. These motifs were initially considered to be the binding site of RasGAP [4]. However, later studies have shown that binding takes place through the N-terminal NTF2-like domain residue 11–133 in human G3BP1 [3]. Recently, RasGAP has been questioned as a genuine binding partner for G3BP [5] and the current understanding of G3BP as a regulator of Ras signaling might, thus, be subject for revision. Regardless of its binding to RasGAP, the G3BP NTF2-like domain is highly conserved between species. It is suggested to play a role in nuclear transport, possibly in a manner similar to nuclear transport factor 2 (NTF2) [2], which is a small 14 kDa protein involved in nuclear-cytoplasmic transport. The NTF2-like domain is furthermore involved in dimer formation of the G3BP protein [6]. In addition to the NTF2-like domain and the PxxP repeats, G3BP also contains a RNA-binding domain and glutamate- and glycine-rich regions. These domains contribute to the multifunctionality of G3BP, that besides regulation of Ras and stress granule assembly also has been implicated in RNA metabolism [7,8] and NFκB signaling [9]. Moreover, human G3BP1 and G3BP2 is overexpressed in cancer tumors [10,11], but the detailed mechanisms and involvement of the various G3BP functions related to this disease scenario remains unclear.

Due to the importance of Ras in *Drosophila* eye development, *Drosophila melanogaster* has in several studies served as a model organism in studies of Ras signaling [12–14]. The *Drosophila* homolog of G3BP, encoded by the *Rin* gene, was identified in the year 2000 and named Rasputin [13]. It shares significant sequence identity over its full length to human G3BPs and is, based on defects in ommatidial polarization in *Rin*−/− mutant flies, suggested to be an effector of Ras and Rho signaling [13]. Furthermore, it is reported that Rasputin, similar to G3BP, is recruited to stress granules [6]. Here we report the crystal structure of the NTF2-like domain of Rasputin, which shares 54.2% and 55.6% sequence identity with the NTF2-like domains of human G3BP1 and G3BP2a, respectively.

* Corresponding author. Fax: +45 3533 6001.
E-mail addresses: tv@farma.ku.dk (T. Vognsen), ok@farma.ku.dk (O. Kristensen).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2012.02.140

2. Materials and methods

2.1. Protein production

Artificial gene synthesis (ShineGene Molecular Biotech Inc., Shanghai) was used to generate an optimized template for *Escherichia* coli expression of the Rasputin (UniProt ID Q9NH72, residues 15–130) NTF2-like domain.

Polymerase chain reaction (PCR) amplification of the DNA was achieved using the following primers: (a) 5′-CATGCTAGCCATATGTCTGTTGGGAGAGAGTTTGTC-3′ and (b) 5′-GCTGTTCGAAGGCGGCCGCTTACTGGTAACGAAAAATATCAT-3′.

The PCR product was digested with NotI and NdeI and cloned into the pET28a(+) expression vector (Novagen). The construct contained a thrombin-cleavable His6-tag leaving four additional vector-derived N-terminal residues (GSHM) after proteolysis. Verification of the recombinant vectors containing the target gene was done by DNA sequencing (Eurofins MWG Operon).

The plasmid was transformed into *E. coli* BL21(DE3) and expressed in ZYM-5052 autoinduction media [15]. The harvested cells were suspended in PBS buffer containing 20 μg/mL RNAse A, 20 μg/mL DNAse I and protease inhibitor (Roche, inhibitor cocktail tablets) before lysis in a cell disruptor (Constant Systems Ltd.). Cell debris was removed by centrifugation (30,000*g*, 30 min) and the supernatant applied to a HisTrap HP column (GE Healthcare) equilibrated with buffer A (20 mM Na_2HPO_4, 30 mM imidazole, 300 mM NaCl, pH 8) and eluted using a linear gradient of buffer B (20 mM Na_2HPO_4, 1 M imidazole, 300 mM NaCl, pH 8). The His6-tag was removed by addition of 2 U thrombin (bovine, Calbiochem) per milligram protein and overnight incubation at room temperature. Size exclusion chromatography (Superdex 75 HR, GE Healthcare) was used as a final purification step (buffer: 10 mM Tris–HCl, 300 mM NaCl, pH 8). The protein concentration was estimated using a Bradford protein assay (Bio-Rad) and the purity checked with SDS–PAGE (Invitrogen).

2.2. Crystallization and data collection

Initial crystallization conditions were based on previously reported results for the NTF2-like domain of human G3BP1 [16]. Rasputin crystals were obtained using hanging drop vapor-diffusion at room temperature and grew with a rectangular prismatic shape to a maximum dimension of 100 μm along the longest edge within 2 weeks. The crystallization drop contained 2 μL of reservoir solution (2.2 M diammonium phosphate and 0.1 M HEPES, pH 7) and 2 μL of protein (2 mg/mL). The crystals were transferred briefly to a cryo solution containing 2 M diammonium phosphate, 0.1 M HEPES, pH 7 and 20% glycerol prior to flash cooling in liquid nitrogen. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, beamline ID14.1 and processed using the XDS [17] pipeline in the xia2 software [18].

2.3. Structure determination and refinement

The structure was initially solved in spacegroup $C222_1$ by molecular replacement using Phaser [19] as implemented in the CCP4 suite [20]. A 1.7 Å structure of the human G3BP1 NTF2-like domain (PDB ID 3Q90, chain B) was used as search model. The obtained model was rebuilt and refined using PHENIX [21]. Post-mortem twinning analysis was performed using PHENIX after data processing in spacegroup P1 [22]. Parallel structure determination and refinement was performed in the subgroups C2 and $P2_1$. Four TLS-groups (chain A: residue 14–63, 64–139 and chain B: residue 14–64, 65–130) were derived from the TLSMD web server [23] and used in refinement. Manual correction of the model was carried out using Coot [24] and the structure was validated with MolProbity [25]. All figures were prepared in PyMol (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC).

2.4. Accession number

Crystallographic coordinates and structure factors have been deposited at the Protein Data Bank with accession code 3ujm.

3. Results and discussion

3.1. Crystal structure of the Rasputin NTF2-like domain

The crystal structure of the Rasputin NTF2-like domain was solved by molecular replacement and refined at a resolution of 2.7 Å with *R* and *R*-free values of 19.9% and 24.2%. Initially, the structure was solved in space group $C222_1$, but stagnation in *R* values during refinement led us to explore potential crystal twinning problems and further refinement in the subgroups C2 and $P2_1$. Our analysis and parallel refinements concluded that the data did not suffer from twinning effects and the final structure was satisfactorily refined in space group $P2_1$ with two molecules per asymmetric unit.

Both polypeptide chains could be traced from residue 14 to 130 including a vector-derived methionine at the N-terminal. In addition, the model contains 34 water molecules and two HEPES molecules. Structure validation showed good overall stereochemistry (Table 1), but notably two residues from each chain, Ser116 and Pro117, were found in the disallowed regions. These residues are located in a tight loop, which might distort the geometry, and despite that somewhat unfavorable conformations persists after

Table 1
Data reduction and refinement statistics. Values in parenthesis refer to the highest resolution bin.

Data collection	
X-ray source, wavelength (Å)	ID14.1/ESRF, 0.93
Space group	$P2_1$
Cell dimensions (Å)	a = 39.20, b = 80.38, c = 39.20 and γ = 92.60
Monomer per asymmetric unit	2
Resolution range (Å)	39–2.74 (2.81–2.74)
Reflections	6272 (477)
Average multiplicity	4.2 (4.3)
Completeness (%)	97.5 (96.6)
$\langle I/\sigma I\rangle$	19.8 (3.1)
R_{merge}[a] (%)	5.3 (43.5)
Refinement	
R_{work}/R_{free}[b] (%)	19.87/24.24
Number of atoms	
Protein	1927
Water	34
HEPES	30
Average B-factor (Å²)	
Protein	63.37
Water	49.22
HEPES	87.62
Rms deviation	
Bond lengths (Å)	0.002
Angles (°)	0.642
Ramachandran plots[c]	
Most favored regions (%)	91.7

[a] $R_{merge} = \Sigma_{hkl}(\Sigma_i(I_{hkl,i} - \langle I_{hkl}\rangle))/\Sigma_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices *h*, *k*, and *l*, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

[b] $R_{work} = \Sigma_{hkl}(F_{obs,hkl} - F_{calc,hkl})/F_{obs,hkl}$, where $F_{obs,hkl}$ and $F_{calc,hkl}$ are the observed and calculated structure factor amplitudes. R_{free} is equivalent to the R_{work}, but calculated with 5% of the reflections omitted from the refinement process.

[c] Values from MolProbity [25].