Vijay Kumar Srivastava,^a
Ajay Kumar Rana,^b Amogh A.
Sahasrabudhe,^a C. M Gupta^a
and J. V. Pratap^{a*}

^aMolecular and Structural Biology Division,
CSIR – Central Drug Research Institute,
B.S/10/1, Sector-10, Janakipuram Extension,
Sitapur Road, Lucknow 226 021, India, and
^bParasitology Division, CSIR – Central Drug
Research Institute, B.S/10/1, Sector-10,
Janakipuram Extension, Sitapur Road,
Lucknow 226 021, India

Correspondence e-mail: jvpratap@cdri.res.in

Received 31 December 2012

Accepted 21 March 2013

Cloning, overexpression, purification and crystallization of the CRN12 coiled-coil domain from *Leishmania donovani*

Leishmania donovani coronin CRN12 is an actin-binding protein which consists of two domains: an N-terminal WD repeat domain and a C-terminal coiled-coil domain. The coiled-coil domain is 53 residues in length. Helix–helix interactions in general and coiled coils in particular are ubiquitous in the structure of proteins and play a significant role in the association among proteins, including supramolecular assemblies and transmembrane receptors that mediate cellular signalling, transport and actin dynamics. The *L. donovani* coronin CRN12 coiled-coil domain (5.8 kDa) was cloned, overexpressed, purified to homogeneity and the N-terminal 6×His tag was successfully removed by thrombin cleavage. Crystals of recombinant *L. donovani* coronin CRN12 coiled-coil domain were grown by vapour diffusion using a hanging-drop setup. Diffraction-quality crystals were obtained and data extending to 2.46 Å resolution were collected at 100 K on BM14, ESRF, Grenoble, France. The crystal belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 118.0$, $b = 50.6$, $c = 46.0$ Å, $\beta = 111.0^\circ$. Matthews coefficient (V_M) calculations suggested the presence of 4–6 molecules in the asymmetric unit, corresponding to a solvent content of ~33–55%, and are consistent with self-rotation function calculations.

1. Introduction

The actin cytoskeleton in a eukaryotic cell mediates a plethora of essential biological processes. It defines the very basis of cell shape, polarity and cellular functions, thereby providing a crucial structural framework (Drubin & Nelson, 1996; Hall, 1998). Actin dynamics provide the sole force for cell division and locomotion (Satterwhite & Pollard, 1992; Stossel, 1993; Mitchison & Cramer, 1996) and are also involved in allied cellular processes such as membrane trafficking, phagocytosis (Chimini & Chavrier, 2000) and fluid-phase endocytosis (Qualmann *et al.*, 2000). The dynamic properties of the actin cytoskeleton are regulated by multiple actin-binding proteins that expand, contract, stabilize, cross-link or sever actin filaments (Pollard, 1990; Stossel, 1993; Welch *et al.*, 1997). However, specific details of the regulation of actin dynamics are currently unclear.

One such actin-associated protein that was originally identified in *Dictyostelium* and found to localize to the actin-rich crown-like structures on the dorsal cell surfaces is coronin (de Hostos *et al.*, 1991). Coronin contains an N-terminal WD (Trp–Asp) repeat and a C-terminal coiled-coil motif. The C-terminus of almost all members of the coronin family is predicted to form a coiled coil, implying the universal importance of this domain (Rappleye *et al.*, 1999). In *Leishmania donovani* coronin CRN12 (Morgan & Fernandez, 2008) the C-terminal domain is 53 residues in length (residues 457–510) and is predicted to form a coiled-coil topology by online coiled-coil identification software such as *COILS* (http://www.ch.embnet.org/software/COILS_form.html; Lupas *et al.*, 1991), *Paircoil2* (<http://groups.csail.mit.edu/cb/paircoil2/>; McDonnell *et al.*, 2006) and *Multicoil* (<http://groups.csail.mit.edu/cb/multicoil/>; Wolf *et al.*, 1997). It contains a heptad-repeat sequence feature (Crick, 1953) typical to coiled coils and acts as an oligomerization domain, forming well defined, stable higher-order multimers, and also performs a dual

polymerizing and depolymerizing role by enhancing the expansion of newly formed actin filaments at one end and dissociating them at the other end (Goode *et al.*, 1999). Thus, in order to obtain structural details of the mechanism of oligomerization and its role in actin dynamics, we cloned, overexpressed, purified and crystallized the coiled-coil domain. We believe that the structure of the *L. donovani* coronin CRN12 coiled-coil domain will help us to understand its regulatory role in actin dynamics.

2. Materials and methods

2.1. Cloning

The CRN12 gene fragment corresponding to its C-terminal 53-residue coiled-coil region was PCR-amplified by using specific primers (forward primer, 5'-AAA AAA **CAT ATG** GTT GAC ATG ACG CAG CAA GAG ATT TTC GAT-3'; reverse primer, 5'-AAG AAG **CTC GAG** TAT CGT CTG A AT ATC CTC TAG CAC TTG TAG-3') and *L. donovani* genomic DNA as template. The 53-amino-acid fragment was cloned in a T/A vector (InsTAclone, MBI Fermentas) and subcloned in the bacterial expression vector pET28a at *Nde*I and *Xho*I restriction sites (bold). The identity of the gene was confirmed by sequencing the clone by Sanger's method.

2.2. Overexpression

The expression vector containing the CRN12 gene fragment was introduced into the *Escherichia coli* RPL1 (DE3) strain. The recombinant strain was cultured in LB medium supplemented with 50 µg ml⁻¹ kanamycin and 25 µg ml⁻¹ chloramphenicol at 310 K and induced at an OD_{600 nm} of 0.6 with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction, the culture was grown for a further 12–14 h at 303 K. The cells were collected by centrifugation; the cell pellet from 3 l culture was suspended in 50 ml 50 mM Tris–HCl buffer containing 300 mM NaCl and 10 mM imidazole pH 8.0 and lysed by sonication using a Heat Systems Ultrasonic processor (New York) with a medium-size probe at 20% output power, 50% duty cycle with a pulse time of 10 min. Before lysis, 200 µl protease-inhibitor cocktail from Sigma–Aldrich was added to the thawed culture. The cell lysate was centrifuged at 21 365g (13 000 rev min⁻¹) in a Heraeus Multifuge X3R for 40 min at 281 K to remove cell debris.

2.3. Purification

The clear supernatant thus obtained was loaded onto an Ni²⁺–IDA column (Amersham) pre-equilibrated with equilibration buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). The column was washed with ten column volumes of wash buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 50 mM imidazole). 10 units of thrombin bovine (Calbiochem) per ml of column volume was added to 50 mM Tris–HCl pH 8.0, 300 mM NaCl buffer, loaded onto the column and left for 16 h at 293 K, after which the protein was collected. The purity, as monitored by running the samples on 15% SDS–PAGE (Fig. 1a), was 99%. The fractions were subsequently pooled and precipitated with 40% ammonium sulfate and the precipitate was dissolved in a minimum volume of size-exclusion buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA) and loaded onto a gel-filtration column (Superdex S-200 HR10/300) pre-equilibrated with size-exclusion buffer on an ÄKTA FPLC system (GE Healthcare, USA). The protein eluted at an elution volume of 15.1 ml (Fig. 1b), corresponding to a higher-order oligomer

(pentamer or hexamer) and was concentrated using a Centricon membrane filter (5 kDa cutoff, Amicon) before crystallization.

2.4. Crystallization and data collection

Preliminary crystallization trials were initiated by the sitting-drop vapour-diffusion method at 295 K in 24-well crystallization plates using Crystal Screen, Crystal Screen 2, Index Screen and Crystal Screen Lite from Hampton Research. 3 µl protein solution was mixed with 1 µl reservoir solution and equilibrated against 500 µl reservoir solution. The initial crystallization conditions were set up with different protein concentrations (5, 7, 7.5 and 8.5 mg ml⁻¹) and the hits were optimized by grid screening. Optimization was carried out by the hanging-drop vapour-diffusion method. For X-ray data collection, crystals were mounted on CryoLoops (Hampton Research), rinsed with cryoprotectant solution [25%(v/v) ethylene glycol in reservoir solution] and flash-cooled directly in a nitrogen stream at 100 K. The diffraction data were collected on BM14 at the ESRF, Grenoble using a MAR 225 CCD detector. The reflections were indexed using the *iMOSFLM* program (Battye *et al.*, 2011) and scaled with *SCALA* (Evans, 2006). The *CTRUNCATE* program

Figure 1
(a) 15% SDS–PAGE under reducing conditions of expressed *L. donovani* coronin (CRN12) coiled-coil domain: lane 1, molecular-weight marker (labelled in kDa); lane 2, 12 µg protein after affinity purification; lane 3, 9 µg coiled-coil protein after size-exclusion chromatography. An identical gel was obtained when run under nonreducing conditions as well. (b) Gel-filtration profile of CRN12 coiled-coil domain. The protein eluted at 15.1 ml.

(French & Wilson, 1978) was used to convert intensities to structure factors. The diffraction data statistics are presented in Table 1.

3. Results and discussion

Purified coronin CRN12 coiled-coil domain was obtained by a two-step purification protocol consisting of affinity and size-exclusion chromatography of protein without a His₆ tag after thrombin cleavage. The yield of the protein was 5 mg per litre of culture. In the initial crystallization trials, 250 commercial screen conditions were tested using the sitting-drop method and subsequently the hanging-drop method. Precipitate formation and crystal growth were

Figure 2
Snapshot of crystal of *L. donovani* coronin (CRN12) coiled coil.

Figure 3
Snapshot of the diffraction pattern of CRN12 coiled coil. The image corresponds to a 1° oscillation with 7 s exposure time and a crystal-to-detector distance of 241 mm at a wavelength of 0.97 Å. The diffraction images were collected on a MAR 225 CCD detector. The resolution at the edge of the diffraction pattern is 2.4 Å.

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Beamline	BM14, ESRF
Wavelength (Å)	0.97
Detector	MAR 225 CCD
Temperature (K)	1000
Space group	C2
Unit-cell parameters (Å, °)	$a = 118.0$, $b = 50.6$, $c = 46.0$, $\beta = 111.0$
Matthews coefficient (Å ³ Da ⁻¹)	1.85–2.77
Solvent content (%)	33–55
Resolution range (Å)	46–2.46 (2.59–2.46)
Total no. of reflections	66123 (9325)
No. of unique reflections	9356 (1346)
Multiplicity	7.1 (6.9)
$\langle I/\sigma(I) \rangle$	21.4 (9.3)
Completeness (%)	99.4 (99.3)
$R_{\text{merge}}^{\dagger}$	0.05 (0.13)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of the i observations.

observed in nine of these conditions in 3 weeks, which were then repeated using the hanging-drop vapour-diffusion method. Four of these nine conditions showed crystal growth. Upon further optimization of these four conditions, a condition from Crystal Screen 2 (0.5 M ammonium sulfate, 0.1 M sodium citrate tribasic dihydrate pH 5.6, 1.0 M lithium sulfate monohydrate) was found to be most promising. This condition was further fine-tuned by varying the precipitant (0.5–2.0 M) and salt (0.2–1.5 M) concentrations, the pH of the buffer (4.6–6.0), the reservoir volume (500 to 1000 μl) and the protein concentration (between 5 and 8.5 mg ml⁻¹). Good diffraction-quality crystals (Fig. 2) appeared in about 2–3 weeks when a drop of 4 μl in volume (3 μl protein solution and 1 μl reservoir solution) was equilibrated against 1000 μl reservoir solution consisting of 0.6 M ammonium sulfate, 0.1 M sodium citrate tribasic dihydrate pH 5.6, 1.0 M lithium sulfate monohydrate with a protein concentration of 7.5 mg ml⁻¹. The crystal diffracted to 2.46 Å resolution (Fig. 3).

Analysis of the diffraction data indicated that the crystal belonged to the monoclinic space group C2, with unit-cell parameters $a = 118.0$, $b = 50.6$, $c = 46.0$ Å, $\beta = 111.0^\circ$. The diffraction data suggested the presence of 4–6 molecules in the asymmetric unit, with a solvent content of 33–55% and a V_M of 1.85–2.77 Å³ Da⁻¹ (Matthews, 1968).

A self-rotation function (SRF) calculated using *MOLREP* (Fig. 4; Vagin & Teplyakov, 2010) from the *CCP4* suite (Winn *et al.*, 2011) suggests the presence of four molecules in the asymmetric unit, consistent with either 222 symmetry or fourfold symmetry. In the 222 symmetry interpretation, the SRF peaks (θ , ϕ , χ) at (147.5, 0.0, 180.0°), (67.7, 50.7, 180.0°) and (67.7, -50.7, 180.0°) can be explained by three mutually perpendicular noncrystallographic twofold axes, with the peak in the $\chi = 90^\circ$ section attributed to the ~45° inclination between the noncrystallographic twofold axes and the crystallographic twofold axis (see Appendix A of Borhani *et al.*, 1999). Alternately, the peak at (147.5, 0, 90°) could be explained by fourfold noncrystallographic symmetry (NCS), with the peaks at (67.7, 50.7, 180.0°) and (67.7, -50.7, 180.0°) arising from the interaction between the fourfold NCS and the crystallographic twofold. Preliminary biochemical studies seem to suggest the possibility of a pentamer or a hexamer in solution (data not shown).

We attempted to solve the structure by molecular-replacement calculations using a synthetic construct of mouse coronin coiled coil (32 residues in length; PDB entry 2akf; Kammerer *et al.*, 2005) and tropomyosin (52 residues in length; PDB entry 2z5h; K. Murakami, K.

Figure 4

Self-rotation function map based on structure-factor amplitudes collected from a crystal of *L. donovani* coronin (CRN12) coiled coil. The crystallographic axes *a* and *b* are parallel to the *x* and *y* axes, respectively. The (a) $\chi = 180^\circ$, (b) $\chi = 120^\circ$, (c) $\chi = 90^\circ$, (d) $\chi = 72^\circ$ and (e) $\chi = 60^\circ$ sections are shown for a self-rotation function calculated with all data in the resolution range 15–3 Å with a radius of integration 18 Å.

Figure 5

Sequence alignment of the CRN12 coiled-coil domain of mouse coronin (PDB entry 2akf; synthetic construct) and the head-to-tail junction of tropomyosin (PDB entry 2z5h). Conserved residues are highlighted in red boxes. The sequence alignment was produced using *ClustalW* (Thompson *et al.*, 1994) and the figure was generated using *ESPrpt* (Gouet *et al.*, 1999).

Nozawa, K. Tomii, N. Kudou, N. Igarashi, Y. Shirakihara, S. Wakatsuki, M. Stewart, T. Yasunaga & T. Wakabayashi, unpublished work) as search models. The sequence alignment of the coiled-coil domain of CRN12 with these two corresponding PDB entries shows that only two residues are identical (Leu18 and Ala31 in the *L. donovani* CRN12 coiled-coil domain) between the pairs of sequences (Fig. 5). We were not able to find an unambiguous solution, probably owing to the low sequence identity between these two proteins. At present, a selenomethionine derivative of the coiled-coil domain is being prepared and soaks with several heavy atoms are also being performed for *ab initio* structure solution.

JVP and VKS thank DBT–Wellcome Trust for access to beamline BM14 at the ESRF, Grenoble, France. We are also grateful to Dr Manjasetty Babu and Dr Hassan Behrlali, beamline scientists at BM14, for their help during data collection. JVP is also grateful to

Dr Pete Dunten, SLAC and Dr P. Sampathkumar, Department of Biochemistry, Albert Einstein College of Medicine, for their helpful suggestions in interpreting the SRF plots. VKS acknowledges the Department of Biotechnology, Government of India, for the fellowship. This manuscript bears CDRI communication No. 8442.

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