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Cloning, expression, purification, crystallization and preliminary crystallographic analysis of the C-terminal domain of Par-4 (PAWR)

Prostate apoptosis response-4 protein is an intrinsically disordered pro-apoptotic protein with tumour suppressor function. Par-4 is known for its selective induction of apoptosis in cancer cells only and its ability to interact with various apoptotic proteins *via* its C-terminus. Par-4, with its unique function and various interacting partners, has gained importance as a potential target for cancer therapy. The C-terminus of the rat homologue of Par-4 was crystallized and a 3.7 Å resolution X-ray diffraction data set was collected. Preliminary data analysis shows the space group to be $P4_12_12$. The unit-cell parameters are $a = b = 115.351$, $c = 123.663$ Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

Prostate apoptosis response-4 protein (Par-4) or PRKC apoptosis WT1 regulator protein (PAWR) is a pro-apoptotic tumour suppressor protein (Zhao & Rangnekar, 2008), the gene for which was found during a screen for genes induced by effectors of apoptosis in prostate cancer cell lines by differential hybridization (Sells *et al.*, 1994). It is a multi-domain protein whose expression is ubiquitous (Boghaert *et al.*, 1997) and it is evolutionarily conserved in vertebrates (Boghaert *et al.*, 1997; Libich *et al.*, 2009). Its N-terminus contains nuclear localization sequences (NLS1 and NLS2) and the unique SAC domain (selective for apoptosis of cancer cells), which is the minimum required fragment necessary for inducing apoptosis selectively in cancer cells (El-Guendy *et al.*, 2003). The C-terminus (amino acids 258–332) of Par-4, which shares homology with the death domains of other apoptotic proteins (Díaz-Meco *et al.*, 1996), includes a putative leucine-zipper (LZ) domain (amino acids 290–332; Sells *et al.*, 1997) with heptad repeats (*abcdefg*)₆ with hydrophobic amino acids at position *a* and predominantly leucines at position *d* (Sells *et al.*, 1997). Par-4 is known to homodimerize (Díaz-Meco *et al.*, 1996) and has been shown to interact with various proteins such as Wilms' tumour suppressor WT1 (Johnstone *et al.*, 1996), ζ PKC and λ/ι PKC (Díaz-Meco *et al.*, 1996), Dlk/ZIP kinase (Page *et al.*, 1999), atypical PKCs and p62 (Chang *et al.*, 2002), THAP (Roussigne *et al.*, 2003), AATF (Guo & Xie, 2004), Akt1/protein kinase B (Goswami *et al.*, 2005), Amida (Boosen *et al.*, 2005), dopamine D2 receptor (Park *et al.*, 2005), β -site APP-cleaving enzyme-1 (Xie & Guo, 2005), E2F1 (Lu *et al.*, 2008) and topoisomerase 1 (Goswami *et al.*, 2008) *via* its LZ domain. The LZ domain has been shown to be natively unfolded at physiological pH and temperature, whereas the coiled coil becomes favoured at low pH and low temperature (Dutta *et al.*, 2001).

Structural analysis of functional domains of Par-4 will provide insights into the mechanism of its interactions and selective induction of apoptosis. In the current report, the crystallization of the C-terminal 240–332 amino acids, referred to as the C-terminal domain, which includes the phosphorylation site of Akt (Ser249; Goswami *et al.*, 2005), the death-domain homologous sequence, the coiled-coil region (254–332) and the LZ domain (292–330), is presented.

Table 1

DNA source and protein information for the C-terminal domain of Par-4.

*Nde*I and *Bam*HI restriction sites are shown in italic for the respective primers and the *Strep*-Tag II sequence is shown underlined. The *Strep*-Tag II in the N-terminus of the protein sequence is shown underlined.

Source organism	<i>Rattus norvegicus</i>
DNA source	Par-4 full-length plasmid 091199A17 (Eidhoff, 2000)
Forward primer	GCGCATATGGCTAGCTGGAGCCACCGCAGTTCGAGAAGGCG- GGAGCCACCGCAGTTCGAGAAGGCGGCTTCAGTAGACA- CAACAGAGATACC
Reverse primer	CCTGGATCCCTACCTGTGCTCAGTGCCCAAC
Expression vector	pET-11a
Expression host	RIL/RP strains of <i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MASWSHPQFEKAGFSRHRDTSAPANFASSTLEKRIEDLEK- EVLRRQENLRLTRLMDQKEEMIGLKKEEIDLLNRDLDDM- EDENEQLKQENKTLTKVVGQLTR

2. Materials and methods

2.1. Cloning, expression and purification of the C-terminal domain of Par-4

The DNA sequence corresponding to the C-terminal amino acids 240–332 of rat Par-4 (UniProt accession No. Q62627) was amplified from the Par-4 full-length plasmid 091199A17 (Eidhoff, 2000) by polymerase chain reaction and cloned into pET-11a vector using *Nde*I and *Bam*HI restriction sites along with an N-terminal *Strep*-tag II. The primer details are shown in Table 1.

The C-terminal domain transformants of RIL or RP strains of *Escherichia coli* BL21(DE3) were selected on an LB-agar plate with 200 µg ml⁻¹ ampicillin. A primary culture of DYT medium supplemented with 200 µg ml⁻¹ ampicillin, which was also maintained in the secondary culture, was set up and allowed to grow overnight at 37°C and 200 rev min⁻¹. The secondary culture, with 750 ml DYT medium per 2 l baffled flask, was inoculated with the primary culture and allowed to grow at 37°C and 110 rev min⁻¹. When the OD₆₀₀ of the culture was 0.6–0.8, overexpression of the C-terminal domain was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continued for 4 h. The culture was harvested at 4000g for 20 min at 4°C. The cell pellet was resuspended in lysis buffer (50 mM Tris buffer pH 7.5, 200 mM MgCl₂) with protease inhibitor (Complete EDTA-free, Roche) and 15 µg ml⁻¹ deoxyribonuclease I (5 g of cell pellet in 50 ml of lysis buffer) and passed through an EmulsiFlex-C3

Figure 1

(a) SDS-PAGE analysis of purification of the C-terminal domain of Par-4. Lane 1, PageRuler Prestained Protein Ladder (labelled in kDa); lane 2, cell lysate; lane 3, flowthrough; lane 4, wash; lanes 5–8, elutions 1–4, respectively; lane 9, pooled C-terminal domain of Par-4; lane 10, C-terminal domain of Par-4 before loading onto a gel-filtration column. (b) Agarose gel electrophoresis of samples from the purification of the C-terminal domain of Par-4. Lane 1, cell lysate 1; lane 2, cell lysate 2; lane 3, flowthrough 1; lane 4, flowthrough 2; lane 5, C-terminal domain; lane 6, 100 bp DNA Ladder (NEB) (labelled in base pairs).

cell disruptor two to three times with a pressure of 103–152 MPa. The lysate was clarified by centrifugation at 57 000g for 45 min at 4°C and the supernatant was loaded onto a gravity-flow column with *Strep*-Tactin Superflow high-capacity resin pre-equilibrated with five bed volumes of equilibration buffer (50 mM Tris buffer pH 7.5 containing 200 mM MgCl₂). The column was washed with four to five bed volumes of the equilibration buffer and the C-terminal domain was eluted by lowering the pH with elution buffer (50 mM potassium phosphate buffer pH 4). The protein fractions were pooled, concentrated and filtered through a 0.22 µm filter. The protein sample was loaded onto a HiLoad 16/60 Superdex 75 gel-filtration prep-grade column pre-equilibrated with the elution buffer. Protein fractions corresponding to the peak were pooled and concentrated.

2.2. Crystallization

The freshly purified protein sample at a concentration of 40–45 mg ml⁻¹ was used for setting up crystallization plates. Crystallization experiments were performed with 100 mM sodium citrate buffer pH 5.9 with 17–42% *tert*-butanol. Different ratios of protein to reservoir buffer were tried. The crystallization plates were incubated at 22°C.

2.3. Data collection and processing

Perfluoropolyether (PFO) or LV CryoOil (LVCO-1) was overlaid on the sitting drop immediately after opening the crystallization experiment. Crystals were picked with cryoloops and soaked in PFO before flash-cooling in a nitrogen cryo-stream at –173°C. Data collection was performed at –173°C on the ID23-1 beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Data were processed with *XDS* (Kabsch, 2010) and analyzed using *POINTLESS* (Evans, 2006, 2011) and the *phenix.xtriage* module of *PHENIX* (Adams *et al.*, 2010).

3. Results and discussion

3.1. Expression and purification of C-terminal domain of Par-4

The C-terminal domain of Par-4 was overexpressed yielding 1.5–2.0 mg purified protein per 1 g of cell pellet. The purified C-terminal domain of Par-4 was analyzed by SDS-PAGE, which shows a single band of protein (Fig. 1a). The molecular weight of the C-terminal domain estimated from SDS-PAGE was 12.2 kDa, which is in good agreement with the value given by mass-spectrometric analysis, *i.e.* 12.26 kDa (data not shown). The SEC (size-exclusion chromatography) profile shows that the protein elutes with an apparent molecular weight of 95 kDa, which could indicate an octamer (12.2 kDa × 8 = 97.6 kDa; data not shown). Considering the fact that the SEC separation is based on Stokes radii (Hong *et al.*, 2012) and the oligomer is predicted to be an anisotropic molecule, there could be an offset in the estimated molecular weight of the oligomer, which was based on a calibration made using standard globular proteins. Fig. 1(b) shows that the protein sample after affinity purification is DNA-free.

3.2. Crystallization

An initial crystallization condition was obtained by screening with commercially available crystallization screens from Hampton Research and Rigaku Reagents (Crystal Screen, Crystal Screen 2, Wizard Classic 1 and 2, and Wizard Cryo 1 and 2). Crystal Screen 2 gave a crystal in the crystallization condition No. 17, *i.e.* 0.1 M sodium citrate tribasic dihydrate pH 5.6, 35% (v/v) *tert*-butanol. A grid screen

Table 2
Crystallization of the C-terminal domain of Par-4.

Method	Vapour diffusion, sitting drop
Plate type	96-well
Temperature (°C)	22.15
Protein concentration (mg ml ⁻¹)	40
Buffer composition of protein solution	50 mM potassium phosphate pH 4.0
Composition of reservoir solution	37–42% <i>tert</i> -butanol, 100 mM sodium citrate pH 5.9
Volume and ratio of drop	1.4 µl, 1:1.3 protein:crystallization buffer
Volume of reservoir (µl)	70

for pH and tertiary butanol concentration gave an improved crystallization condition (Kubicek, 2005). The improved crystallization condition for the C-terminal domain of Par-4 consisted of 37–42% *tert*-butanol, 100 mM sodium citrate/citric acid pH 5.9. Immediately after the addition of crystallization buffer protein precipitation was observed, but the sample usually cleared within an hour. In some experiments the protein remained in the precipitated form. Phase transformation of precipitated protein to a gel-like phase was observed when a 24-well plate with 1 ml reservoir buffer and 7–10 µl sitting-drop volume was used. For the smaller sitting-drop volumes of 0.6–1.5 µl the gel-like phase was observed only infrequently.

Crystals appeared randomly from the clear crystallization droplet as well as from the gel-like phase within a time range of 3 d to a couple of weeks. The protein mostly formed tetragonal bipyramidal crystals (Fig. 2). Crystallization details are given in Table 2.

3.3. Crystal cooling and data collection

The obtained crystals are very sensitive with respect to the loss of volatile crystallization buffer components upon opening the experiment. After opening the experiment under the microscope the crystals could be seen to move before collapsing to precipitate within 5–10 s. The diffraction of the crystals of the C-terminal domain of Par-4 was usually limited to medium resolution, extending to around 3.7 Å resolution at –173°C. Including different concentrations of a cryoprotectant, *e.g.* 2-methyl-2,4-pentanediol (MPD), in the crystallization buffer or soaking the crystal in a cryoprotectant was not helpful as the time required for crystal collection was sufficient for the crystal to be destroyed. Overlaying PFO on the sitting drop immediately after opening the experiment helped to slow down the evaporation. Crystals were picked up and immersed in PFO before plunging into the cryostream. This method of cooling helped in obtaining the diffraction data beyond 3.7 Å resolution (Fig. 3).

Figure 2
Crystal of the C-terminal domain of Par-4 used for data collection.

Table 3
Data collection and processing.
Values in parentheses are for the outer shell.

Diffraction source	Synchrotron
Wavelength (Å)	0.972400
Temperature (°C)	–173
Detector	ADSC
Crystal-to-detector distance (mm)	458.842
Rotation range per image (°)	2.0
Total rotation range (°)	182
Exposure time per image (s)	0.9
Space group	<i>P</i> 4 ₁ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	115.351, 115.351, 123.663
α , β , γ (°)	90.000, 90.000, 90.000
Mosaicity (°)	0.185
Resolution range (Å)	49.27–3.71
Total No. of reflections	130664
No. of unique reflections	9350
Completeness (%)	99.7 (99.4)
$\langle I/\sigma(I) \rangle$	27.56 (10.57)
<i>R</i> _{meas} (%)	6.4 (27.6)
Overall <i>B</i> factor from Wilson plot (Å ²)	90.178
Twinning analysis	
Statistics independent of twin laws	
$\langle I^2 \rangle / \langle I \rangle^2$	2.204
$\langle F \rangle^2 / \langle F^2 \rangle$	0.775
$\langle E^2 - 1 \rangle$	0.759
$\langle L \rangle$, $\langle L^2 \rangle$	0.476, 0.307
Multivariate <i>Z</i> -score <i>L</i> -test	1.082

The data exhibited severe anisotropy as indicated by the *Diffraction Anisotropy Server* at UCLA MBI (Strong *et al.*, 2006), as a consequence of which the data had to be cut at 3.7 Å resolution [for the 3.6 Å shell $\langle I/\sigma(I) \rangle = 5.24$ and *R*_{meas} = 46.6%]. Data analysis (Table 3) predicted the best possible space group to be *P*4₁2₁2. No twin laws are possible for this space group and the data statistics are shown in Table 3. Unit-cell content analysis suggested the asymmetric unit contains seven molecules (solvent content of 51.1% and *V*_M = 2.51 Å³ Da⁻¹; Matthews, 1968). The presence of an odd number of molecules in the asymmetric unit cell in spite of dimeric coiled-coil

Figure 3
X-ray diffraction image of the C-terminal domain of Par-4 with resolution rings.

formation indicates that the true number could be either six or eight. It could also be possible with three dimers and a crystallographic dimer. Selenomethionine labelling of the protein is aimed at obtaining experimental phases.

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