Crystallization and preliminary X-ray crystallographic analysis of the CARD domain of apoptosis repressor with CARD (ARC)

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Apoptosis repressor with caspase-recruiting domain (ARC) is an apoptosis repressor that inhibits both intrinsic and extrinsic apoptosis signalling. Human ARC contains an N-terminal caspase-recruiting domain (CARD domain) and a C-terminal proline- and glutamic acid-rich (P/E-rich) domain. The CARD domain in ARC is the domain that is directly involved in inhibition of the extrinsic pathway. In this study, the N-terminal CARD domain of ARC was overexpressed, purified and crystallized. X-ray diffraction data were collected to a resolution of 2.1 Å and the crystals were found to belong to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = 98.28$, $b = 98.28$, $c = 51.86$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Apoptosis is a cellular suicide mechanism that eliminates potentially dangerous and unnecessary cells in tissue homeostasis, development and immune responses (Rathmell & Thompson, 2002; Nagata, 1997). Failure to control apoptosis leads to serious diseases, including various cancers and neurodegenerative diseases (Andersen *et al.*, 2005; Evan & Vousden, 2001). There are two main pathways that lead to apoptosis: the intrinsic (mitochondrial) and extrinsic (cytoplasmic) pathways (Green, 1998, 2000).

Apoptosis repressor with caspase-recruiting domain (ARC) is a multifunctional modulator of apoptosis. This protein is known to be involved in various cancers and heart diseases upon overactivation (Zaiman *et al.*, 2011; Mercier *et al.*, 2008). ARC performs its function on both the intrinsic and extrinsic apoptosis pathways, which is unique (Ludwig-Galezowska *et al.*, 2011; Nam *et al.*, 2004). ARC inhibits the extrinsic pathway by interfering with DISC formation. The ARC caspase-recruiting domain (CARD domain) directly interacts with the death domains (DDs) of Fas and FADD, and with the death-effector domain (DED) of procaspase-8 (Nam *et al.*, 2004). ARC also inhibits the intrinsic pathway by direct interaction with the pro-apoptotic BCL-2 family member BAX (Gustafsson *et al.*, 2004). Human ARC contains an N-terminal CARD domain and a C-terminal proline- and glutamic acid-rich (P/E-rich) domain. The CARD domain is a protein-interaction module belonging to the death-domain superfamily, which includes the death domain (DD), death-effector domain (DED) and pyrin domain (PYD) (Bae & Park, 2011; Park, 2011, 2012).

Although structural information on ARC is important for understanding the regulatory mechanism of both the intrinsic and extrinsic apoptosis pathways, the structure of ARC has not been determined to date. In the current study, we overexpressed, purified and crystallized the N-terminal CARD domain of ARC as a first step towards elucidating its molecular structure and regulatory mechanism. X-ray diffraction data were collected to a resolution of 2.1 Å and the crystals were found to belong to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = 98.28$, $b = 98.28$, $c = 51.86$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$. Details of the high-resolution structure of this protein should enable us to understand the mechanism of inhibition of apoptosis by interfering with DISC formation.

Table 1
Macromolecule-production information.

Source organism	Human
DNA source	GenBank AAC34993
Forward primer	5'-GGGCATATGATGGGAAATGCTCAAGAACG-3'
Reverse primer	5'-GGGCTCGAGATGCTGCCAATCCCAAGC-3'
Cloning vector	pET-24a
Expression vector	pET-24a
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	MGNAQERPSSETIDRERMRLVETLQADSGLLLDALLARGVLTG-PEYEALDLPDAERRVRLRLVQKGGEAACQELLRCQAQR-TAGAPDPAWDWQHLEHHHHH

2. Materials and methods

2.1. Macromolecule production

The ARC CARD domain (amino-acid residues 1–95) was expressed in *Escherichia coli*. Full-length human ARC (GenBank ID AAH12798.1) was amplified by PCR using gene-specific primers containing NdeI and XhoI sites (Table 1). The PCR fragments were subsequently digested and ligated into the pET-24a vector containing a C-terminal hexahistidine tag. The sequences of the cloned genes were verified by DNA sequencing. Construction of the ARC CARD domain adds an eight-residue tag that includes six C-terminal histidine residues (LEHHHHHH). The resulting plasmids were individually transformed into *E. coli* BL21 (DE3) competent cells, after which the cells were plated onto Luria–Bertani (LB) medium and incubated for 24 h at 310 K. Individual colonies were inoculated into 5 ml LB medium and incubated overnight at 310 K with shaking. Cultured cells were then moved to 1 l LB medium and incubated for 4 h at 310 K with shaking, after which expression was induced by treating the bacteria with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 20 h at 293 K. Following induction, the bacteria were collected, resuspended and lysed by sonication in 50 ml lysis buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 10 mM imidazole). The bacterial lysate was subsequently centrifuged at 16 000 rev min^{−1} for 30 min at 277 K, after which the supernatant was applied onto a gravity-flow column (Bio-Rad) packed with 2 ml Ni–NTA affinity resin (Qiagen). The unbound bacterial proteins were subsequently removed from the column using 100 ml washing buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 60 mM imidazole). The target protein was eluted from the column using elution buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 250 mM imidazole), after which 0.6 ml elution fractions were collected over a total of 4.8 ml. Fractions containing greater than 90% homogenous protein as judged by SDS–PAGE were then selected and combined, after which the protein purity was further improved using a Superdex 200 10/30 size-exclusion column (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl pH 8.0, 150 mM NaCl. The target molecule, which eluted at 17–18 ml, was collected and concentrated to 8–9 mg ml^{−1}. The concentration was measured using a protein-assay kit (Bio-Rad) and was determined using the method of Bradford (1976). The extinction coefficient of the ARC CARD (amino-acid residues 1–95) is 12 615 M^{−1} cm^{−1}. Cloning details are provided in Table 1.

2.2. Crystallization

The initial conditions for crystallization were screened at 293 K by the hanging-drop vapour-diffusion method using screening kits from Hampton Research (Crystal Screen, Crystal Screen 2, Index HT, SaltRX, Natrix, MembFac and Crystal Screen Cryo) and from Rigaku (Wizard I, II, III and IV). 24-well crystallization plates from Hampton Research were used in the crystallization. Initial crystals were grown on a siliconized cover slip by equilibrating a mixture consisting of 1 μ l

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plates (Hampton Research)
Temperature (K)	293
Protein concentration (mg ml ^{−1})	8–9
Buffer composition of protein solution	20 mM Tris–HCl pH 8.0, 150 mM NaCl
Composition of the reservoir solution	0.6 M ammonium phosphate, 0.1 M imidazole pH 8.2
Volume and ratio of drop	2 μ l, 1:1
Volume of reservoir (μ l)	400

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	5C (SB II), PAL
Wavelength (Å)	0.97760
Temperature (K)	110
Detector	ADSC Quantum 315r
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	<i>P</i> 6 ₁ or <i>P</i> 6 ₃
<i>a</i> , <i>b</i> , <i>c</i> (Å)	98.28, 98.28, 51.86
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.5
Resolution range (Å)	50–2.1 (2.15–2.10)
Total No. of reflections	171665
No. of unique reflections	16721
Completeness (%)	99.1 (100)
Multiplicity	10.3 (8.2)
$\langle I/\sigma(I) \rangle$	63.94 (5.4)
$R_{\text{c.i.m}}^{\dagger}$ (%)	2.7 (24.2)

$\dagger$ Estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity.

protein solution (8–9 mg ml^{−1} protein in 20 mM Tris pH 8.0, 150 mM NaCl) and 1 μ l reservoir solution consisting of 1.0 M ammonium phosphate, 0.1 M imidazole pH 8.2 against 0.4 ml reservoir solution (Wizard I condition No. 36). The crystals were further refined by modifying the concentration and the pH of the precipitant. The final crystal used for data collection was produced in 0.6 M ammonium phosphate, 0.1 M imidazole pH 8.2. A summary of the crystallization is provided in Table 2.

2.3. Data collection and processing

For data collection, the crystals were cooled in liquid nitrogen. A level of 40% glycerol in the crystallization solution was sufficient to act as a cryoprotectant. Diffraction data sets were collected on beamline 5C (SB II) at the Pohang Accelerator Laboratory (PAL), Republic of Korea. The data set was indexed and processed using *HKL*-2000 (Otwinowski & Minor, 1997). Diffraction data statistics are given in Table 3.

3. Results and discussion

ARC, a multifunctional modulator of apoptosis, contains a CARD domain at the N-terminus. As mentioned in §1, the ARC CARD domain directly interacts with the death domain (DD) of Fas and FADD, and with the death-effector domain (DED) of procaspase-8. ARC CARD-mediated inhibition of DISC formation is an important process for controlling apoptosis. To understand ARC CARD-mediated protein interaction and its roles in the inhibition of apoptosis, we expressed and purified the ARC CARD domain using affinity chromatography followed by size-exclusion chromatography. The quick two-step chromatography produced >95% pure target

Figure 1
Purification of the ARC CARD domain by size-exclusion chromatography. Superdex S200 size-exclusion chromatographic analysis of the ARC CARD domain. An SDS-PAGE of fractions from the peak is shown.

Figure 2
Crystals of the ARC CARD domain. The crystals grew in 3 d in the presence of 0.6 M ammonium phosphate, 0.1 M imidazole pH 8.2. The approximate dimensions of the crystals were $0.2 \times 0.2 \times 0.1$ mm.

Figure 3
A diffraction image from an ARC CARD domain crystal with a 2.1 Å resolution limit.

protein, which was analyzed by SDS-PAGE (Fig. 1). ARC CARD is dimeric in solution, eluting at around 17 ml from a Superdex 200 size-exclusion column (Fig. 1). A size-exclusion standard (Bio-Rad) consisting of a mixture of molecular-weight markers [thyroglobulin (670 000 Da), globulin (158 000 Da), ovalbumin (44 000 Da), myoglobin (17 000 Da) and vitamin B₁₂ (1350 Da)] was used for size calibration.

Diffracting crystals of ARC CARD were successfully obtained owing to the generation of constructs of various lengths. The production of many different ARC CARD constructs were attempted, most of which were not well expressed. Only the construct that contained amino acids 1–95 expressed well. An initial crystal was obtained from 1.0 M ammonium phosphate, 0.1 M imidazole pH 8.2. The crystals were further refined by modifying the concentration of precipitant and the pH over a wide range. The best crystal was finally produced using 0.6 M ammonium phosphate, 0.1 M imidazole pH 8.2 (Fig. 2). The optimized crystals grew to dimensions of $0.2 \times 0.2 \times 0.1$ mm in 3 d and diffracted to 2.1 Å resolution (Fig. 3). The crystals were found to belong to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = 98.28$, $b = 98.28$, $c = 51.86$ Å, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 120^\circ$. Diffraction data statistics are shown in Table 3. The data set was indexed and processed using *HKL-2000* (Otwinowski & Minor, 1997).

Assuming the presence of one dimer in the crystallographic asymmetric unit, the Matthews coefficient (V_M) was calculated to be $3.07 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 59.99% (Matthews, 1968). The molecular-replacement (MR) phasing method was conducted with *Phaser* (McCoy, 2007) using the structure of the RAIDD CARD (PDB entry 3crd; Chou *et al.*, 1998), which has 21% amino-acid sequence homology with the ARC CARD, as a search model. We also conducted single-wavelength anomalous dispersion (SAD) phasing using *PHENIX* (Adams *et al.*, 2010), which is a more powerful phasing method for solving structures of CARD domains (Jang *et al.*, 2013). The structure of ARC CARD will provide valuable information regarding the mechanism of inhibition of apoptosis by interfering with DISC formation and the function of BAX.

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