Purification, crystallization and preliminary X-ray crystallographic studies of Drep2 CIDE domain

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Drep2 is a novel nuclease from the fruit fly that might have a similar function in apoptosis to DFF40 and DFF45, which are primary players in apoptotic DNA fragmentation. Drep2 contains a conserved CIDE domain of ~90 amino-acid residues that is involved in protein–protein interaction. In this study, the Drep2 CIDE domain was purified and crystallized by the hanging-drop vapour-diffusion method. X-ray diffraction data were then collected to a resolution of 2.3 Å. The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 50.28$, $b = 88.70$, $c = 113.37$ Å.

1. Introduction

Chromatin condensation and cleavage are hallmarks of apoptotic cells (Nagata, 2000; Liu *et al.*, 1997; Enari *et al.*, 1998; Park *et al.*, 2007). It is well known that DNA undergoes fragmentation and is cleaved into regular fragments with unit lengths of ~180 bp during apoptosis (Enari *et al.*, 1998; Liu *et al.*, 1997; Park, 2012). Apoptotic DNA fragmentation is primarily executed by DNA fragmentation factor DFF40 (CAD is the mouse orthologue), which is a novel nuclease that is inhibited by DFF45 (ICAD is the mouse orthologue) *via* tight interactions. Interestingly, DFF45 seems to function as a chaperone for DFF40 during its synthesis (Park, 2009). The N-termini of DFF40 and DFF45 contain a conserved CIDE domain, which is a protein-interaction module composed of about 90 amino-acid residues (Lugovskoy *et al.*, 1999; Wu *et al.*, 2008). CIDE-A, CIDE-B and CIDE-3 (also called FSP27 in the mouse) have been identified as CIDE-domain-containing proteins. Although all CIDE-domain-containing proteins are known to function in apoptosis, the function of CIDE-containing proteins has recently been broadened by the revelation of new roles in energy metabolism (Yonezawa *et al.*, 2011).

A search for CIDE-domain-containing proteins in *Drosophila melanogaster* revealed four proteins: Drep1, Drep2, Drep3 and Drep4 (Inohara & Nuñez, 1999). Although the functions of Drep proteins in the fruit fly have not been clearly identified, several studies have shown that Drep1 and Drep4 might be DFF45 and DFF40 orthologues, respectively (Lee & Park, 2014; Sakahira *et al.*, 1999). Recently, nuclease activity of Drep2 and Drep2-inhibitory activity of Drep3 have been reported (Park & Park, 2012). Unlike other CIDE domains, the Drep2 CIDE domain forms a large homooligomer in solution (Lee & Park, 2013) and it has been reported that Drep2 can interact with both Drep1 and Drep3 *via* the CIDE domain (Lee & Park, 2013). Structural studies of protein-interaction domains are particularly important in understanding the molecular pathways mediated by protein interactions (Park, 2011; Bae & Park, 2011). Although the interactions mediated by the CIDE domain are important in understanding the regulatory mechanism of apoptotic DNA fragmentation, structural information regarding the CIDE domains and their complexes is limited. To date, two CIDE-domain structures have been determined by NMR, including a complex structure between the DFF40 and DFF45 CIDE domains, which shows an α/β roll with two α -helices and five β -strands (Lugovskoy *et al.*, 1999; Otomo *et al.*, 2000). Despite the availability of the NMR structures, further structural studies of the CIDE domain are important to aid understanding of apoptotic DNA fragmentation. Structural investigations of the Drep2 CIDE domain will be particularly interesting because the Drep2 CIDE domain can form a

Table 1

Macromolecule-production information.

Source organism	<i>D. melanogaster</i>
DNA source	GenBank AF149795
Forward primer†	5'-GGGCATATGATGGCCAGAGAGGAGTCTCG-3'
Reverse primer‡	5'-GGGCTCGAGCCAGCGCTGCGCCTGCTTA-3'
Cloning vector	pET-26b
Expression vector	pET-26b
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	MAREESRGKRPLKIWDSWRNVRKGVVGTFEELLVRGKDLG-VPASEPVRVVLECDGTQIEDGEYFRTLANNVTLLLLRQGE-RWLEHHHHHHH

† The *NdeI* site is underlined. ‡ The *XhoI* site is underlined.

homo-oligomer, which has never been reported for other CIDE domains. In the current study, we overexpressed, purified and crystallized the Drep2 CIDE domain as a first step towards elucidating its molecular structure and regulatory mechanism. X-ray diffraction data were collected to a resolution of 2.3 Å and the crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 50.28$, $b = 88.70$, $c = 113.37$ Å. Details regarding the structure of this domain will hopefully enable us to understand the regulatory mechanism of apoptotic DNA fragmentation and the homo-oligomerization mechanism of the CIDE domain.

2. Materials and methods

2.1. Macromolecule production

Drep2 CIDE (amino-acid residues 1–84) was amplified by PCR using gene-specific primers containing *NdeI* and *XhoI* sites (Table 1). Full-length Drep2 from *D. melanogaster* (GenBank ID AF149795) was used as the DNA template. PCR fragments were subsequently digested and ligated into the pET-26b vector containing a C-terminal hexahistidine tag. The recombinant sequence was verified by DNA sequencing. Construction of the Drep2 CIDE domain adds an eight-residue tag that includes six C-terminal histidine residues (LEHHHHHH). The expression vector was transformed into *Escherichia coli* BL21 (DE3) competent cells. Individual colonies were then inoculated into 5 ml LB medium and incubated overnight

Figure 1

Purification of Drep2 CIDE domain by gel-filtration chromatography. Superdex S-200 gel-filtration column analysis of Drep2 CIDE domain. An SDS-PAGE of the fractions from the peak of the Drep2 CIDE domain is shown.

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plates from Hampton Research
Temperature (°C)	20
Protein concentration (mg ml ⁻¹)	6–8
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 500 mM NaCl
Composition of reservoir solution	0.4 M formate dihydrate, 0.2 M NDSB-221, 0.1 M bis-tris pH 5.0
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (µl)	500

at 37°C with shaking. Cultured cells were then transferred to 1 l LB medium and incubated for 4 h at 37°C with shaking, after which expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 h at 20°C. 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 4°C, 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 subsequently eluted from the column using elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 250 mM imidazole) and 0.6 ml elution fractions were collected over a total of 6 ml. Fractions containing greater than 90% homogeneous proteins, based upon SDS-PAGE analysis, were pooled and loaded onto a Superdex 200 gel-filtration column 10/30 (GE Healthcare) pre-equilibrated with a solution consisting of 20 mM Tris-HCl pH 8.0, 500 mM NaCl. Purification procedures are given in Table 1.

2.2. Crystallization

The initial conditions for crystallization were screened at 20°C 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 the deCODE Biostructure Group (Wizard I, II, III and IV). 24-Well crystallization plates from Hampton Research were used and initial crystals were grown on a siliconized coverslip by equilibrating a mixture consisting of 1 µl protein solution (6–8 mg ml⁻¹ protein in 20 mM Tris-HCl pH 8.0, 500 mM NaCl) and 1 µl reservoir solution

Figure 2

Crystal of Drep2 CIDE domain. The crystal grew in 3 d in the presence of 0.4 M formate dihydrate, 0.2 M NDSB-221, 0.1 M bis-tris pH 5.0. The approximate dimensions of the crystal were 0.05 × 0.05 × 0.3 mm.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	5C (SBII) at PAL
Wavelength (Å)	0.97951
Temperature (°C)	−163
Detector	ADSC Quantum 315r
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	2
Space group	$P2_12_12_1$
a, b, c (Å)	50.28, 88.70, 113.37
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.6
Resolution range (Å)	50–2.3
Total No. of reflections	60122
No. of unique reflections	10489
Completeness (%)	95.4 (98.3)
Multiplicity	9.7 (5.9)
$\langle I/\sigma(I) \rangle$	55.8 (3.8)
R_{merge}	5.6 (28.4)

against 0.5 ml reservoir solution. The condition 0.1 M bis-tris pH 5.5, 0.3 M formate dihydrate produced an initial crystal but it did not grow properly and diffracted to only 8–10 Å resolution. Optimization of the buffer and the precipitant and the use of an additive screen (Hampton Research) finally produced a long, stick-shaped crystal with 0.1 M bis-tris pH 5.0, 0.4 M formate dihydrate, and 0.2 M non-detergent sulfobetaine 221 (NDSB-221), a zwitterionic compound, as an additive. Crystals appeared in 3 d and grew to maximum dimensions of 0.05 × 0.05 × 0.3 mm. This crystal diffracted to 2.3 Å resolution. A summary of the crystallization is provided in Table 2.

2.3. Data collection and processing

For data collection, the crystals were flash-cooled in liquid nitrogen using 35% glycerol in the crystallization solution as a cryoprotectant.

Figure 3

A diffraction image from the Drep2 CIDE domain crystal with a 2.3 Å resolution limit.

Diffraction data sets were collected on beamline 5C (SB II) of 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

To understand CIDE-mediated protein interaction and its roles in apoptotic DNA fragmentation and energy metabolism, we expressed and purified the Drep2 CIDE domain using affinity chromatography followed by gel-filtration chromatography. The quick two-step chromatography produced highly pure Drep2 CIDE which showed no contaminating bands on SDS-PAGE (Fig. 1). Drep2 CIDE elutes at around 120 kDa from a Superdex 200 gel-filtration column calibrated with a gel-filtration standard (Bio-Rad) containing a mixture of thyroglobulin (670 000 Da), globulin (158 000 Da), ovalbumin (44 000 Da), myoglobin (17 000 Da) and vitamin B₁₂ (1350 Da) (Fig. 1). Assuming that the complex bands contain ~20 µg protein and the detection limit of SDS-PAGE is 0.1 µg, the purity of the complex was >99%. Since the calculated molecular weight of monomeric Drep2 CIDE, including the additional residues at the C-terminus, was 10 864.4 Da, it is clear that Drep2 CIDE exists as a large oligomeric complex in solution (Fig. 1).

An initial crystal was obtained from 0.1 M bis-tris pH 5.5, 0.3 M formate dihydrate, although it only diffracted poorly to 8–10 Å resolution. Better crystals were obtained using an additive screen (Hampton Research), and the use of 0.2 M NDSB-221 produced long, stick-shaped crystals (Fig. 2) that grew to dimensions of 0.05 × 0.05 × 0.3 mm in 3 d and diffracted to 2.3 Å resolution (Fig. 3). The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 50.28$, $b = 88.70$, $c = 113.37$ Å. Diffraction data statistics are shown in Table 3.

Molecular replacement with *Phaser* (McCoy *et al.*, 2007) and the CIDE domain of human CIDE-B (PDB entry 1d4b; 29% sequence identity to Drep2 CIDE; Lugovskoy *et al.*, 1999) as a search model gave a clear solution including four chains in the asymmetric unit. This corresponds to a Matthews coefficient (V_M) of 2.77 Å³ Da^{−1} and a solvent content of 55.6% (Matthews, 1968). Preliminary refinement with *REFMAC5* (Murshudov *et al.*, 2011) using the initial *Phaser* model gave an R_{work} of 32.8% and an R_{free} of 36.7%. Further structural refinement is in progress.

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