Filament-like DREP4 CIDE domain: characterization and preliminary X-ray crystallographic studies

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DREP4 is a nuclease from fruit fly that is involved in apoptotic DNA fragmentation. DREP4 contains a conserved CIDE domain that acts as a protein-interaction module and is critical for its function. In this study, it was found that DREP4 CIDE domains form filament-like structures in solution. The length of the highly ordered filament-like structure is dependent on the salt concentration. By adjusting the salt concentration the DREP4 CIDE domain could be crystallized, and X-ray diffraction data were collected to a resolution of 1.9 Å. The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 53.08$, $b = 76.58$, $c = 174.59$ Å.

1. Introduction

Chromatin cleavage is a hallmark of apoptotic cells (Nagata, 2000; Liu *et al.*, 1997; Enari *et al.*, 1998). During apoptosis, DNA undergoes fragmentation, producing regular fragments with unit lengths of ~180 base pairs (Enari *et al.*, 1998; Liu *et al.*, 1997). Although several nucleases have been identified, DNA fragmentation factor DFF40, a novel nuclease inhibited by DFF45 *via* tight interactions, is the main nuclease responsible for apoptotic DNA fragmentation. Under normal conditions, the nuclease activity of DFF40 is inhibited by its inhibitor DFF45 through direct interaction. However, once the apoptosis process has been triggered, DFF45 is cleaved and dissociates from DFF40. Free DFF40 oligomerizes into its active form, which fragments DNA (Liu *et al.*, 1997, 1999). The N-termini of DFF40 and DFF45 contain a conserved CIDE domain, which is a protein-interaction module (Lugovskoy *et al.*, 1999; Wu *et al.*, 2008). The CIDE domain in DFF proteins is important for both hetero-oligomerization between DFF40 and DFF45 for inhibitory processes, and homo-oligomerization of DFF40 to generate nuclease activity (Liu *et al.*, 1997).

DREP4 is the fruit-fly orthologue of DFF40; the nuclease activity of DREP4 is inhibited by DREP1, which is the fruit-fly orthologue of DFF45 (Inohara & Nuñez, 1999; Mukae *et al.*, 2000). DREP4 and DREP1 also interact *via* their CIDE domains. In addition to DREP4 and DREP1, two further CIDE domain-containing proteins, DREP2 and DREP3, have been identified in the fruit fly (Lee & Park, 2013, 2014). Although the functions of DREP2 and DREP3 in the cell remain unclear, a recent study showed that DREP2 has nuclease activity and is inhibited by DREP3 by tight interactions *via* the CIDE domain (Park & Park, 2012).

Unlike other CIDE domains, the DREP4 CIDE domain forms a large homo-oligomer in solution (Lee & Park, 2014). Although CIDE-domain-mediated control of the nuclease

Table 1

Macromolecule-production information.

Source organism	<i>D. melanogaster</i>
DNA source	GenBank AAF03220
Forward primer†	5'-GGGCATATGGAATCTCGTGGTACCGCG-3'
Reverse primer‡	5'-GGGCTCGAGTTTTCGAATTCGAAGTCCG-3'
Cloning vector	pOKD5
Expression vector	pOKD5
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	SRGTAGAPMRGYKVTDNERTKYGIGANSLEMLI-AKAKSKFPLLEPHLYLASDGFVSDDEYLKSL-PAQTLFIVSGPDAVITTDADFEFEKMLEHHHH-HHH

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

involved in apoptotic DNA fragmentation is important in understanding the regulatory mechanism of this process, structural information regarding the CIDE domain and its complexes is limited. Although several CIDE-domain structures showing an α/β roll with two α -helices and five β -strands have been reported, further structural studies, particularly of higher-ordered oligomeric structures, are important in order to understand the activity of DFF and its role in apoptotic DNA fragmentation. Structural studies of the DREP4 CIDE domain are of particular interest because the DREP4 CIDE domain forms a homo-oligomer. Determining this structure would provide insight into the homo-oligomerization-based control of DREP4.

In the current study, the purified DREP4 CIDE domain was characterized and X-ray crystallographic analysis was performed using crystals of the DREP4 CIDE domain. The DREP4 CIDE domain formed a higher-order homo-oligomeric state in solution; oligomerization was dynamically regulated by the salt concentration. Further electron-microscopic analysis showed that the DREP4 CIDE domain forms a filament-like structure. The homo-oligomeric complex of the DREP4 CIDE domain, the size of which could be adjusted by the salt concentration, was crystallized and X-ray diffraction data were collected to a resolution of 1.9 Å. The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 53.08$, $b = 76.58$, $c = 174.59$ Å.

2. Materials and methods

2.1. Macromolecule production

The DREP4 CIDE domain (amino-acid residues 39–130) was amplified by PCR using gene-specific primers containing NdeI and XhoI sites (Table 1). Full-length DREP4 from *Drosophila melanogaster* (GenBank ID AAF03220) was used as the DNA template. PCR fragments were digested and ligated into a home-made pOKD vector. Construction of the DREP4 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 inoculated into 6 ml LB medium and incubated overnight at 37°C with shaking. Cultured cells were then moved 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-thiogalactopyranoside for 18 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, 25 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, Hercules, California, USA) packed with 2 ml Ni-NTA affinity resin (Qiagen, Hilden, Germany). The unbound bacterial proteins were washed out with 50 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 8 ml. Fractions containing greater than 95% homogenous protein, based on SDS-PAGE analysis, were pooled and loaded onto a Superdex 200 gel-filtration column 10/30 (GE Healthcare, Little Chalfont, England) pre-equilibrated with 20 mM Tris-HCl pH 8.0 and 150 mM NaCl (or 500 mM NaCl). The target protein was concentrated using an Amicon filter (Merck Millipore, Billerica, Massachusetts, USA) until the concentration of the protein reached 10 mg ml⁻¹. Expression details are given in Table 1.

2.2. Multi-angle light scattering

The molar mass of the DREP4 CIDE domain was determined by multi-angle light scattering (MALS). The purified DREP4 CIDE domain was reloaded onto a Superdex 200 HR 10/30 gel-filtration column (GE Healthcare) that had been pre-equilibrated in buffer consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl or 20 mM Tris-HCl pH 8.0, 1 M NaCl. An ÄKTA chromatography system (GE Healthcare) was coupled to a MALS detector (mini-DAWN TREOS) and a refractive-index detector (Optilab DSP; Wyatt Technology, Woburn, Massachusetts, USA).

2.3. Electron microscopy

The DREP4 CIDE domain protein sample after size-exclusion chromatography purification that contained 150 mM NaCl was diluted to 0.1 mg ml⁻¹. For negative staining, 10 μ l of sample was placed onto a glow-discharged copper grid, stained with 1% uranyl formate pH 4.5 for 30 s and air-dried. The grids were imaged using a Tecnai G² Spirit BioTWIN transmission electron microscope (FEI Company, Hillsboro, Oregon, USA) and were recorded with a 2k CCD camera (Advanced Microscopy Techniques, Woburn, Massachusetts, USA) at the Harvard Medical School Electron Microscopy Facility.

2.4. Crystallization

Crystallization was performed at 293 K by the hanging-drop vapour-diffusion method using various screening kits (Crystal Screen, Crystal Screen 2, Index HT, SaltRX, Natrix, MembFac and Crystal Screen Cryo from Hampton Research, Aliso Viejo, California, USA, and Wizard I, II, III and IV from

deCODE Biostructure Group, Reykjavik, Iceland). 24-well crystallization plates were used and the initial crystals were grown on a siliconized cover slip by equilibrating a mixture consisting of 1 μ l protein solution (10 mg ml⁻¹ protein in 20 mM Tris pH 8.0, 500 mM NaCl) and 1 μ l reservoir solution (condition No. 48 of Index HT from Hampton Research) against 0.5 ml reservoir solution. A reservoir condition consisting of 0.1 M Tris-HCl pH 8.5, 20 mM ammonium phosphate monobasic produced a good-quality crystal for further structural analysis. Crystals appeared in 3 d and grew to maximum dimensions of 0.5 $\times$ 0.2 $\times$ 0.2 mm. The crystal diffracted to 1.9 Å resolution. A summary of the crystallization conditions is provided in Table 2.

2.5. Data collection and processing

For data collection, the crystals were flash-cooled in liquid nitrogen using 40% glycerol in the crystallization solution as a cryoprotectant. A diffraction data set was collected on beamline 5C (SB II) of Pohang Accelerator Laboratory (PAL), Republic of Korea. The data set was indexed and

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plates from Hampton Research
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris pH 8.0, 500 mM NaCl
Composition of reservoir solution	0.1 M Tris-HCl pH 8.5, 20 mM ammonium phosphate monobasic
Volume and ratio of drop	2 μ l, 1:1
Volume of reservoir (μ l)	500

processed using *HKL-2000* (Otwinowski & Minor, 1997). Diffraction data statistics are shown in Table 3.

3. Results and discussion

To understand the functional correlation between CIDE domain-mediated oligomerization and the activity of apoptotic DNA fragmentation factors, the DREP4 CIDE domain was expressed and purified using a rapid two-step

Figure 1
(a) Size-exclusion chromatography profiles of various CIDE domains. (b, c) Multi-angle light scattering (MALS) results for the DREP4 CIDE domain under a low-salt condition (150 mM NaCl) (b) and a high-salt condition (1 M NaCl) (c). The red line indicates the experimental molecular weight. (d) Electron-microscopy image of negatively stained DREP4 CIDE domain.

chromatography method consisting of affinity chromatography and size-exclusion chromatography. The DREP4 CIDE domain eluted from a Superdex 200 gel-filtration column at approximately 400 kDa, indicating that the DREP4 CIDE domain exists as a highly homo-oligomeric complex in solution (Fig. 1*a*).

The stoichiometry of CIDE domains in solution varies in a protein-dependent manner and DREP4 is the most highly oligomeric form (Fig. 1*a*). During the characterization of the DREP4 CIDE domain for structural analysis, the oligomeric state of the DREP4 CIDE domain was found to alter in a salt concentration-dependent manner; higher oligomers formed at a low salt concentration (150 mM NaCl), while lower oligomers formed at a high salt concentration (1 M NaCl). Accurate oligomeric verification was conducted by MALS. The calculated molecular weight of the monomeric DREP4 CIDE domain, including the C-terminal His tag, was 11.5 kDa and the experimental molecular weights from MALS were 463.9 kDa (0.5% fitting error) in the 150 mM NaCl condition and 61.5 kDa (1.3% fitting error) in the 1 M NaCl condition (Figs. 1*b* and 1*c*). Size-exclusion chromatography and MALS analyses indicated that the DREP4 CIDE domain formed a higher oligomeric complex (containing approximately 40

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 5C (SB II), PAL
Wavelength (Å)	1.0000
Temperature (K)	110
Detector	ADSC Quantum 315r
Crystal-to-detector distance (mm)	200
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)	53.08, 76.58, 174.59
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.6
Resolution range (Å)	50–1.90 (1.93–1.90)
Total No. of reflections	410359
No. of unique reflections	56928
Completeness (%)	100 (100)
Multiplicity	7.2 (7.3)
$\langle I/\sigma(I) \rangle$	35.1 (3.6)
$R_{\text{merge}}^\dagger$ (%)	8.4 (70.6)

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

molecules) under the 150 mM NaCl condition and that the oligomer size was reduced on increasing the salt concentration (to approximately five molecules in 1 M NaCl). This oligomeric alteration may be specific to the CIDE domain. The most highly oligomeric DREP4 CIDE domain under the 150 mM NaCl condition was evaluated by electron microscopy to determine the overall structure of the homo-oligomeric complex. As shown in Fig. 1(*d*), the homo-oligomeric complex appeared as a filament structure, which agrees with previous analyses showing that DFF40 is activated by homo-oligomerization.

Based on this preliminary characterization of the DREP4 CIDE domain, the domain was crystallized at various salt

Figure 2

Crystallization of the DREP4 CIDE domain. (*a*) Profile from size-exclusion chromatography performed in a buffer consisting of 20 mM Tris-HCl, 500 mM NaCl. A stained SDS-PAGE gel including the peak elution peaks is shown. (*b*) Crystals grew in 3 d in the presence of 0.1 M Tris-HCl pH 8.5, 20 mM ammonium phosphate monobasic. The approximate dimensions of the crystals were $0.5 \times 0.2 \times 0.2$ mm.

Figure 3

Diffraction image from a DREP4 CIDE domain crystal, with a 1.9 Å resolution limit.

concentrations. As expected, the best crystal was obtained using the protein sample solubilized in a buffer containing a relatively high salt concentration of 500 mM NaCl (Fig. 2*a*) from a screening solution consisting of 0.1 M Tris–HCl pH 8.5, 20 mM ammonium phosphate monobasic. The crystal grew to dimensions of $0.5 \times 0.2 \times 0.2$ mm in 3 d (Fig. 2*b*). A diffraction data set was then collected to 1.9 Å resolution (Fig. 3). The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 53.08$, $b = 76.58$, $c = 174.59$ Å. Diffraction data statistics are shown in Table 3.

Assuming the presence of six molecules in the crystallographic asymmetric unit, the Matthews coefficient was calculated to be $2.53 \text{ Å}^3 \text{ Da}^{-1}$, with a solvent content of 51.4% (Matthews, 1968). Molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the CIDE domain of human CIDE-B (PDB entry 1d4b; 32% sequence identity to DREP4 CIDE; Lugovskoy *et al.*, 1999) as a search model gave a clear solution including six chains in the asymmetric unit. Preliminary refinement with *REFMAC5* (Mushudov *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 under way.

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References

- Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. & Nagata, S. (1998). *Nature (London)*, **391**, 43–50.
- Inohara, N. & Núñez, G. (1999). *Cell Death Differ.* **6**, 823–824.
- Lee, S. M. & Park, H. H. (2013). *FEBS Lett.* **587**, 854–859.
- Lee, S. M. & Park, H. H. (2014). *Apoptosis*, **19**, 428–435.
- Liu, X., Zou, H., Slaughter, C. & Wang, X. (1997). *Cell*, **89**, 175–184.
- Liu, X., Zou, H., Widlak, P., Garrard, W. & Wang, X. (1999). *J. Biol. Chem.* **274**, 13836–13840.
- Lugovskoy, A. A., Zhou, P., Chou, J. J., McCarty, J. S., Li, P. & Wagner, G. (1999). *Cell*, **99**, 747–755.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Mukae, N., Yokoyama, H., Yokokura, T., Sakoyama, Y., Sakahira, H. & Nagata, S. (2000). *J. Biol. Chem.* **275**, 21402–21408.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst. D* **67**, 355–367.
- Nagata, S. (2000). *Exp. Cell Res.* **256**, 12–18.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Park, O. K. & Park, H. H. (2012). *FEBS Lett.* **586**, 3085–3089.
- Wu, C., Zhang, Y., Sun, Z. & Li, P. (2008). *BMC Evol. Biol.* **8**, 159.