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Crystallization and preliminary X-ray crystallo-
graphic studies of the CIDE-N domain of CIDE-3

The CIDE-3 protein plays a critical role in lipid metabolism by its involvement in lipid droplet formation. CIDE-3 contains two conserved cell-death-inducing DFF45-like effector (CIDE) domains (CIDE-N at the N-terminus and CIDE-C at the C-terminus) of ~90 amino-acid residues that are involved in protein–protein interaction. In this study, the CIDE-N domain of CIDE-3 was purified and crystallized by the hanging-drop vapour-diffusion method and X-ray diffraction data were collected from the crystals to a resolution of 2.0 Å. The crystals were found to belong to space group $P3_2$, with unit-cell parameters $a = b = 63.35$, $c = 37.60$ Å, $\gamma = 120^\circ$.

1. Introduction

The CIDE domain is a protein-interaction module of ~90 amino-acid residues that is particularly involved in apoptosis (Yonezawa *et al.*, 2011). The apoptotic DNA fragmentation proteins DFF40 and DFF45 are well known CIDE-containing proteins (Park, 2009; Liu *et al.*, 1999) that execute DNA fragmentation, which is a biochemical hallmark of apoptotic cell death. DNA fragmentation factor DFF40 (mouse homologue CAD) is a novel nuclease, the activity of which is inhibited by DFF45 (mouse homologue ICAD) *via* a tight interaction using a CIDE domain (Zhou *et al.*, 2001; Thomas *et al.*, 2000; Lee *et al.*, 2013).

CIDE-3 (also known as FSP27 in mouse) is a member of the CIDE-containing protein family that was initially found to activate apoptosis (Liang *et al.*, 2003). Overexpression of CIDE-3 results in its localization to the mitochondria, where it induces DNA fragmentation and apoptosis in mammalian cells (Liang *et al.*, 2003). Interestingly, it has recently been revealed that CIDE-3 is tightly linked to energy metabolism, insulin sensitivity and lipid storage (Zhou *et al.*, 2003; Yonezawa *et al.*, 2011). CIDE-3 is highly expressed in white adipose tissue and is found in lipid droplets (LDs) and the endoplasmic reticulum (ER) (Gummesson *et al.*, 2007). In agreement with this finding, the most recent studies have shown that CIDE-3 is increased in a PPAR (peroxisome proliferator-activated receptor) γ -dependent manner during adipocyte differentiation (Puri *et al.*, 2008). In addition, overexpression of CIDE-3 induced the accumulation of this protein at LDs and enhancement of the LD size, while knockout of CIDE-3 decreased the LD size (Nishino *et al.*, 2008; Kim *et al.*, 2008). These results indicate that CIDE-3 is particularly important for the growth of LDs in adipocytes for energy metabolism and lipid storage processes. Two distinct functions of CIDE-3 in apoptosis and metabolism might be derived by its cellular localization.

CIDE-3 contains two conserved CIDE domains at the N-terminus (CIDE-N) and C-terminus (CIDE-C). It is well known that the CIDE-N domain of CIDE-3 is sufficient for LD growth (Lugovskoy *et al.*, 1999). CIDE-3 can also directly interact with CIDE-A (another CIDE-containing protein) *via* the CIDE-C domain, but not *via* the CIDE-N domain (Lugovskoy *et al.*, 1999). Several CIDE domain structures have been identified by NMR, including the structure of the complex 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).

In the current study, we overexpressed, purified and crystallized the CIDE-N domain of mouse CIDE-3 (also called FSP27) as a first

Table 1

Diffraction data for crystals of the CIDE-N domain of CIDE-3.

Values in parentheses are for the outermost resolution shell.

No. of crystals	1
Beamline	5C (SB II), PAL
Wavelength (Å)	0.97942
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
Resolution range (Å)	50–2.0
Space group	$P3_2$
Unit-cell parameters (Å, °)	$a = b = 63.35$, $c = 37.60$, $\gamma = 120$
Mosaicity (°)	0.8
Total No. of measured intensities	57364
No. of unique reflections	11299
Multiplicity	5.1 (5.0)
Mean $I/\sigma(I)$	27.5 (2.9)
Completeness (%)	98.9 (99.4)
$R_{\text{merge}}^{\dagger}$ (%)	12.6 (78.8)

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

step towards the elucidation of its molecular structure and its functions in apoptosis and energy metabolism. X-ray diffraction data were collected to a resolution of 2.0 Å and the crystals were found to belong to space group $P3_2$, with unit-cell parameters $a = b = 63.35$, $c = 37.60$ Å, $\gamma = 120^\circ$. Details of the high-resolution structure of CIDE-3 should enable us to understand the molecular mechanism involved in apoptosis *via* apoptotic DNA fragmentation and drive advances in therapeutic intervention in metabolic disorders such as diabetes and obesity.

2. Materials and methods

2.1. Protein expression and purification

The CIDE-N domain of CIDE-3 was expressed in *Escherichia coli*. The CIDE-N domain of CIDE-3 (corresponding to amino-acid residues 32–120) was amplified by PCR using gene-specific primers containing *NdeI* and *XhoI* sites, after which the PCR fragments were digested and ligated into a home-made pOKD5 vector (Dzivenu *et al.*, 2004) containing a C-terminal hexahistidine tag. The sequences of

the cloned genes were then verified by DNA sequencing. Construction of the CIDE-N domain of CIDE-3 adds an eight-residue tag that includes six C-terminal histidine residues (LEHHHHHH). The resulting plasmids were transformed into *E. coli* BL21 (DE3) competent cells individually, which were subsequently plated onto Luria–Bertani (LB) medium and incubated for 24 h at 310 K. Single colonies were then inoculated in 5 ml LB medium and incubated overnight at 310 K with shaking. Next, cultured cells were moved to 1 l LB medium and incubated for 4 h at 310 K with shaking. Expression was then induced by treating the bacteria with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 18 h at 293 K. Next, 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 40 min at 277 K, after which the supernatant fraction 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 80 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) and 0.6 ml elution fractions were collected over a total of 6 ml. Fractions containing more than 95% homogeneous proteins (as shown by SDS–PAGE) were then selected and combined, after which the protein purity was further improved using a Superdex 200 gel-filtration column 10/30 (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl pH 8.0, 500 mM NaCl. A gel-filtration standard (Bio-Rad) containing a mixture of molecular-mass markers (thyroglobulin, 670 000 Da; globulin, 158 000 Da; ovalbumin, 44 000 Da; myoglobin, 17 000 Da; vitamin B₁₂, 1350 Da) was used for size calibration.

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 Emerald BioSystems (Wizard I, II, III and IV). Initial crystals were grown on a siliconized cover slip by equilibrating a mixture consisting of 1 μ l protein solution (7.5 mg ml^{−1} protein in 20 mM Tris pH 8.0, 500 mM NaCl) and 1 μ l reservoir solution consisting of 25% PEG 1500, MMT buffer/NaOH pH 6.5 (screening solution No. 19 of Wizard IV from Emerald BioSystems) against 0.5 ml reservoir solution. This crystal did not grow properly and diffracted only poorly. The crystals were further refined by changing the concentration of the precipitant and the pH. The best crystal was grown using 28% PEG 1500, MMT buffer/NaOH pH 6.2. Under these conditions, crystals appeared in 3 d and grew to maximum dimensions of 0.05 $\times$ 0.05 $\times$ 0.5 mm. This crystal diffracted to 2.0 Å resolution.

2.3. Crystallographic data collection

The crystals were cooled in liquid nitrogen for subsequent data collection. A level of 30% glycerol in the crystallization solution was sufficient for use as a cryoprotectant. 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 1.

Figure 1
Purification of the CIDE-N domain of CIDE-3 by gel-filtration chromatography. The positions of the molecular-mass markers are labelled in kDa. Inset, SDS–PAGE of fractions from the peak of the CIDE-N domain of CIDE-3.

Figure 2

Crystal of the CIDE-N domain of CIDE-3. Crystal grew in 3 d in the presence of 28% PEG 1500, MMT buffer/NaOH pH 6.2. The approximate dimensions of the crystal were $0.05 \times 0.05 \times 0.5$ mm.

3. Results and discussion

Proteins from the death domain superfamily, including death domains (DDs), death effector domains (DEDs), caspase-recruitment domains (CARDs), pyrin domain (PYDs) and CIDE domains, are well known protein-interaction modules (Bae & Park, 2011; Park, 2011; Park *et al.*, 2007). CIDE-domain-containing proteins, such as CIDE-A, CIDE-B, CIDE-3, DFF40 and DFF45, function specifically in the processes of apoptosis and energy metabolism. To understand CIDE-mediated protein interaction and its roles in apoptotic DNA fragmentation and energy metabolism, we expressed and purified the CIDE-N domain of CIDE-3 using affinity chromatography followed by gel-filtration chromatography. Quick two-step chromatography produced the CIDE-N domain of CIDE-3 with 95% purity as analyzed by SDS-PAGE (Fig. 1). The CIDE-N domain of CIDE-3 was detected as a dimer in solution, eluting at around 18–20 kDa from a Superdex 200 gel-filtration column (Fig. 1). No contaminating bands were visible upon SDS-PAGE analysis. Since the calculated

molecular weight of the monomeric CIDE-N domain of CIDE-3, including the additional residues at the C-terminus, was 10 184 Da, the elution peak from gel-filtration chromatography suggests that the CIDE-N domain of CIDE-3 exists as a dimer in solution (Fig. 1). The purified target protein was used in crystallization trials with many crystal screening solutions. The success in obtaining diffracted crystals of the CIDE-N domain of CIDE-3 was the result of producing constructs of various lengths. Many different CIDE-N domains of CIDE-3 constructs were attempted, most of which led to crystals that diffracted to around 6–7 Å resolution. An initial diffracting crystal was obtained from 25% PEG 1500, MMT buffer/NaOH pH 6.5 (screening solution No. 19 of Wizard IV from Emerald BioSystems). The crystals were further refined by modifying the precipitant concentration and the pH. A suitable crystal was finally produced using 28% PEG 1500, MMT buffer/NaOH pH 6.2 (Fig. 2). The optimized crystals grew to dimensions of $0.05 \times 0.05 \times 0.5$ mm in 3 d and diffracted to 2.0 Å resolution (Fig. 3). The crystals were found to belong to space group $P3_2$, with unit-cell parameters $a = b = 63.35$, $c = 37.60$ Å, $\gamma = 120^\circ$. Diffraction data statistics are shown in Table 1.

Assuming the presence of one homodimer in the crystallographic asymmetric unit, the Matthews coefficient (V_M) was calculated to be $2.17 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 43.35% (Matthews, 1968). The diffraction data statistics are given in Table 1. The data set was indexed and processed using *HKL-2000* (Otwinowski & Minor, 1997). The molecular-replacement (MR) phasing method was used with the program *Phaser* (McCoy, 2007) and a CIDE domain from human CIDE-B (PDB entry 1d4b; Lugovskoy *et al.*, 1999), which has 31% amino-acid sequence homology to the CIDE-N domain of CIDE-3, as a search model. Probable MR solutions with rotation-function and translation-function Z-scores of 6.1 and 10.2, respectively, for one monomer and of 6.8 and 21.8, respectively, for the second monomer were initially obtained. Initial refinement with *REFMAC5* (Murshudov *et al.*, 2011) using the initial *Phaser* model gave an R_{work} of 38.9% and an R_{free} of 43.5%. The structure was further refined and is reported in Lee *et al.* (2013).

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Figure 3

A diffraction image from the CIDE-N domain of the CIDE-3 crystal with a 2.0 Å resolution limit.

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