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Expression, purification, crystallization of fragments from the C-terminal region of DFF45/ICAD

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DFF45/ICAD, which forms a heterodimer with DFF40/CAD as its DNase inhibitor and chaperone, plays a key role in nuclei DNA fragmentation in apoptosis. Several fragments from the C-terminal region of DFF45/ICAD have been cloned and expressed in *Escherichia coli* as His-tagged proteins. After purification to homogeneity, the recombinant proteins of three fragments were crystallized by the hanging-drop vapor-diffusion method. Of these, a crystal of DFF45c1 diffracted to 3.4 Å in a capillary at 277 K and crystals of DFF45c2 diffracted to 3.2 Å at cryotemperature using synchrotron radiation.

1. Introduction

Degradation of the nuclear DNA into nucleosomal fragments is considered to be an important part of the apoptosis cascade. This event is induced by DNA fragmentation factor (DFF), which is a heterodimeric protein consisting of two subunits: the inhibitor of DFF40/CAD, named DFF45/ICAD (45 kDa), and DNA fragmentation factor, which is a caspase-activated deoxyribonuclease named DFF40/CAD (40 kDa) (Liu *et al.*, 1997; Enari *et al.*, 1998). DFF45, which is also a molecular chaperone of DFF40, assists the folding DFF40 into the correct form as well as inhibiting its nuclease activity (Liu *et al.*, 1999). Caspases, especially the member caspase-3, are upper apoptosis factors which can cleave the 331-residue DFF45 into three fragments at the sites of residues 117 and 224. The cleavage dissociates DFF45 from DFF40; DFF40 then forms an oligomeric protein to digest the nuclei DNA into 50–300 kbp fragments (Liu *et al.*, 1997, 1999). These three fragments of DFF45 are recognized as separate conserved domains. The structure of the N-terminal domain (NTD) of DFF45 (amino acids 1–116) has been solved (Zhou *et al.*, 2001). The result showed that the NTD of DFF45 interacts with the NTD of DFF40 and initiates the correct folding of the DFF40–DFF45 complex. The solution structure of an extreme C-terminal domain of DFF45 (amino acids 225–307) was also solved recently, revealing a molecular surface with many negatively charged residues with the potential to interact with the positively charged nuclease catalytic domain of DFF40 (Fukushima *et al.*, 2002). Until now, the structure of the middle DFF45 domain (animo acids 117–224) has remained unknown.

It is most likely that the DFF45 C-terminal domain (amino acids 117–331) plays a key role in inhibiting the nuclease function of DFF40 (McCarty *et al.*, 1999*a*,*b*). Thus, structure information on the DFF45 C-terminal region will be helpful in discovering the relationship of the DFF45–DFF40 complex and deciphering the inhibitor and chaperone function of DFF45.

In this study, we report the bacterial expression and the purification of different fragments of the DFF45 C-terminal region. We observed different behaviors of the various fragments with regard to protein stability in solution, crystallization and X-ray diffraction. The information will assist in our efforts towards the structure solution of DFF45.

2. Results and discussion

2.1. Selection of stable protein fragments

ctdDFF45 (residues 121–331) was cloned into plasmid pET11a-DEST and expressed in *Escherichia coli* by previously described methods (Ding *et al.*, 2002). The FPLC-purified recombinant ctdDFF45 protein was stored at 277 K for two months and was found to have degraded to a stable fragment (Fig. 1*a*), of which the first five amino-acid residues are Gly -Leu-Lys-Trp-Lys at the N-terminal end (sequenced at Life Sciences College, Peking University, People's Republic of China) and which has a molecular weight of 20 644 Da (determined at Instrumental Analysis Center, Academy of Military Medical Sciences, People's Republic of China). This fragment, designated dctdDFF45 (residues 121–304), was then cloned and expressed as follows. The purified dctdDFF45 was kept at 277 K for 40 d and was further degraded to yield two fragments (Fig. 1*b*): one has an approximate molecular weight of between 11 105 and 11 662 Da and the first eight residues at the N-terminal end were Gly-Ser-His-Met-Gly-Leu-Lys-Trp (the first four residues were from

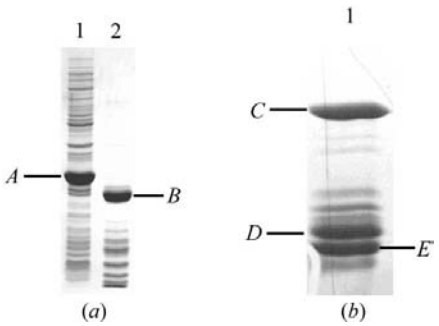

Figure 1
Fragment analysis of DFF45: (*a*) Lane 1, supernatant of *E. coli* expression. The recombinant ctdDFF45 is indicated by *A*. Lane 2, the FPLC-purified ctdDFF45 protein after being degraded to the fragment dctdDFF45, indicated by *B*. (*b*) Lane 1, FPLC-purified dctdDFF45 (labeled *C*), the large fragment (labeled *D*) and the small fragment (labeled *E*) groups degraded from the dctdDFF45 protein.

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mevtgdagvpesgeirtlkpcllrrnysreqhgvaascledlrskacdil 50

aidksltpvtlvlaedgtivddddyflclpsntkfvalasnekwaynnsd 100

ggtawisqesfdvdetdsga glkwknvarqlkedlssiillseedlqm 148
                    N1
lvdapcsdlaqelrqscatvqrlqhtlqqvldqreevrqskqllqlylqa 198

lekegsllskqeeskaaf ge evd avdtgisretssdvalashil 242
                  N2 N3 N4
talrekqapelslssqdlelvtkedpkalavalnwdikktetvqeacere 292

lalrlqqtqslh slrsisaskasppgdlqnpkrarqdpt          331
           C
```

Figure 2
The elucidation of stable fragments in the context of the amino-acid sequences of DFF45. The sequence of ctdDFF45 is shown with a gray background. The results of mass spectroscopy and the N-terminal sequence determination identifying the fragment dctdDFF45 are included within the N1 and C sites. dctdDFF45 was further split into two parts at sites N2, N3 or N4.

the fusion peptide in the expression vector). The other includes smaller fragments of approximate molecular weights of between 9157 and 9829 Da and with three different sequences for the first five residues at the N-terminal end: Gly-Glu-Glu-Val-Asp, Glu-Val-Asp-Ala-Val and Ala-Val-Asp-Thr-Gly (the results of the mass-spectrum analysis were from the Department of Pharmacology and Toxicology and the results of the N-terminal sequence determination from the Protein Analysis Laboratory, University of Alabama at Birmingham, USA). Based on these data, we concluded that dctdDFF45 might contain two tightly folded domains that are separated by residues 216–222 (Fig. 2).

2.2. Protein expression and purification

dctdDFF45 (residues 121–304) was cloned into expression vector pET15b (Novagen, USA). Three smaller fragments, DFF45c1 (residues 121–218), DFF45c2 (residues 121–216), DFF45c3 (residues 219–304) and DFF45c4 (residues 222–304), were also cloned into vector pET28b (Novagen, USA) for protein expression. The recombinant proteins were expressed in *E. coli* strain BL21(DE3). Cells with the expression plasmid were grown at 310 K in Luria–Bertani broth until the OD_{600} reached 0.7; the expression was then induced with 1 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 5 h at 310 K. Harvested cells were resuspended in binding buffer (20 m*M* Tris–HCl, 5 m*M* imidazole, 500 m*M* NaCl pH 7.9) and broken using a 550 sonic dismembrator (Fisher Scientific, USA). Cell debris was removed by centrifugation for 50 min at 15 000*g* and 277 K. The supernatant containing the recombinant protein was purified through a nickel-affinity column and the N-terminal histidine tag was removed as described in Ding *et al.* (2003). The resulting sample was loaded onto a Hiprep 26/10 desalting column (Amersham Biosciences, USA) and exchanged into buffer *A* (20 m*M* Tris pH 8.0). The desalted sample was applied onto a Hitrap Q column (Amersham Biosciences, USA) pre-equilibrated with buffer *A* and the proteins were eluted with a linear gradient of 0–500 m*M* sodium chloride in buffer *A*. The fractions containing the recombinant protein were pooled. Further purification with an FPLC size-exclusion column (Superdex 75, Pharmacia) and concentration of the protein solution before crystallization followed the protocol reported in Ding *et al.* (2003). DFF45c1 and DFF45c2 were purified without the Hitrap Q step. Finally, dctdDFF45 was concentrated to 8.8 mg ml^{-1}, DFF45c1 to 20.0 mg ml^{-1}, DFF45c2 to 26.0 mg ml^{-1}, DFF45c3 to 10.5 mg ml^{-1} and DFF45c4 to 37.1 mg ml^{-1} in a buffer consisting of 10 m*M* HEPES, 0.01% sodium azide pH 7.5.

2.3. Crystallization

Crystallization was initially screened using commercially available screening kits (Hampton Research/Emerald Biostructures, USA). Trials were set up using the hanging-

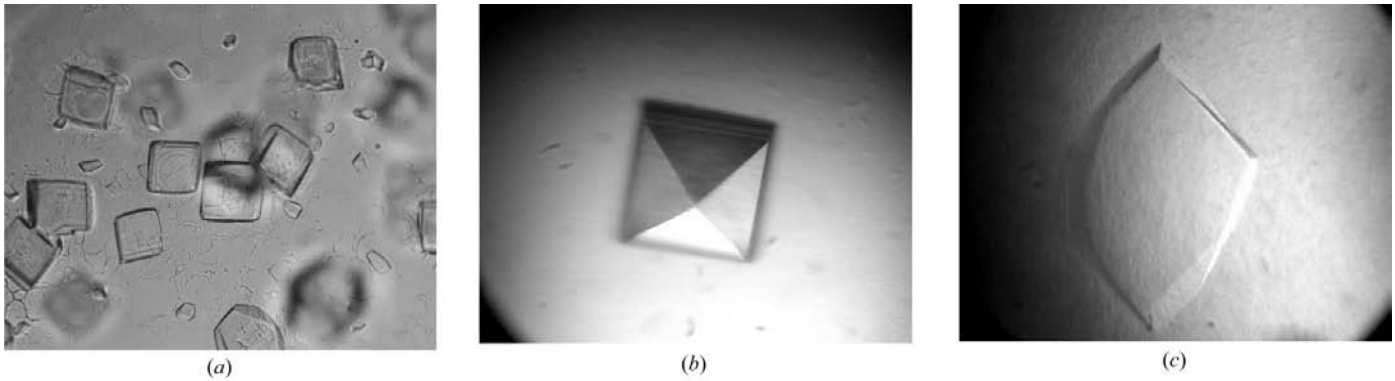

Figure 3
Crystals of different fragments of DFF45: (*a*) dctdDFF45, (*b*) DFF45c1 and (*c*) DFF45c2. Approximate sizes are 0.12 × 0.12 × 0.12, 0.40 × 0.40 × 0.40 and 0.40 × 0.40 × 0.10 mm for (*a*), (*b*) and (*c*), respectively.

Table 1
Data-collection parameters and statistics of DFF45c1.

Values in parentheses refer to the last resolution shell.

Space group	$P4_n22$†
Unit-cell parameters (Å)	$a = b = 162.5$, $c = 113.0$
Resolution range (Å)	40.0–3.96 (4.10-3.96)
Average mosaicity (°)	0.073
Total No. of reflections	69729
No. of unique reflections	12728
Completeness (%)	92.9 (82.2)
Redundancy	5.5 (5.5)
Average $I/\sigma(I)$	20.9 (4.2)
$R_{\rm merge}$‡	0.085 (0.392)

† $n = 0, 1, 2$ or 3. ‡ $R_{\rm merge} = \sum |I - \langle I\rangle| / \sum I$, where $\langle I\rangle$ is the mean intensity.

drop vapor-diffusion method in 24-well VDX plates (Hampton Research, USA) at 295 K. 1 µl of protein solution mixed with 1 µl of commercial buffer was equilibrated against 600 µl of commercial buffer in the reservoir. Crystals of dctdDFF45 were obtained from condition 13 of the Natrix kit (Scott *et al.*, 1995). Crystals of DFF45c1 appeared in condition 26 of the Natrix kit. Crystals of DFF45c2 grew in condition 7 of Crystal Screen I (Hampton Research, USA). Optimization involved varying the pH and the type of buffer and the concentration of precipitants and salts, as well as the addition of low concentrations of detergents. The optimal conditions for growing crystals of dctdDFF45 were 1.5 *M* ammonium sulfate, 0.1 *M* sodium cacodylate, 5 m*M* magnesium acetate, 210 m*M* potassium nitrate, 0.5%(*w*/*v*) *n*-octyl-β-D-glucopyranoside pH 5.9 for 40 d. Crystals of DFF45c1 were obtained from 5%(*w*/*v*) polyethylene glycol (PEG) 8000, 50 m*M* sodium cacodylate, 0.2 *M* potassium chloride, 0.1 *M* magnesium acetate, 25 m*M* potassium thiocyanate pH 6.4 in 5 d. Crystals of DFF45c2 grew from 1.4 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.5 in 2 d (Fig. 3).

2.4. X-ray analysis

Crystals of dctdDFF45 and DFF45c2 were soaked in mother liquor with the addition of 30% glycerol and flash-cooled in liquid nitrogen prior to data collection. Crystals of DFF45c1 were mounted in quartz capillaries.

Crystals of dctdDFF45 diffracted weakly to around 8 Å resolution using laboratory X-ray equipment (CBSE, University of Alabama at Birmingham, USA) as well as synchrotron radiation (SSRL). The diffraction pattern was highly mosaic and the data could not be indexed properly. The diffraction experiments of DFF45c1 and DFF45c2 were performed at Stanford Linear Accelerator Center, USA on beamline BL9-2 for DFF45c1 and BL9-1 for DFF45c2, using a Quantum315 CCD area detector (ADSC) for DFF45c1 and a Quantum4 CCD area detector for DFF45c2. X-ray diffraction data from DFF45c1 crystals were collected with a 1° oscillation at 277 K, a crystal-to-detector distance of 400 mm, an exposure time of 5–20 s and a wavelength of 0.992 Å. No suitable cryoprotectant was identified for the DFF45c1 crystals. Data from the DFF45c2 crystals were collected at cryo-temperature, with a crystal-to-detector distance of 270 mm, and were exposed for 2 min with a wavelength of 0.984 Å. The native crystals of DFF45c1 in capillaries diffracted X-rays to beyond 3.4 Å resolution. The crystals of DFF45c2 diffracted X-rays to a resolution of 3.2 Å (Fig. 4). A total of 73 X-ray diffraction images from 12 crystals of DFF45c1 were processed with *HKL*2000 (Otwinowski & Minor, 1997). The results of this partial data set are listed in Table 1. Self-rotation functions using program *GLRF* (Tong & Rossmann, 1997) and the preliminary data processed as *P*4 suggest that DFF45c1 has *P*4/*mmm* Laue symmetry. The final data set was processed using *P*4/*mmm* symmetry and the space group was determined to be $P4_n22$ in which

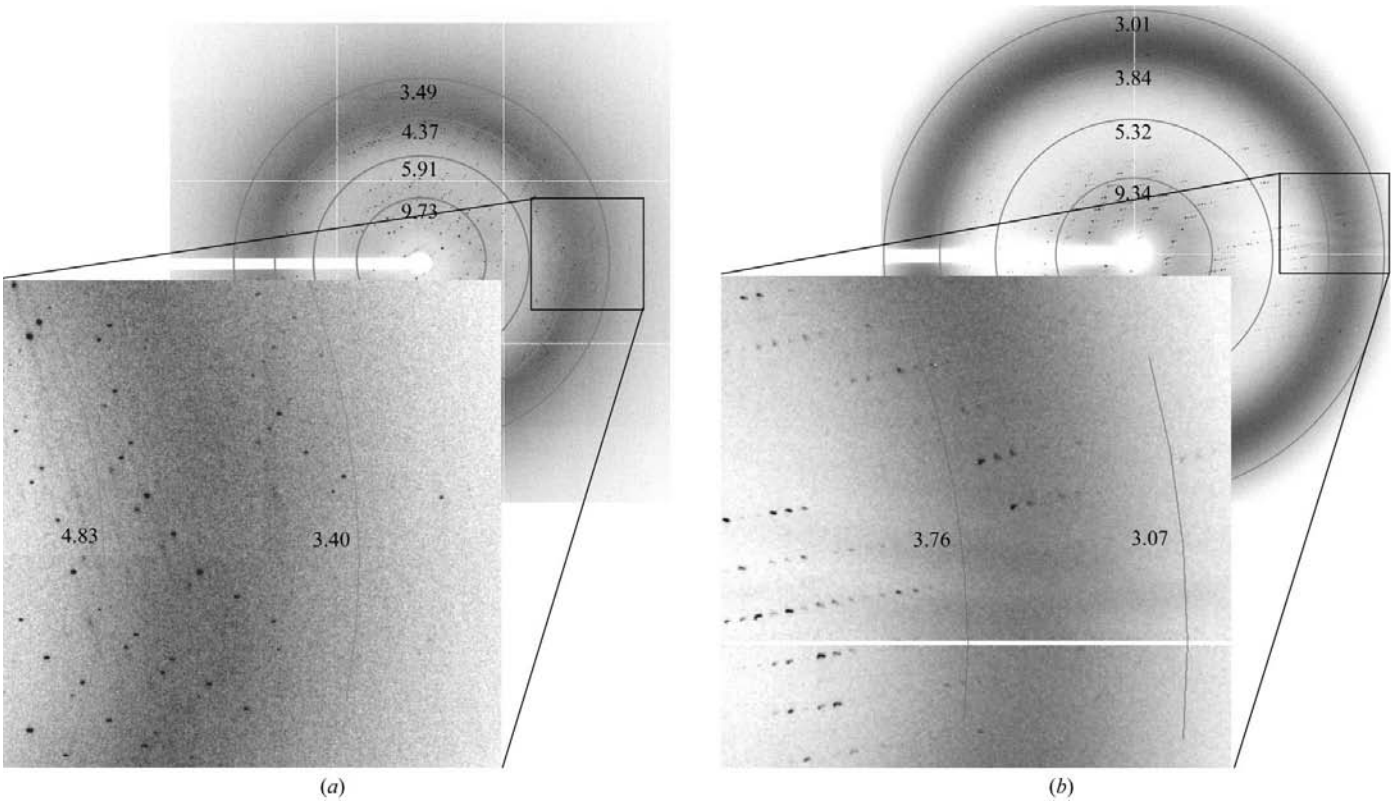

Figure 4
X-ray diffraction patterns of (*a*) DFF45c1 and (*b*) DFF45c2.

n could not be assigned owing to a lack of sufficient 00*l* reflections (Table 1).

In attempts to process DFF45c2 data, we noticed significant overlaps between spots at high resolution owing to high mosaicity. No successful indexing of the diffraction pattern was achieved. The morphology of DFF45c2 crystals appears to be different from that of DFF45c1 crystals. Meanwhile, the content of the hanging drops containing crystals of DFF45c2 were checked by SDS–PAGE and the results showed that this fragment was further degraded during crystallization.

DFF45c1 protein appeared to be more stable during crystallization based on SDS–PAGE analysis of the crystallization drops. Cryoprotectant solutions composed of the mother solution of DFF45c1 crystallization mixed with 30% glycerol, 30% 2-methyl-2,4-pentanediol (MPD), 30% ethylene glycol, 30% PEG 400 or 30%(*w*/*v*) sucrose failed to protect the crystals during flash-freezing. Recently, small crystals of DFF45c1 were grown under similar conditions to those used for the previous DFF45c1 crystals except that the reservoir contained 25% glycerol and 10%(*w*/*v*) PEG 8000. These crystals could be cryofrozen directly. The space group and the resolution of the frozen crystals appear to be similar to those of the room-temperature data. However, the diffraction pattern was very weak because of the limited size of the crystals.

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