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DFF, a Heterodimeric Protein That Functions Downstream of Caspase-3 to Trigger DNA Fragmentation during Apoptosis

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Summary

We have identified and purified from HeLa cytosol a protein that induces DNA fragmentation in coincubated nuclei after it is activated by caspase-3. This protein, designated DNA Fragmentation Factor (DFF), is a heterodimer of 40 kDa and 45 kDa subunits. The amino acid sequence of the 45 kDa subunit, determined from its cDNA sequence, reveals it to be a novel protein. Caspase-3 cleaves the 45 kDa subunit at two sites to generate an active factor that produces DNA fragmentation without further requirement for caspase-3 or other cytosolic proteins. In cells undergoing apoptosis, the 45 kDa subunit is cleaved in the same pattern as it is cleaved by caspase-3 in vitro. These data delineate a direct signal transduction pathway during apoptosis: caspase-3 to DFF to DNA fragmentation.

Introduction

Apoptosis is executed through a suicide program that is built into all animal cells (reviewed by Wyllie, 1995; White, 1996). Cells undergoing apoptosis show distinctive morphological changes, including membrane blebbing, cytoplasmic and nuclear condensation, chromation aggregation, and formation of apoptotic bodies (Wyllie, 1980). The biochemical hallmark of apoptosis is the cleavage of chromatin into nucleosomal fragments (Wyllie, 1980).

Multiple lines of evidence indicate that apoptosis can be triggered by the activation of a family of cysteine proteases with specificity for aspartic acid residues, including CED-3 of C. elegans, CPP32/Yama/Apopain of humans, and DCP-1 of Drosophila (Yuan et al., 1993; Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995; Xue et al., 1996; Song et al., 1997). Recently, these proteins have been designated as caspases (Alnemri et al., 1996).

The most intensively studied apoptotic caspase is caspase-3, previously called CPP32/Yama/Apopain (Fernandes-Alnemri, et al., 1994; Nicholson, et al., 1995; Tewari, et al., 1995). Caspase-3 normally exists in the cytosolic fraction of cells as an inactive precursor that is activated proteolytically when cells are signaled to undergo apoptosis (Schlegel et al., 1996; Wang et al., 1996). Multiple apoptotic signals, including serum withdrawal, activation of Fas, treatment with granzyme B, ionizing radiation, and a variety of pharmacological agents, activate caspase-3 (Chinnaiyan et al., 1996; Darmon, et al., 1996; Datta et al., 1996, 1997; Erhardt and Cooper, 1996; Hasegawa et al., 1996; Jacobson et al., 1996; Martin et al., 1996; Schlegel et al, 1996). A caspase-3-specific tetrapeptide inhibitor, Ac-DEVD-CHO, can abolish the ability of cytosol from apoptotic cells to induce apoptosis in normal nuclei and block the initiation of the cellular apoptotic program in response to apoptotic stimuli (Nicholson et al., 1995; Dubrez, et al., 1996; Jacobson et al., 1996). Deletion of caspase-3 from the mouse genome through homologous recombination results in excessive accumulation of neuronal cells, owing to a lack of apoptosis in the brain (Kuida et al., 1996). Addition of active caspase-3 to normal cytosol activates the apoptotic program (Enari et al., 1996). These data indicate that caspase-3 is both necessary and sufficient to trigger apoptosis.

The identified substrates of caspase-3 include poly (ADP-ribose) polymerase (PARP) (Nicholson et al., 1995; Tewari et al. 1995), sterol-regulatory element–binding proteins (SREBPs) (Wang et al., 1995a, 1996), the U1-associated 70 kDa protein (Caciola-Rosen et al., 1996), D4-GDI (Na et al., 1996), huntingtin (Goldberg et al., 1996), and the DNA-dependent protein kinase (Casciola-Rosen et al., 1996; Song et al., 1996). It is not known whether the cleavage of any of these substrates plays a causal role in apoptosis.

Our laboratory recently established an experimental system in which DNA fragmentation characteristic of apoptosis can be triggered in vitro by incubation of normal nuclei with activated cytosolic extracts (Liu et al., 1996b). The activation occurred in two stages: first, cytosolic caspase-3 was cleaved and activated in a reaction that was triggered by cytochrome c released from mitochondria; and second, activated caspase-3 interacted with other cytosolic proteins to generate DNA fragmentation when added to isolated nuclei (Liu et al., 1996b; Yang et al., 1997).

In the current study, we report the purification, characterization, and cDNA cloning of the downstream factor that is activated by caspase-3 and, in turn, induces nuclear DNA fragmentation. We call this factor DNA Fragmentation Factor (DFF).

Results

Identification of DFF

To elucidate the molecular events leading to DNA fragmentation, we set up an in vitro DNA fragmentation assay in which normal nuclei from hamster liver were incubated with active recombinant caspase-3 together with Hela cell S-100 cytosol (Figure 1). Caspase-3 alone was not able to induce DNA fragmentation in the coincubated nuclei (Figure 1, lane 1); neither were the HeLa cell cytosolic or nuclear extracts (Figure 1, lanes 2 and 3).

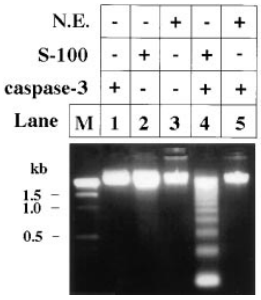

Figure 1. Identification of DFF, a Caspase-3-Dependent DNA Fragmentation Factor

Recombinant caspase-3, hamster liver nuclei, HeLa cell S-100, and nuclear extract were prepared as described in Experimental Procedures. An aliquot (50 μl) of HeLa cell S-100 (250 μg) or nuclei extract (250 μg) was incubated in the absence (lanes 2 and 3) or presence of 150 ng of caspase-3 (lane 4,5) with 7 μl of hamster liver nuclei for 2 hr at 37°C in a final volume of 60 μl adjusted with buffer A. Lane 1 is caspase-3 alone incubated with hamster liver nuclei. The genomic DNA from these samples was isolated as described in Experimental Procedures and subjected to 2% agarose gel electrophoresis. The DNA was visualized by ethidium bromide staining.

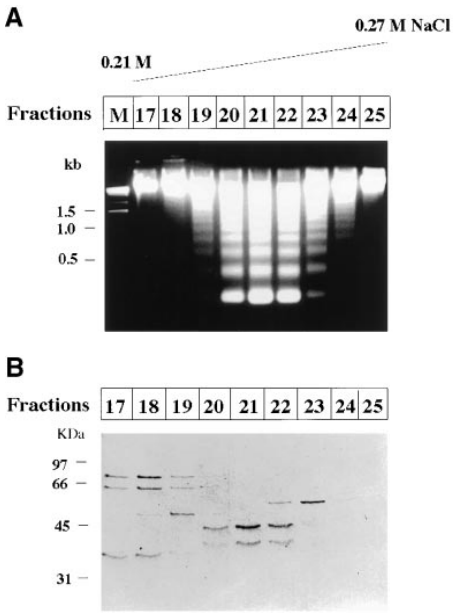

Figure 2. Mono Q Column Purification of DFF

The DFF activity was purified through the Mono Q column as described in Experimental Procedures.

(A) Aliquots of 2 μl of Mono Q column fractions were incubated with aliquots of 0.3 μg of caspase-3 and 7 μl of hamster liver nuclei at 37°C for 2 hr in a final volume of 60 μl of buffer A. DNA was isolated, analyzed, and visualized as described in Experimental Procedures.

(B) Aliquots (50 μl) of the indicated Mono Q column fractions were subjected to 10% SDS–PAGE, followed by Coomassie brilliant blue staining.

However, when caspase-3 and the HeLa S-100 fraction were incubated together with the nuclei, DNA fragmentation occurred (Figure 1, lane 4). These data indicate the existence of a DNA fragmentation factor(s) in HeLa cell cytosol that induces DNA fragmentation in the presence of caspase-3. No DFF activity was detected in the nuclear extract (Figure 1, lane 5).

Purification of DFF

Using caspase-3-dependent DNA fragmentation as an assay, we purified DFF from HeLa cell S-100 in an eight step procedure (see Table 1 and Experimental Procedures). The results of the last step of the purification, a Mono Q column chromatographic step, are shown in Figure 2. DFF activity eluted from the Mono Q column at about 250 mM NaCl (Figure 2A, fractions 20–22). These fractions were subjected to SDS–PAGE, followed by Coomassie brilliant blue staining (Figure 2B). Two polypeptides with molecular masses of 45 kDa and 40 kDa were observed to coelute with the DFF activity. We used the Coomassie stain because the 45 kDa protein stained poorly with silver (X. L. and X. W., unpublished data). No other proteins were detected in fractions containing the peak of DFF activity (fraction 21).

Purified DFF from the Mono Q column induced DNA fragmentation in coincubated nuclei in a fashion that was dependent on its concentration and the concentration of caspase-3 (Figure 3, lanes 1–7). As increasing amounts of purified DFF were added to the reaction, the extent of DNA fragmentation increased as demonstrated by the increase in the intensity of small-sized nucleosomal DNA fragments (~180 base pairs/nucleosome) and

Table 1. Purification of DFF from HeLa Cells

Step	Fraction	Protein	Specific Activity	Total Activity	Purification	Recovery
		mg	units/mg	unit	-fold	%
1	S-100	3750	526	1972500		100
2	SP-Sepharose	968	1466	1419355	2.8	72
3	Phenyl-Sepharose	275	3040	835820	5.8	42
4	50% Ammonium-Sulfate Precipitation	88	8333	733333	16	37
5	Superdex-200	20	34285	685714	65	35
6	Mono S	0.675	227920	153846	433	7.8
7	Hydroxyapatite	0.2	328947	65789	625	3.3
8	Mono Q	0.015	4000000	60000	7604	3.0

S-100 was purified from 100 liters of HeLa cells as described in Experimental Procedures. An aliquot of each fraction was dialyzed against buffer A, and the DFF activity was assayed at five concentrations of protein. The results were quantified by IS-1000 Digital Imaging System (Alpha Innotech Corporation). One unit of activity was arbitrarily defined as one unit of the intensity of the single nucleosomal DNA.

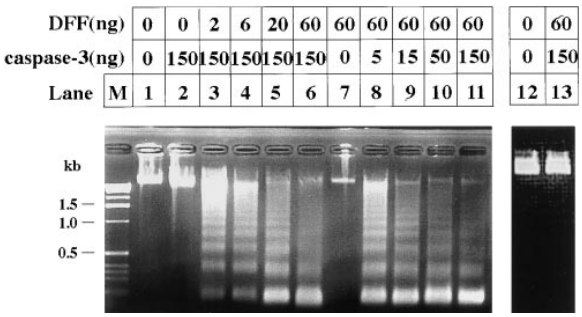

Figure 3. Reconstitution of DNA Fragmentation Activity by Purified DFF and Caspase-3

Hamster liver nuclei and caspase-3 were prepared as described in Experimental Procedures. Aliquots (7 μl) of hamster liver nuclei were incubated alone (lane 1), or with 0.15 μg of caspase-3 and the indicated amounts of purified DFF (lanes 2–6), or with 60 ng of purified DFF and the indicated amount of caspase-3 (lanes 7–11) at 37°C for 2 hr in a final volume of 60 μl of buffer A. The DNA was isolated, analyzed, and visualized as described in Experimental Procedures. In lanes 12 and 13, λ phage DNA was isolated using a Wizard Lambda Preps DNA Purification System (Promega) and incubated alone (lane 12) or with 60 ng of purified DFF plus 0.15 μg of caspase-3 at 37°C for 2 hr.

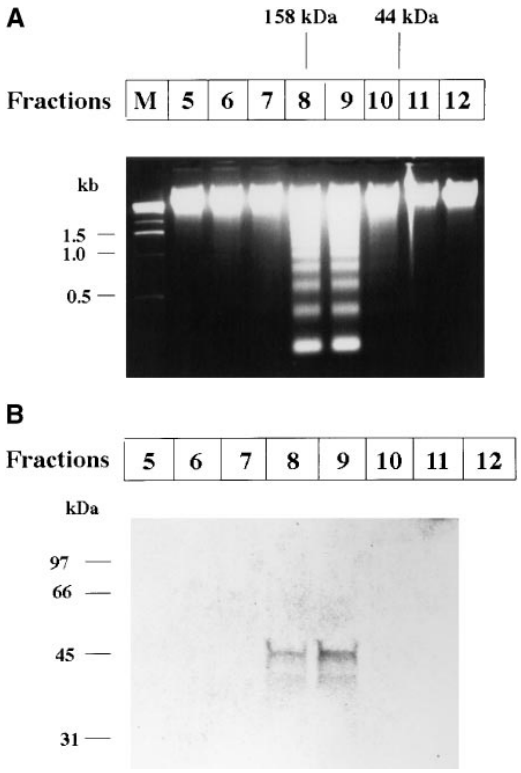

Figure 4. Gel Filtration Column Profile of DFF

The peak fraction of DFF (fraction 21) from the Mono Q column (see Figure 2) was loaded onto a Superdex 200 10/30 gel filtration column (Pharmacia). The column was equilibrated and eluted with buffer A. Fractions of 1 ml were collected.

(A) Aliquots (10 μl) of the gel filtration fractions were assayed for DFF activity as described in Experimental Procedures.

(B) Aliquots (1 ml) of the indicated fractions were concentrated using a Centricon 10 (amicon) to a final volume of 90 μl and then subjected to a 10% SDS–PAGE, followed by Coomassie blue staining. Arrows denote the positions of elution of molecular weight standards.

the decrease of large molecular weight genomic DNA. At the highest concentration used, almost all of the DNA was cleaved into the size of single nucleosomes after 2 hr (Figure 3, lane 6). No DNA fragmentation was observed when caspase-3 was omitted from the reaction (Figure 3, lane 7). A similar pattern, although not as linear, was observed when increasing amounts of caspase-3 were added to the reactions with a fixed amount of DFF (Figure 3, lanes 8–11). Interestingly, DFF plus caspase-3 showed no detectable nuclease activity when incubated with naked DNA (Figure 3, lanes 12–13).

The relative linear increase in single nucleosomal fragments in response to increasing concentrations of DFF enabled us to quantitatively estimate the degree of purification of DFF. Table 1 shows estimates of the quantitative parameters for the purification of DFF starting with S-100 fractions from 100 liters of suspension-cultured HeLa cells. The DFF was purified more than 7000-fold to apparent homogeneity through the Mono Q step with an overall recovery of 3% activity.

To confirm the association of the 45 kDa and 40 kDa polypeptides, we applied the purified DFF from the Mono Q column step to a Superdex-200 gel filtration column (Pharmacia). The column fractions were collected and assayed for DFF activity (Figure 4A). DFF activity appeared at fractions 8 and 9 with an estimated molecular mass of 85 kDa relative to the molecular weight standard (Figure 4A). The same fractions were also subjected to SDS–PAGE, followed by Coomassie blue staining (Figure 4B), and the 45 kDa and 40 kDa polypeptides were observed to coelute with DFF activity.

Characterization of DFF

Several known substrates of caspase-3 are nuclear proteins, including PARP, lamin B1, 70 kDa U1 RNP protein, and DNA-dependent kinase. To test the possibility that the function of DFF is to facilitate the transport of caspase-3 into nuclei, we studied the time course of cleavage of lamin B1 by caspase-3 in the absence or presence of DFF. As shown in Figure 5A, caspase-3 alone was not able to induce DNA fragmentation. In the presence of DFF, nuclear DNA started to fragment after 15 min of incubation and the extent of fragmentation increased with time (Figure 5A, lanes 7–11). However, the rate of lamin B1 cleavage by caspase-3 remained the same with or without DFF (Figure 5B, lanes 2–11). These data indicate that caspase-3 cuts the nuclear substrate with or without DFF. The same conclusion was obtained when the rate of cleavage of PARP was measured (data not shown).

DFF Functions Downstream of Caspase-3

We next conducted an experiment to determine whether caspase-3 was needed for DNA fragmentation after it had activated DFF, or whether the sole requirement for caspase-3 is to activate DFF. To distinguish these possibilities, we employed a caspase-3-specific tetrapeptide aldehyde inhibitor, Ac-DEAD-CHO (Wang et al., 1995a). As shown in Figure 6, incubation of caspase-3 and DFF

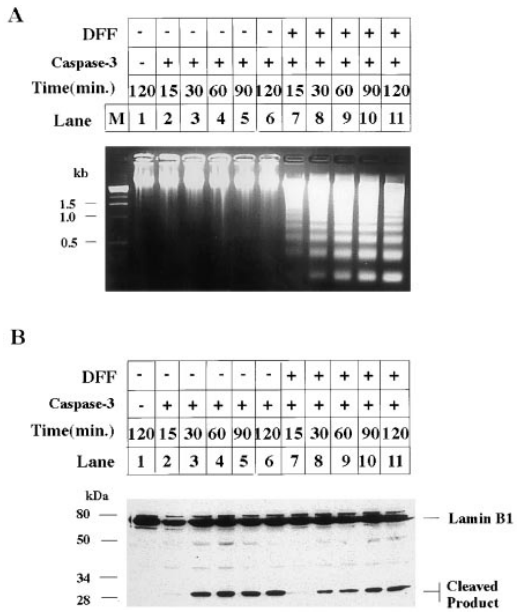

Figure 5. Lamin B1 Cleavage in the Absence and Presence of DFF

Aliquots (14 μl) of HeLa cell nuclei were incubated at 37°C alone for 2 hr (lane 1), or with 0.6 μg of caspase-3 alone (lanes 2–6), or with 0.6 μg of caspase-3 and 0.12 μg of purified DFF (lanes 7–11) for the indicated times in a final volume of 120 μl of buffer A.

(A) Aliquots (60 μl) of these reactions were digested with proteinase K, and the DNA was isolated, analyzed, and visualized as described in Experimental Procedures.

(B) Aliquots (30 μl) of each reaction were subjected to a 12% SDS–PAGE and transferred to a nitrocellulose filter. The filter was probed with monoclonal anti-lamin B1 antibody, and the antigen–antibody complex was visualized by the ECL method as described in Experimental Procedures. The filter was exposed to a Kodak X-OMAT AR X-ray film for 30 sec.

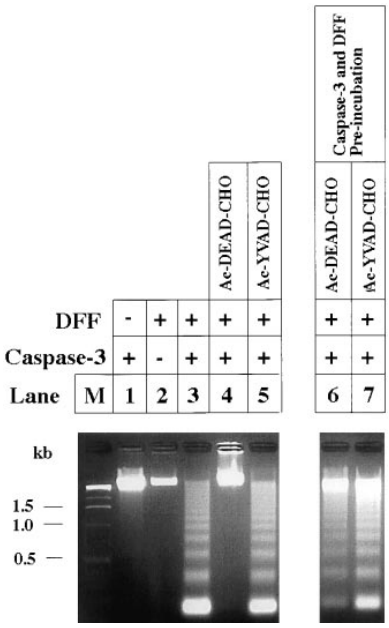

Figure 6. Activation of DFF by Caspase-3

Aliquots (7 μl) of hamster liver nuclei were incubated with caspase-3 (50 ng) alone (lane 1), DFF alone (60 ng) (lane 2), or caspase-3 and DFF together (lane 3) for 2 hr at 37°C. In lanes 4 and 5, aliquots (50 ng) of caspase-3 were incubated with 1 μM Ac-DEAD-CHO (lane 4) or 1 μM Ac-YVAD-CHO (lane 5) at 37°C for 5 min, followed by the addition of purified DFF (60 ng) and 7 μl of hamster liver nuclei. Reactions were incubated for another 2 hr in a final volume of 60 μl of buffer A. In lanes 6 and 7, aliquots of caspase-3 (50 ng) were incubated with purified DFF (60 ng) at 37°C for 1.5 hr, followed by the addition of Ac-DEAD-CHO (lane 6) or Ac-YVAD-CHO (lane 7) to a final concentration of 1 μM. After incubation for 5 min, aliquots of hamster liver nuclei (7 μl) were added to each reaction, followed by an additional 2 hr incubation. The reactions were stopped by proteinase K digestion. The DNA was isolated, analyzed, and visualized as described in Experimental Procedures.

together induced DNA fragmentation (lane 3), while caspase-3 or DFF alone failed to do so (lanes 1 and 2). When Ac-DEAD-CHO was included in the reaction, DNA fragmentation was inhibited, demonstrating that activation of DFF requires active caspase-3 (lane 4). Under the same conditions, the ICE-specific inhibitor, Ac-YVAD-CHO, did not inhibit DNA fragmentation (lane 5). In contrast, when caspase-3 and DFF were preincubated for 2 hr, followed by the addition of nuclei and Ac-DEAD-CHO (lane 6), there was no longer inhibition of DNA fragmentation reaction (lane 6). It appears that caspase-3 activity is no longer required for DNA fragmentation once DFF is activated.

The above protocol also provided an opportunity to determine whether the cleavage of nuclear substrates by caspase-3, such as PARP and lamin B1, are necessary for DNA fragmentation. It had not been clear whether the cleavage of PARP or lamins is a required step for DNA fragmentation. To directly test this requirement, we preincubated purified DFF with caspase-3 and then added nuclei in the presence of Ac-DEAD-CHO (Figures 7A–7C, lanes 5–7), or the control inhibitor Ac-YVAD-CHO (Figure 7A–7C, lanes 8–10). Neither Ac-DEAD-CHO nor Ac-YVAD-CHO had any effect on DNA fragmentation when added after DFF and caspase-3 had been preincubated (Figure 7A). However, Ac-DEAD-CHO still blocked the cleavage of both PARP and lamin B1 in the coincubated nuclei (Figures 7B and 7C, lanes 5–7). It therefore appears that DNA fragmentation does not require cleavage of nuclear substrates such as PARP and lamin B1.

The 45 kDa Subunit of DFF Is a Substrate for Caspase-3

Inasmuch as activation of DFF by caspase-3 is inhibited by Ac-DEAD-CHO, it is likely that this activation involves the cleavage of one or both of the subunits of DFF. To test this hypothesis, we labeled purified DFF with biotin and incubated the biotinylated DFF with caspase-3. After incubation, the samples were subjected to SDS–PAGE and visualized by chemiluminescence using streptavidin-conjugated peroxidase. As shown in Figure 8, both subunits of DFF were labeled with biotin (lane 1). Incubation with caspase-3 resulted in the cleavage of the 45 kDa subunit into fragments of molecular masses of 30 kDa and 11 kDa that were separated by SDS–PAGE. The 40 kDa subunit remained intact (lanes 1 and 2).

To confirm that DFF-45 is cleaved and activated in vivo in cells undergoing apoptosis, we performed immunoblot analysis of DFF-45 using extracts from human

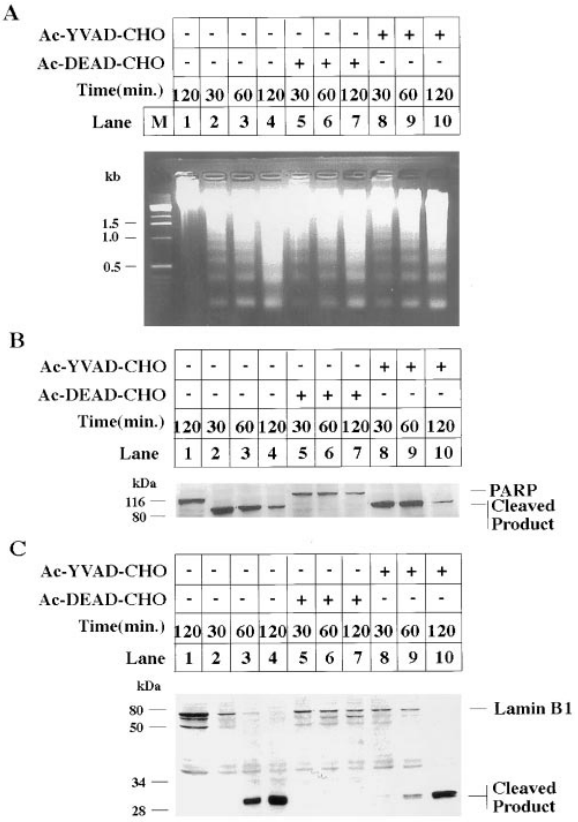

Figure 7. DNA Fragmentation Does Not Require the Cleavage of PARP and Lamin B1

Aliquots (0.12 μg) of purified DFF were incubated with aliquots of caspase-3 (100 ng) at 37°C for 1.5 hr in a final volume of 120 μl of buffer A, followed by the addition of Ac-DEAD-CHO (lanes 5–7) or Ac-YVAD-CHO (lanes 8–10) to a final concentration of 1 μM. After incubation at 37°C for 5 min, aliquots of 7 μl of HeLa cell nuclei were added to each reaction and incubated at 37°C for 2 hr. In lane 1, nuclei alone were incubated with buffer A.

(A) Aliquots (60 μl) of each reaction were stopped by proteinase K digestion, and the DNA was isolated, analyzed, and visualized as described in Experimental Procedures.

(B and C) Aliquots (30 μl) of each reaction were subjected to 10% (B) or 12% (C) SDS–PAGE. After electrophoresis, the gels were transferred to nitrocellulose filters. The filters were probed either with a monoclonal anti-PARP antibody (B) or a monoclonal anti-lamin B antibody (C). The antigen–antibody complexes were visualized by the ECL method as described in Experimental Procedures. The filters were exposed to a Kodak X-OMAT AR X-ray film for 1 min (B) or 30 sec (C).

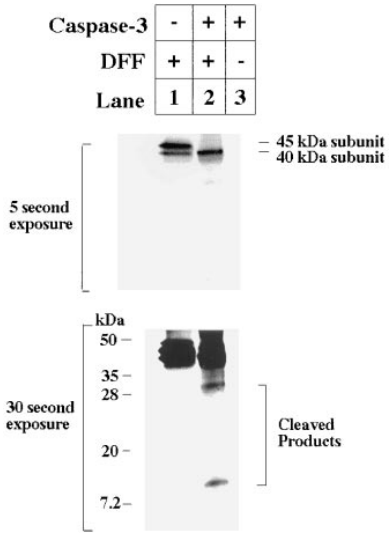

Figure 8. DFF-45 Is a Substrate for Caspase-3

DFF was biotinylated as described in Experimental Procedures. The biotinylated DFF was incubated alone (lane 1) or with 0.3 μg of caspase-3 (lane 2) for 1.5 hr at 37°C. Caspase-3 was also incubated with the inactivated biotinylation buffer under the same reaction conditions (lane 3). The samples were subjected to 12% SDS–PAGE and transferred to a nitrocellulose filter. The filter was probed with streptavidin, and the biotin–streptavidin complex was visualized by the ECL method as described in Experimental Procedures. The filters were exposed to a Kodak X-OMAT AR X-ray film for 5 sec and 30 sec, respectively.

monocytic U937 cells undergoing apoptosis induced by staurosporine. As shown in Figure 9, DFF-45 exists as ~45 kDa precursor in growing cells. After a 2 hr treatment with staurosporine, DFF-45 was cleaved into fragments of 30 kDa and 11 kDa. At later time points, the 30 kDa fragment was reduced and the 11 kDa fragment increased. The cleavage of DFF-45 into fragments of 30 kDa and 11 kDa has also been observed in other cell types, such as HeLa cells and human fibroblast SV589 (A. Lutschg and X. W., unpublished data). Similar results were obtained when U937 cells were induced to undergo apoptosis with etoposide (data not shown). The data suggest that there are multiple caspase-3 cleavage sites in DFF-45. The 30 kDa fragment is an intermediate that is further cleaved to an 11 kDa form. The time of appearance of the 11 kDa fragments correlated well with the fragmentation of chromatin in these U937 cells (Figure 9B). Protein sequencing analysis of DFF-45 (Figure 10A) and DFF-40 (data from four tryptic peptides ranging from 7 to 16 amino acids, 50 amino acids total) revealed that both are previously uncharacterized proteins. We isolated a cDNA clone encoding the 45 kDa subunit based on the protein sequence generated from tryptic digestion of DFF-45 followed by Edman degradation (Figure 10A, underlines). The cDNA contains an open reading frame of 331 amino acids with no obvious homology to any known proteins in the data base (Figure 10A). The translated product of this cDNA in a rabbit reticulocyte lysate runs at the identical position as purified DFF-45 in SDS–PAGE (data not shown). The cDNA cloning of DFF-40 is not yet complete.

To map the caspase-3 cleavage sites in DFF-45, we expressed the protein with a six-histidine tag at the NH_2-terminus (MGSSHHHHHHSSGLVPRGSH). The fusion protein migrates at about 45 kDa on SDS–PAGE after purification on a nickel affinity column (Figure 10B, lane 1). Incubation of this fusion protein with caspase-3 resulted in its cleavage into three fragments of 30 kDa, 18 kDa, and 11 kDa at the early time point, and the 30 kDa fragment was further cleaved into ~11 kDa fragments with longer incubation. These fragments were separated by SDS–PAGE and electroblotted onto Immobilon Psq for automated Edman degradation. The results revealed that the NH_2-terminal residues of the

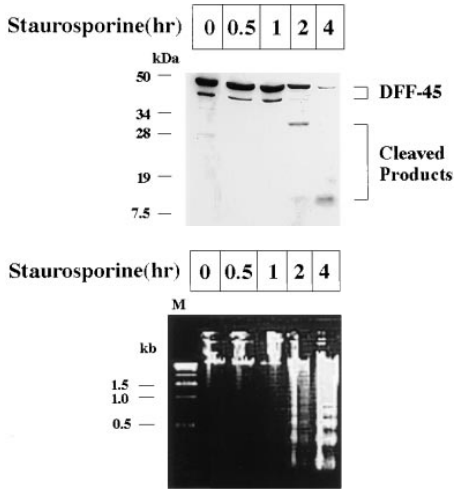

Figure 9. Activation of DFF-45 in Cells Undergoing Apoptosis

Human monoblastic U937 cells were set up at 5×10^5 cells per/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. After incubation at 37°C for 48 hr in 5% CO_2 incubator, the cells were treated with staurosporine at a final concentration of 1 μM for indicated times. The cells were then collected by centrifugation (1,000 × g for 10 min at 4°C). After one wash with ice-cold PBS, the cell pellets were resuspended in 5 volumes of buffer A and incubated on ice for 15 min. The cells were disrupted by passage 15 times through a G22 needle. After centrifugation in a microcentrifuge for 5 min at 4°C, the pellets were resuspended in 2 volumes of buffer A containing 500 mM NaCl and rotated at 4°C for 30 min. The nuclear debris was spun down in a microcentrifuge for 20 min at 4°C, and the supernatants were analyzed by immunoblot using antibody against DFF-45.

(A) Aliquots of 30 μg of U937 cell extracts prepared from cells treated with staurosporine at indicated times were subjected to 15% SDS–PAGE, and were transferred onto a nitrocellulose filter. The filter was probed by rabbit anti-DFF-45 anti-serum (1:10000 dilution), and the antigen–antibody complexes were visualized by an ECL method as described in Experimental Procedures. The filter was exposed to a Fuji reflection film for 30 sec.

(B) Aliquots (5 ml) of U937 cells treated with staurosporine for the indicated times were used for genomic DNA isolation, and the DNA was analyzed as described in Experimental Procedures.

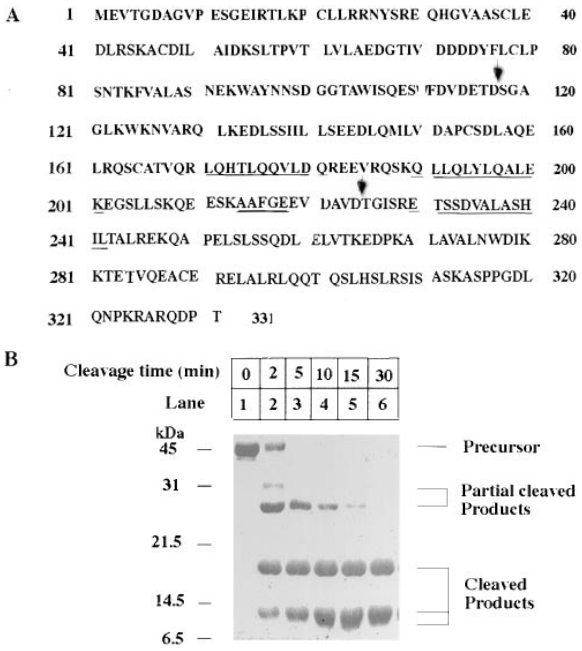

Figure 10. Mapping the Cleavage Sites of Caspase-3 in DFF-45 Protein

(A) This panel shows the translation of the longest open reading frame from a DFF-45 cDNA clone (Hela λ Exlox). The peptide sequences obtained from purified DFF-45 protein are underlined. The arrows denote the cleavage sites of caspase-3 that were determined by automated sequencing and mass spectrometry analysis of caspase-3-cleaved recombinant DFF-45.

(B) Recombinant DFF-45 fusion protein was prepared as described in Experimental Procedures. Aliquots of 50 μg of recombinant DFF-45 protein were mixed with 1 μg of caspase-3 in a final volume of 50 μl of buffer A. Incubation was carried out at 37°C. The reactions were stopped at the indicated time points, and an aliquot (10 μl) of each sample was analyzed by 15% SDS–PAGE. The samples were subsequently transferred to a nitrocellulose filter and stained with Ponceau S.

fragments were Gly-2 (30 kDa and 18 kDa), Ser-138 (11 kDa), and Thr-245 (11 kDa), respectively, suggesting that caspase-3 had cleaved between Asp-137 and Ser-138 and between Asp-244 and Thr-245. This generates two different fragments of ~11 kDa that could not be separated on SDS–PAGE. The 18 kDa fragment is probably generated as the result of the His-tag at the NH_2 terminus of the fusion protein, which runs at ~11 kDa if it was from the native DFF-45.

To confirm that these are the principal sites of cleavage, unfractionated samples of digested DFF-45 were subjected to electrospray mass spectrometry. This analysis revealed protein fragments with masses of 23,732, 14,833, 12,036, and 11,713 Da. These values corresponded well with those calculated for the peptides expected from the results of Edman degradation, namely Ser-138 to Thr-351 (23,736), Gly-2 to Asp-137 (14,836), Ser-138 to Asp-244 (12,039), and Thr-245 to Thr-351 (11,715). The NH_2-terminal fragment of DFF-45 runs abnormally slowly on SDS–PAGE for unknown reasons. The NH_2-terminal sequence analysis and the mass spectrometry of the cleavage products revealed that cleavage occurs at the sequences DETD (aa 117) and DAVD (aa 224). These cleavage sites are consistent with the known cleavage sites for caspase-3, such as DEVD for PARP and DEPD for SREBP-2 (Nicholson et al., 1995; Wang et al., 1995a).

Discussion

DFF Is a Cytosolic Factor That Mediates DNA Fragmentation

DNA fragmentation into nucleosomal fragments is the most recognizable biochemical feature of apoptosis (Wyllie, 1980). The identification and purification of DFF revealed a direct pathway from cytosol to nuclei, leading to DNA fragmentation. Remarkably, active caspase-3 and DFF seem to be sufficient to reconstitute the cytosolic events leading to DNA fragmentation. Purified DFF consists of two subunits of 45 kDa and 40 kDa, of which the 45 kDa subunit can be cleaved by caspase-3 into smaller polypeptides. In cells undergoing apoptosis, a similar pattern of cleavage of DFF-45 was observed

(Figure 9A). Once DFF-45 is cleaved and activated, caspase-3 activity is no longer required for DNA fragmentation, although it is required for cleavage of nuclear substrates such as lamin B1 and PARP. These findings raise the possibility that the sole required function of caspase-3 to mediate DNA fragmentation is to cleave the 45 kDa subunit of DFF. The cleavage of other substrates of caspase-3 either may be needed to mediate some other apoptosis-related events, such as cleavage of lamin B1 to facilitate the fragmentation of nuclei, or has no active role in apoptosis. The latter may be true for the cleavage of PARP since PARP knockout mice show no detectable defect in apoptosis (Wang et al., 1995b).

DFF appears unrelated to a recently discovered 50 kDa mitochondria-derived factor (AIF) that also causes isolated nuclei to undergo apoptosis (Susin et al., 1996). AIF was released from mitochondria through the opening of "permeability transition (PT) pores" that are regulated by Bcl-2 protein (Susin et al., 1996). Once released, AIF was sufficient to induce nuclear apoptotic events in coincubated nuclei (Susin et al., 1996; Zamzami et al., 1996). The activity of AIF was not sensitive to caspase-3-specific tetrapeptide inhibitor (Zamzami et al., 1996). One difference between the work of Susin et al. and the current studies is that Susin et al. start with mitochondria undergoing permeability transition induced by the treatment of atractyloside (Susin et al., 1996). In our studies, cytosolic extracts are derived from nonapoptotic cells, and caspase-3 is activated by overexpression in bacteria. It is possible that cells have two systems by which mitochondria activate apoptosis: (1) by release of cytochrome c, which leads to activation of caspase-3, which in turn activates DFF; and (2) by direct release of AIF, which acts independently of caspase-3. We have no evidence that DFF is derived from mitochondria, and we have found the same amount of latent DFF in cytosol from wild-type cells and from those overexpressing Bcl-2 (X. L. and X. W., unpublished data).

Activation of DFF

The activation of DFF is dependent on the activation of caspase-3. Caspase-3, in turn, is activated through cleavage at two aspartic residues that cleave the 32 kDa precursor into a 20 kDa and a 10 kDa heterodimeric fragment (Nicholson et al., 1995; Wang et al., 1996). Apoptotic signals such as serum withdrawal, activation of Fas, and treatment with ionizing radiation and a variety of pharmacological agents induce the activation of caspase-3 (Darmon, et al., 1996; Chinnaiyan et al., 1996; Dubrez, et al., 1996; Erhardt and Cooper, 1996; Hasegawa et al., 1996; Jacobson et al., 1996; Schlegel et al, 1996). In the case of cytotoxic T cells, caspase-3 in the target cells is activated directly by granzyme B (Darmon et al., 1996; Martin et al., 1996; Quan et al., 1996). During Fas-induced apoptosis, another member of the caspase family, caspase-8 (Mach/Flice/Mch5), is proposed to be activated by interacting directly with the Fas signaling complex on the cell membrane (Boldin et al., 1996; Muzio et al., 1996). The activated caspase-8 is able to activate caspase-3, either directly or through a caspase cascade (Muzio et al., 1997). Overexpression of Bcl-2, an antiapoptosis protein that is mainly located on the outer membrane of mitochondria, prevents the activation of caspase-3 in response to a variety of apoptotic signals (Armstrong et al., 1996; Boulakia et al., 1996; Chinnaiyan et al., 1996; Erhardt and Cooper, 1996; Ibrado et al., 1996; Monney et al., 1996). The recent identification of cytosolic cytochrome c as a necessary component for the activation of caspase-3 sheds light on the possible mechanism by which Bcl-2 prevents activation of caspase-3 (Liu et al., 1996b). In cells overexpressing Bcl-2, the release of cytochrome c from mitochondria in response to apoptotic stimuli is blocked, aborting the activation of caspase-3 (Kluck, et al., 1997; Yang et al., 1997).

The cleavage of DFF-45 during apoptosis presents a clear example of activation through caspase-3 cleavage. In the cases of lamin B1 and PARP, however, cleavage by caspase-3 may result in the destruction of these proteins.

How Does DFF Induce DNA Fragmentation?

It is unlikely that DFF is a nuclease that directly cleaves chromatin DNA. Indeed, DFF showed no DNase activity when incubated with naked DNA (Figure 3, lanes 11–12). It is therefore likely that DFF activates a nuclease(s) that resides in nuclei. One candidate is a Ca^{++}/Mg^{++}-dependent nuclease that was described previously (Gaido and Cidlowski, 1991; Nikonova et al., 1993; Peitsch et al., 1993). Interestingly, incubation of nuclei with high concentrations of Ca^{++} (5 mM) is sufficient to induce DNA fragmentation indistinguishable from that induced by DFF, even though the activity of DFF does not require additional Ca^{++} (McConkey, 1996; X. L. and X. W., unpublished data). To activate a nuclear DNase, DFF might either translocate into the nuclei or interact with a specific protein(s) on the outer surface of the nuclear envelope, to trigger a signal transduction pathway that activates a nuclease. All of these questions should be answerable now that purified DFF is available.

Experimental Procedures

General Methods and Materials

We obtained Ac-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) from Biochem and Bioscience Inc.; Ac-Asp-Glu-Ala-Asp-aldehyde (Ac-DEAD-CHO) as described in Wang et al. (1995a); proteinase K and DNase-free RNase A from Worthington; and Coomassie brilliant blue, molecular weight standards for SDS–PAGE, and gel filtration chromatography from Bio-Rad. Protein concentrations were determined by the Bradford Method. General molecular biology methods were as in Sambrook et al. (1989). HeLa cell cytosol was prepared as described in Liu et al. (1996b).

Assay for DFF

Caspase-3 was prepared as described in Liu et al. (1996a). The purified enzyme was stored in buffer A (20 mM HEPES–KOH (pH 7.5), 10 mM KCL, 1.5 mM MgCl, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM PMSF) containing 20% glycerol and 1 mg/ml bovine serum albumin (BSA) in multiple aliquots at −80°C. Hamster liver nuclei were prepared as described in Liu et al. (1996b); HeLa cell nuclei and nuclear extract were prepared as in Wang et al. (1993). Purified nuclei were resuspended in buffer B (10 mM PIPES [pH 7.4], 80 mM KCl, 20 mM NaCl, 5 mM sodium EGTA, 250 mM sucrose, and 1 mM DTT) at 8.5×10^7 nuclei/ml and stored in multiple aliquots at −80°C. The DNA fragmentation was assayed by incubating an aliquot (7 μl) of hamster liver or HeLa cell nuclei and 6 μl of caspase-3 with the indicated enzyme fractions at 37°C for 2

hr in a final volume of 60 μl adjusted with buffer A. After incubation, 330 μl of buffer C (100 mM Tris–HCl [pH 8.5], 5 mM EDTA, 0.2 M NaCl, 0.2% w/v SDS, and 0.2 mg/ml proteinase K) was added to each reaction and incubated at 37°C overnight. NaCl was then added to a final concentration of 1.5 M, and the nuclear debris was spun down for 15 min in a microcentrifuge at room temperature. The DNA in the supernatant was precipitated with an equal volume of 100% ethanol. The DNA precipitate was washed once with 70% (v/v) ethanol and resuspended in 40 μl of buffer D (10 mM Tris–HCl [pH 7.5], 1 mM sodium EDTA, and 200 μg/ml DNase-free RNase A). After incubation at 37°C for 2 hr, the DNA was loaded onto a 2% agarose gel, and electrophoresis was conducted at 50 V for 2 hr in 0.5 × Tris–borate–EDTA (TBE) buffer (1 × TBE buffer contains 90 mM Tris–borate/2 mM EDTA). The gel was stained with 2 μg/ml ethidium bromide for 15 min, destained with water for 1 hr, and visualized under UV light.

Purification of DFF from HeLa S-100

All purification steps were carried out at 4°C. All chromatography steps except the SP-Sepharose column and the phenyl-Sepharose column were carried out using an automatic fast protein liquid chromatography (FPLC) station (Pharmacia).

HeLa S-100 (750 ml) from 100 liters of suspension-cultured HeLa cells were applied to a SP-Sepharose column (200 ml bed volume) equilibrated with buffer A. The column was washed with three column volumes of buffer A and eluted with two column volumes of buffer A containing 0.5 mM NaCl. Ammonium sulfate (1 M) was added directly to the Sp-Sepharose 0.5 M eluate. After rotating at 4°C for 1 hr, the sample was centrifuged at 15,000 rpm for 30 min in a Sovall JA-600 rotor. The supernatant was directly loaded onto a 100 ml phenyl-Sepharose column equilibrated with buffer A containing 1 M ammonium sulfate and 0.5 M NaCl. The column was washed with three bed volumes of buffer A containing 1 M ammonium sulfate and 0.5 M NaCl, and the bound material was eluted with two bed volumes of buffer A. Ammonium sulfate was added to the phenyl-Sepharose eluate to 50% saturation. After stirring at 4°C for 5 hr, the sample was centrifuged at 15,000 rpm for 15 min in a Sovall JA-600 rotor. The pellet was resuspended in buffer A and loaded onto a Superdex-200 16/60 gel filtration column (Pharmacia), equilibrated with buffer A, and eluted with the same buffer. Fractions of 4 ml were collected and assayed for DFF activity. The active fractions from the gel filtration column were pooled and loaded onto a Mono S 10/10 column (Pharmacia) equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with a 200 ml 0–0.2 M linear NaCl gradient. Fractions of 4 ml were collected and assayed for DFF activity. The active fractions from the Mono S column were pooled and loaded onto a 1 ml hydroxyapatite column (Bio-Rad) equilibrated with buffer A. The column was washed with 10 ml of buffer A, and the bound material was eluted with 0–0.25 M linear phosphate gradient. Fractions of 1 ml were collected and assayed for DFF activity. The active fractions were pooled and loaded onto a Mono Q 5/5 column (Pharmacia) equilibrated with buffer A. The column was washed with 10 ml of buffer A containing 0.1 M NaCl, and DFF was eluted from the column with a 30 ml 0.1–0.3 M linear NaCl gradient. Fractions of 1 ml were collected and assayed for DFF activity.

Western Blot Analysis

A monoclonal antibody against human PARP (c-2–10) was used as described in Kaufmann et al. (1993). A monoclonal antibody against human lamin B1 was from Calbiochem. Anti-DFF-45 anti-serum was generated by immuning rabbits with a recombinant DFF-45 fusion protein (see below). Immunoblot analysis was performed with the horseradish peroxidase–conjugated goat anti-mouse (PARP and lamin B1) or goat anti-rabbit (DFF-45) immunoglobulin G using Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagents (Amersham).

Biotinylation of DFF

The biotinylation of DFF was carried out using an ECL protein biotinylation kit (Amersham) with modifications. Briefly, 0.6 μg of purified DFF was incubated with 10 μl of biotinylation reagent in 120 μl of 40 mM bicarbonate buffer at room temperature for 1 hr. Then 20 μl of 1 M Tris–HCl (pH 8.0) was added to the reaction, followed by incubation at room temperature for 1 hr. The sample was then dialyzed against buffer F (20 mM Tris–HCl [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium DGTA, 1 mM DTT, 1 mM PMSF) at 4°C overnight.

cDNA Cloning of DFF-45

Hela Poly(A)$^+$ mRNA was purified using Rapid mRNA Purification kit (Pharmacia). First-strand cDNA synthesis was carried out using a First-Strand cDNA Synthesis kit with oligo(dT) primers (Pharmacia). The cDNA was amplified with 40 pmol oligonucleotide 5′-GGCACGAGCTCCAGATGCTTGTT-3′ and 40 pmol oligonucleotide 5′-CAGCCAATGCTTTGGGGTCTTCC-3′ designed from an EST clone (#116412) that encodes one of the DFF-45 peptide sequences (Figure 10A, underlined). A 395 bp PCR product was subcloned into the PCR II vector using the TA cloning kit (Invitrogen) and sequenced. The 395 bp PCR product was subsequently labeled with [^{32}P]dCTP using *redi* prime RANDOM Primer Labelling kit (Amersham) and used to screen a Hela λgt11 cDNA library by hybridizing duplicate filters at 42°C for 3 hr in Rapid-hyb buffer (Amersham). The filters were washed twice with 1× saline citrate (SSC)/ 0.1% SDS for 15 min at room temperature and once with 0.5 × SSC/0.1% SDS for 10 min at 65°C. Out of 8 × 10^5 plaques screened, a 1.3 kb partial length clone was identified and subcloned into the EcoRI site of PCRII vector (In Vitro Gene). A 1.0 kb EcoRI/BamHI fragment was excised from the 5′ end of the 1.3 kb insert and labeled with dCTP as described above. A Hela λ Exlox library (Yokoyama et al., 1993) was screened with this 1 kb cDNA fragment as described above. In 6 × 10^5 plaques screened, 30 positive clones were identified. A 1.6 kb clone that contains the longest open reading frame was sequenced in both strands in an automated sequencer.

Production of DFF-45 Fusing Protein

The primers 5′-CCCACCTTGTGGCATATGGAGGTGACCGGGA-3′ and 5′-TGTAATTAGAGGAGGCTCGAGATTGTTGGTGCAGCTAT ATCATT- 3′ were designed to PCR amplify the DFF-45 cDNA open reading frame, and the amplified cDNA was subcloned in-frame into the NdeI/XhoI sites of the bacterial expression vector pET-15b (Novagen). The expression plasmid was transformed into bacteria BL21(DE3). In a typical DFF-45 preparation, a 10 ml overnight-cultured bacteria containing DFF-45 expression vector was added into a 500 ml LB broth and cultured for 3 hr by shaking at 220 rpm in 37°C, and then isopropyl-1-thio-B-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and cultured for another 2 hr. The bacterial pellet was resuspended in 10 ml of buffer A and broken by sonication. After centrifugation at 4000 × g for 15 min, the supernatant was loaded onto a nickel affinity column (6 ml). The column was washed with 30 ml of buffer A containing 1 M NaCl, followed by 20 ml of buffer A. The column was eluted with buffer A containing 250 mM imidazole. About 10 mg of DFF-45 protein was purified from a 500 ml culture.

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GenBank Accession Number

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