

Activation of the Apoptotic Endonuclease DFF40 (Caspase-activated DNase or Nuclease)

OLIGOMERIZATION AND DIRECT INTERACTION WITH HISTONE H1*

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DNA fragmentation factor (DFF) is a heterodimeric protein composed of 45-kDa (DFF45) and 40-kDa (DFF40) subunits, a protein that mediates regulated DNA fragmentation and chromatin condensation in response to apoptotic signals. DFF45 is a specific molecular chaperone and an inhibitor for the nuclease activity of DFF40. Previous studies have shown that upon cleavage of DFF45 by caspase-3, the nuclease activity of DFF40 is relieved of inhibition. Here we further investigate the mechanism of DFF40 activation. We demonstrate that DFF45 can also be cleaved and inactivated by caspase-7 but not by caspase-6 and caspase-8. The cleaved DFF45 fragments dissociate from DFF40, allowing DFF40 to oligomerize to form a large functional complex that cleaves DNA by introducing double strand breaks. Histone H1 directly interacts with DFF, confers DNA binding ability to DFF, and stimulates the nuclease activity of DFF40 by increasing its K_{cat} and decreasing its K_m .

Chromatin condensation and DNA fragmentation into nucleosomal fragments are the best recognized biochemical events of apoptosis (1, 2). Such events are mediated by the activation of DFF,¹ a heterodimeric protein consisting of a 40-kDa (DFF40/CPAN/CAD) and a 45-kDa (DFF45/ICAD) subunit (3–8). DFF45 is a dual function protein subunit that serves both as a specific molecular chaperone to mediate the correct folding of DFF40 and as an inhibitor of the DFF40 nuclease when complexed with it (4, 6–8). DFF45 and its mouse homologue named ICAD are substrates for caspase-3 and become cleaved in cells undergoing apoptosis (3, 4). The dual functions of DFF45 ensure that DFF will only become activated through the cleavage by caspases, the apoptotic pro-

teases that only become activated when cells receive apoptotic signals (9). In cell extracts prepared from *DFF45* knock out mice, DNA fragmentation activity is completely abolished (8). In response to apoptotic stimuli, splenocytes, thymocytes, and granulocytes from *DFF45* mutant mice are resistant to DNA fragmentation, and splenocytes and thymocytes are also resistant to chromatin condensation (8). Interestingly, unlike other nucleases, the activity of DFF40 can be markedly stimulated by the abundant chromatin-associated proteins such as histone H1, HMG-1, and HMG-2 (7, 10), which are known to be located at the nucleosomal linker regions in chromatin (11, 12). We have hypothesized that such a stimulatory effect facilitates the generation of uniform nucleosomal fragments and also provides an efficient way to disassemble complex chromatin structures (7).

In this communication, we report a novel mechanism for DFF40 activation that involves DFF40 oligomerization and its direct interaction with histone H1.

EXPERIMENTAL PROCEDURES

General Methods and Materials—We obtained [³⁵S]methionine from Amersham Pharmacia Biotech, histone H1 and anti-histone H1 antibody from Roche Molecular Biochemicals and DNase I and micrococcal nuclease from Worthington. Plasmids were purified using a Megaprep kit from Qiagen.

Production and Purification of Recombinant DFF—The expression plasmid containing both *DFF45* and *DFF40* was constructed as described in Ref. 7 and transformed into bacteria BL21(pLysS) (Novagen). DFF was induced and purified as described in Ref. 7.

Production of Recombinant Caspase-3, -6, -7, and -8—A pET15b vector containing coding regions for caspase-3, caspase-6, caspase-7, or a pET21a containing coding regions for caspase-8 was used to transform bacteria BL21 (DE3). The bacterial cultures (1 liter for each plasmid) were grown at 37 °C until the density reached an A_{600} reading of 0.6. Isopropyl-1-thio- β -D-galactopyranoside was then added to a final concentration of 2 mM. After a 3-h induction, the bacteria were pelleted by centrifugation and lysed in buffer A by sonication. After centrifugation, the supernatants were loaded onto two 3-ml nickel-Sepharose (Qiagen) columns equilibrated with buffer A. The columns were washed with 10 ml of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) followed by 200 ml of buffer A containing 500 mM NaCl and again with 10 ml of buffer A. The fusion proteins were then eluted with buffer A containing 250 mM imidazole.

In Vitro Translation of Procaspase-3—A pET 15b vector (Novagen) containing a PCR fragment encoding amino acids 29–277 of hamster caspase-3 was translated in a TNT T7 transcription/translation kit in the presence of [³⁵S]methionine according to the manufacturer's instructions. The translated protein was passed through a 1-ml nickel affinity column (Qiagen) equilibrated with buffer A. After washing the column with 10 ml of buffer A, the translated caspase-3 was eluted with buffer A containing 250 mM imidazole.

Nuclease Assay for DFF—An aliquot of recombinant DFF was incubated with 60 ng of caspase-3 and 3 μ g of pcDNA3 (Invitrogen) at 37 °C for 30 min in a final volume of 30 μ l adjusted with buffer A containing 4 mM MgCl₂. The reactions were stopped by adding EDTA to a final concentration of 5 mM, and the samples were loaded onto 2% agarose

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¹ The abbreviations used are: DFF, DNA fragmentation factor; DFF45, 45-kDa subunit of DFF; DFF40, 40-kDa subunit of DFF; CAD, caspase-activated DNase; ICAD, inhibitor of CAD; CPAN, caspase-activated nuclease; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; BSA, bovine serum albumin; HMG, high mobility group.

gels containing 2 $\mu\text{g/ml}$ ethidium bromide. Electrophoresis was conducted at 50 V for 1 h in 0.5 \times Tris borate/EDTA buffer (1 \times Tris borate/EDTA buffer contains 90 mM Tris borate, 2 mM EDTA). Gels were visualized under UV light.

Immunoprecipitation—An aliquot of 1 ml of protein G-agarose (Santa Cruz) was incubated with an aliquot of 200 μg of anti-histone H1 antibody at 4 $^{\circ}\text{C}$ overnight. The protein G-agarose beads were pelleted by centrifugation and washed five times with buffer A. The beads were then incubated with 1 ml of bovine calf serum and 1 mg of BSA for 1 h at room temperature. The beads were pelleted by centrifugation and washed with buffer A five times. The beads were resuspended in 1 ml of buffer A. Aliquots of 3 μg of DFF were incubated alone, with 3 μg of histone H1, or with 3 μg of histone H1 plus 5 μg of pcDNA3 in the absence or presence of 1 μg of caspase-3 at room temperature for 30 min in a final volume of 300 μl of buffer A followed by the addition of 50 μl of antibody protein G-agarose beads prepared above. The samples were incubated at room temperature for another hour, and the agarose beads were pelleted by centrifugation and washed five times with buffer A. 60 μl of 1 \times SDS loading buffer was added to the beads, and the samples were boiled at 100 $^{\circ}\text{C}$ for 3 min before being subjected to SDS-PAGE and Western blot analysis.

Immunoprecipitation with anti-DFF45 antibody was carried out by incubating an aliquot of 500 μl of protein A-agarose with 500 μl of preimmune or immune serum of DFF45 at 4 $^{\circ}\text{C}$ overnight. The antibody protein A-agarose beads were pelleted by centrifugation and washed five times with buffer A. The beads were then incubated with 1 ml of bovine calf serum (1 mg/ml) for 1 h at room temperature, pelleted by centrifugation, and washed with buffer A five times. The beads were resuspended in 500 μl of buffer A. An aliquot of 50 μl of preimmune protein A-agarose or immune protein A-agarose beads was incubated with 3 μg of DFF and 10 μg of histone H1 at room temperature for 1 h. The beads were harvested by centrifugation and washed five times with buffer A. 60 μl of 1 \times SDS loading buffer was added to the beads, and the samples were boiled at 100 $^{\circ}\text{C}$ for 3 min before being subjected to SDS-PAGE and Western blot analysis.

Western Blot Analysis—Polyclonal antibodies against DFF45 and DFF40 were produced as described (3, 7). A monoclonal antibody against histone H1 was purchased from Roche Molecular Biochemicals. Immunoblot analysis was performed with the horseradish peroxidase-conjugated goat anti-mouse (histone H1) or goat anti-rabbit (DFF45, DFF40) immunoglobulin G using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech).

RESULTS

Activation of DFF by Caspase-3 and -7—To characterize the enzymatic activity of DFF40, we generated recombinant DFF through a bacterial double expression vector and purified the recombinant DFF to apparent homogeneity through a nickel affinity column followed by a Mono S column. Purified DFF exhibits a 1:1 ratio of DFF45 and DFF40, which further proves that DFF40 and DFF45 form a stable complex (Fig. 1A).

It has been reported that in cells lacking caspase-3, DFF45 still gets cleaved in response to apoptotic stimuli, suggesting that other caspases may also cleave DFF45 (13, 14). Therefore we tested whether apoptotic caspases other than caspase-3 could also activate DFF using the purified recombinant DFF. As shown in Fig. 1B, both caspase-3 and caspase-7 activated the nuclease activity of DFF (lanes 3 and 7). However, caspase-6 did not activate DFF (lanes 5 and 11), and caspase-8 only weakly activated DFF (lane 9). All these caspases did not show any nuclease activity by themselves (lanes 2, 4, 6, and 8). Although caspase-6 and -8 were unable to activate DFF, these enzymes were indeed active as demonstrated by their ability to cleave procaspase-3, a known substrate for these caspases (lanes 11 and 12).

Oligomerization of DFF40 upon Activation by Caspase-3—DFF migrates on a gel filtration column as a heterodimer with a molecular mass of ~ 85 kDa (3). When subjected to a Superdex 200 gel filtration chromatography, the DFF peak was observed at fraction 15, corresponding to the inactive heterodimeric form (Fig. 2A). The DNase activity of DFF was readily detected when the column fractions were incubated with caspase-3 (Fig. 2A, lower panel). Strikingly, when DFF

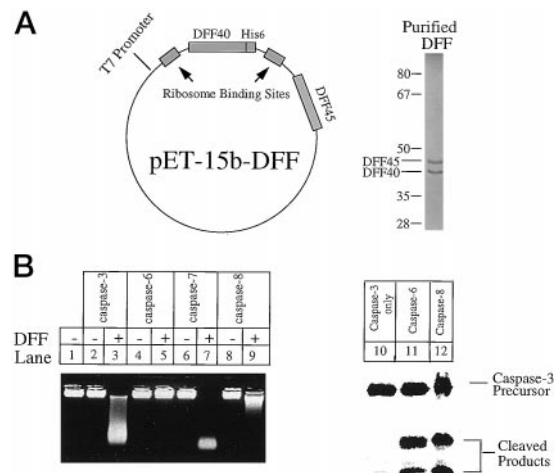


FIG. 1. Activation of DFF by different caspases. A, the bacterial double expression vector (in pET15b) for DFF (left) and a Coomassie Blue stained gel of purified recombinant DFF (right). B, aliquots of 60 ng of caspase-3, caspase-6, caspase-7, or caspase-8 were incubated with 3 μg of pcDNA3 in the presence or absence of 300 ng of DFF at 37 $^{\circ}\text{C}$ for 30 min in a final volume of 30 μl adjusted with buffer A containing 4 mM MgCl_2 . The reaction samples were loaded onto a 2% agarose gel containing 2 mg/ml ethidium bromide (left). Aliquots of 60 ng of caspase-6 and caspase-8 were incubated with 3 μl of *in vitro* translated, [^{35}S]methionine-labeled procaspase-3 at 37 $^{\circ}\text{C}$ for 10 min in 20 μl of buffer A (right). The samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a film for 16 h at room temperature.

was activated by incubating with caspase-3 before being loaded on the gel filtration column, a caspase-3-independent DNase activity was now observed at fraction 10 after chromatography (Fig. 2B, lower panel), corresponding to a complex with a size larger than the exclusion volume of this column (>1.3 million daltons). This DNase activity co-migrated with one of the DFF40 peaks as detected by Western blot analysis (Fig. 2B, middle panel). The second DFF40 peak at fraction 16 showed no DNase activity, indicating that only the large complex form of DFF40 is active. The cleaved fragments of DFF45 now migrated at fractions 16–19, and no DNase activity was detected in these fractions. This large DFF40 complex is responsive to histone H1 stimulation, and including histone H1 during DFF activation does not interfere with the formation of this large functional DFF40 complex (data not shown).

Interaction of Histone H1 and DFF—Previously, we have shown that the endonuclease activity of DFF40 can be stimulated by chromatin-associated proteins such as histone H1 and HMG proteins (7, 10). To elucidate the mechanism of this stimulatory effect, we tested whether histone H1 could directly interact with DFF. The recombinant DFF was incubated with histone H1 in the absence (Fig. 3A, lanes 1–3) or presence (lanes 4–6) of caspase-3. The protein complexes were immunoprecipitated with a monoclonal anti-histone H1 antibody and analyzed by Western blotting using antibodies against DFF45, DFF40, and histone H1 (Fig. 3A). When caspase-3 was not included in the reaction, both DFF40 and DFF45 were found to co-precipitate with histone H1 (Fig. 3A, lane 2). However, in the presence of caspase-3, only DFF40 was co-precipitated with histone H1 (Fig. 3A, lane 4). Inclusion of DNA in the reaction did not increase the binding between DFF and histone H1 (Fig. 3A, lanes 3 and 6). As a control, DFF alone could not be precipitated by the anti-histone H1 antibody (Fig. 3A, lanes 1 and 4). Furthermore, the cleaved fragments of DFF45 did not co-precipitate with histone H1 and resided exclusively in the supernatant as detected by Western blot analysis (data not shown).

To confirm such interaction between histone H1 and DFF,

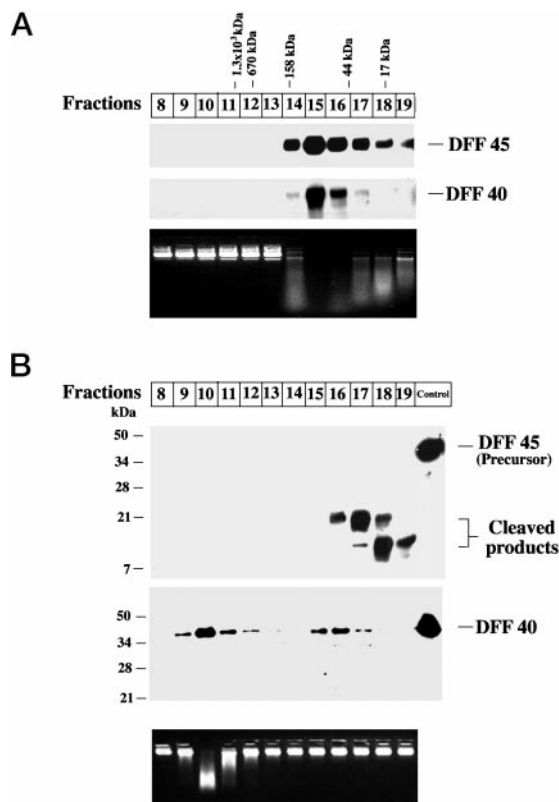


FIG. 2. Oligomerization DFF40 is required for activation. In *A*, an aliquot of 5 μ g of recombinant DFF was fractionated by a Superdex-200 gel filtration column using a Smart System (Amersham Pharmacia Biotech). Fractions of 100 μ l were collected. Aliquots of 30- μ l column fractions were subjected to 12% SDS-PAGE followed by Western blot analysis using a polyclonal antibody against DFF40 (1:2000) and a polyclonal antibody against DFF45 (1:5000). The antigen-antibody complexes were visualized by an ECL method as described under "Experimental Procedures." The filters were exposed to x-ray film for 1 min (*upper* and *middle* panels). In the *lower panel*, aliquots of 15- μ l column fractions were incubated with aliquots of 50 ng of bacteria-expressed caspase-3, 300 ng of histone H1, 400 ng of BSA, and 500 ng of plasmid DNA and $MgCl_2$ at a final concentration of 4 mM in 30 $^\circ$ C in a final volume of 25 μ l in buffer A. After 1 h of incubation, the samples were analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide staining. In *B*, an aliquot of 5 μ g of recombinant DFF complex was preincubated with an aliquot of 200 ng of bacteria-expressed caspase-3 at 30 $^\circ$ C for 1 h in a final volume of 70 μ l in buffer A. After incubation, the reaction mixture was fractionated in the gel filtration column as in *A*. Aliquots of 30- μ l column fractions were subjected to 12% SDS-PAGE followed by Western blot analysis as in *A*. The filters were exposed to x-ray film for 1 min (*upper* and *middle* panels). In the *lower panel*, aliquots of 20- μ l column fractions were incubated with 300 ng of histone H1, 400 ng of BSA, and 500 ng of plasmid DNA and $MgCl_2$ at a final concentration of 4 mM at 30 $^\circ$ C in a final volume of 30 μ l in buffer A. After 1 h of incubation, the samples were analyzed as in *A*.

we also carried out the reciprocal immunoprecipitation experiment using an antiserum against DFF45. Both DFF40 and histone H1 were co-precipitated by the anti-DFF45 antibody (Fig. 3A, *lane* 8). In contrast, the preimmune serum did not precipitate either protein (Fig. 3A, *lane* 7).

Because DFF is located in the nucleus (7, 15), it was of interest to see whether DFF could directly bind to DNA. We tested DNA binding by conducting an immunoprecipitation experiment using the anti-DFF45 antibody. As shown in Fig. 3B, in the absence of histone H1, very little DNA was co-precipitated even with the highest amount of DFF used. However, in the presence of histone H1, significant amounts of DNA were co-precipitated with DFF in a dose-dependent fashion.

To evaluate the effect of histone H1 on the kinetics of nuclease activity of DFF, we measured the nuclease activity of DFF

in the absence or presence of histone H1 using different concentrations of sheared DNA as substrates. The velocities of the reactions were determined by the Kunitz method (16). As shown in Fig. 3C, histone H1 causes a 2-fold increase on K_{cat} and a 4-fold decrease on K_m of the nuclease activity of DFF.

DFF Cleaves DNA by Introducing Double Strand Breaks—To determine how DFF cleaves double-stranded DNA, we compared the cleavage products of DFF with that of DNase I by two-dimensional gel electrophoresis. As shown in Fig. 4c, after two-dimensional alkaline electrophoresis, a horizontal distribution of single-stranded DNA was observed for DNase I digestion, confirming that DNase I introduces single strand nicks into double-stranded DNA (17). However, the DNA cleaved by DFF showed a diagonal distribution on two-dimensional gel electrophoresis that was different from the pattern generated by DNase I (Fig. 4, *a* and *c*), indicating that DFF did not generate single strand nicks on double-stranded DNA. Inclusion of histone H1 in the reaction did not change the diagonal distribution of cleavage products on the two-dimensional gel (Fig. 4b), suggesting that histone H1 stimulates the nuclease activity of DFF without changing its mode of DNA cleavage.

DISCUSSION

Caspases have been suggested to be the core apparatus of the execution of cell death (9). The cleavage of DFF45 by caspase-3, which liberates the nuclease activity of DFF40, supports this suggestion. In addition to caspase-3, DFF can also be activated by caspase-7 and weakly activated by caspase-8 but not by caspase-6 (Fig. 1B). This is consistent with previous studies on the substrate preferences of caspases, with caspase-3 and caspase-7 preferring the DXXD motif that fits the caspase cleavage sites DETD and DAVD on DFF45. On the other hand, caspase-6, -8, and -9 prefer the (L/V)EXD motif that is absent in DFF45 (18, 19).

The cleavage of DFF45 by caspases, interestingly, does not just dissociate the fragments of DFF45 from DFF40 to release its inhibition; in addition, liberated DFF40 oligomerizes to form a large protein complex that elutes at the exclusion volume of a Superdex 200 gel filtration column (Fig. 2). This large DFF40 oligomer is by itself an active DNase and is responsive to histone H1 stimulation. The formation of this large DFF40 oligomer probably requires the chaperoning function of DFF45 because the expression of DFF40 alone cannot generate functional DNase both in cultured cells and in *DFF45* knock out mice (4, 6–8).

Purified DFF exhibits relatively weak nuclease activity in the presence of caspase-3, whereas the same amount of protein can actively induce DNA fragmentation in nuclei (3). Based on this observation, we have identified additional protein factors, such as histone H1 and HMG-1/2, that enhance the nuclease activity of DFF (7, 10). Histone H1 and HMG proteins are "architectural" proteins that are located in the internucleosomal linker regions of chromatin (11, 12). The association of DFF with these chromatin-associated proteins therefore targets DFF to the linker regions. Moreover, the direct interaction of DFF with histone H1 also confers the DNA binding ability to DFF (Fig. 3B).

Previously, we have shown that histone H1 stimulates the nuclease activity of DFF more than 10-fold (7). Kinetic analysis indicates that histone H1 enhances the nuclease activity of DFF by increasing K_{cat} and decreasing K_m (Fig. 3C). We postulate that HMG proteins may have a role similar to histone H1. Of the HMG proteins tested, HMG-1, HMG-2, and HMG-14 but not HMG1/Y stimulated the nuclease activity of DFF (data not shown). One possibility is that HMG1/Y does not interact directly with DFF although histone H1 and HMG-1, -2, and -14 do. Therefore histone H1 and HMG proteins not only recruit

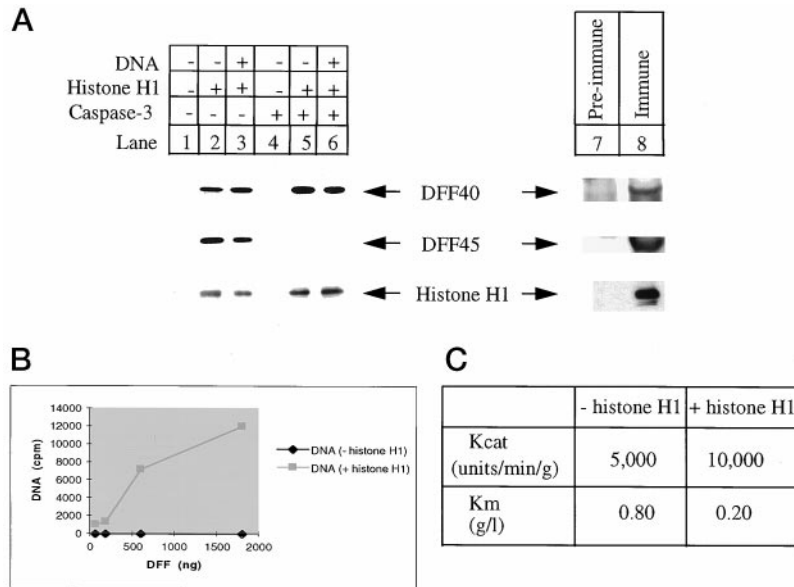


FIG. 3. Histone H1 directly interacts with DFF40. *A*, aliquots of 3 μ g of DFF were incubated alone (*lanes 1 and 4*), with 3 μ g of histone H1 (*lanes 2 and 5*), or with 3 μ g of histone H1 plus 5 μ g of pcDNA3 (*lanes 3 and 6*) in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of 1 μ g of caspase-3 at room temperature for 30 min in a final volume of 300 μ l adjusted with buffer A. In *A*, aliquots of 70 μ l of protein G-agarose coupled with anti-histone H1 antibody were included in each reaction (*left*). After 1 h of incubation at room temperature, the agarose beads were precipitated and washed five times with 500 μ l of buffer A. 60 μ l of 1 \times SDS loading buffer was added to the agarose beads and heated at 100 $^{\circ}$ C for 3 min. After spinning at 14,000 rpm in a microcentrifuge for 1 min, aliquots of 20 μ l of the resulting supernatant were subject to SDS-PAGE, and Western blot analysis was carried out using anti-DFF45, anti-DFF40, or anti-histone H1 antibody, and the antigen-antibody complexes were visualized by an ECL method as described under “Experimental Procedures.” The filters were exposed to x-ray film for 1 min. In *A*, an aliquot of 3 μ g of DFF was incubated with 10 μ g of histone H1 at room temperature for 30 min in a final volume of 300 μ l adjusted with buffer A (*right*). Then aliquots of 50 μ l of protein A-agarose beads coupled with either the preimmune serum (*lane 7*) or the anti-DFF45 serum (*lane 8*) were added to half of the reaction mixture, and the incubation was continued for 1 h. The agarose beads were washed five times with 500 μ l of buffer A. 60 μ l of 1 \times SDS loading buffer was added to the agarose beads and heated at 100 $^{\circ}$ C for 3 min. Aliquots of 20 μ l were subject to SDS-PAGE, and Western blot analysis was carried out using anti-DFF45, anti-DFF40, or anti-histone H1 antibody. In *B*, two primers were used to PCR amplify a 1-kilobase DNA fragment containing the DFF45 coding region in the absence or presence of [32 P]dATP. The PCR products were purified by passing through a PCR purification column (Qiagen). An aliquot of 3 μ g of the unlabeled PCR product was mixed with 100,000 cpm of the same [32 P]-labeled PCR product and incubated with the indicated amounts of DFF (60, 180, 600, and 1800 ng) in the absence or presence of 3 μ g of histone H1 at room temperature for 30 min. Then 50 μ l of the DFF45 antibody-coupled protein A-agarose was added to each reaction. After 30 min of incubation at room temperature, the agarose beads were pelleted by centrifugation and washed five times with 1 ml of buffer A. The radioactivity in the agarose beads was detected by scintillation counting. *C*, aliquots of 1.5 μ g of DFF were incubated with 1.0 μ g of caspase-3 at 37 $^{\circ}$ C for 5 min with a final volume of 100 μ l of buffer A. Then aliquots of various amounts of sheared DNA (10, 15, 25, 40, 50, and 65 μ g) were added to each reaction in the absence or presence of 10 μ g of histone H1 with a final volume of 0.5 ml of buffer A containing 4 mM MgCl₂. The absorbances of the reaction mixtures at 260 nm (A_{260}) were recorded at different times, and the slope of the linear increase on the A_{260} was defined as the initial velocity of the reaction. The concentration of DNA (g/liter) in the reaction was defined as substrate concentration(s). The K_{cat} and K_m values were determined by plotting 1/velocity against 1/substrate concentration.

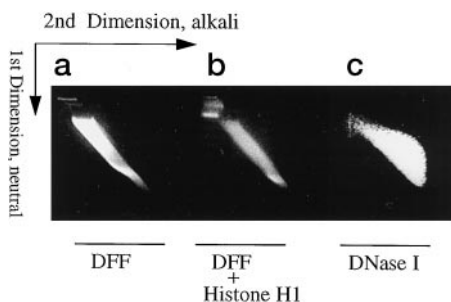


FIG. 4. DFF40 cleaves DNA by introducing double-stranded DNA breaks. 3 μ g of pcDNA3 and 150 ng of caspase-3 were incubated with 100 ng of DFF (*a*) or 20 ng of DFF plus 0.5 μ g of histone H1 (*b*) at 37 $^{\circ}$ C for 15 min in a final volume of 40 μ l adjusted with buffer A containing 4 mM MgCl₂. In *c*, 3 μ g of pcDNA3 were incubated with 0.05 unit of DNase I at 37 $^{\circ}$ C for 5 min in a final volume of 30 μ l adjusted with buffer B (50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, and 1 mM CaCl₂). Samples were run in 0.5 \times TAE (40 mM Tris-acetate, 1 mM EDTA) agarose gel (*1st dimension, neutral*). After treatment with denaturing buffer (50 mM NaOH, 1 mM EDTA), the gels were turned 90 $^{\circ}$ and run in the same buffer (*2nd dimension, alkaline*).

DFF to the internucleosomal linker regions of chromatin but also stimulate the nuclease activity of DFF40 oligomer. Recently, Halenbeck *et al.* (6) stated that carrier proteins such as BSA also enhance the endonuclease activity of DFF40/CPAN in

the presence of higher concentrations of Mg²⁺. However, our results indicate that low concentrations of BSA have no effect on DFF40 nuclease activity (7), and only when high concentrations of BSA (1 mg/ml) were included in the reaction buffer did the BSA effect become obvious (data not shown). The stimulatory effect of histone H1 on DFF40 nuclease is at least two orders of magnitude higher than that of BSA (data not shown). Furthermore, histone H1 and HMG-1/2 still stimulated the nuclease activity of DFF40 when we used the same assay conditions as described by Halenbeck *et al.* (6) (data not shown). Therefore the stimulatory effect of chromatin-associated proteins on DFF is different from that of BSA.

Unlike DNase I, DFF40 did not generate single strand nicks when cleaving double-stranded DNA (Fig. 4), indicating that DFF40 cleaves DNA by introducing double strand breaks. The break points are either blunt ends or one-base 5'-overhangs with 5'-phosphate and 3'-hydroxyl groups (data not shown). Such a property eliminates the possibility for single strand repair enzymes to relegate the broken points. In addition, because double-stranded DNA breaks are themselves apoptotic signals, the cleavage of genomic DNA by DFF40 may trigger an amplification cycle that marks an irreversible step for apoptosis.

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REFERENCES

1. Wyllie, A. H. (1980) *Nature* **284**, 555–556
2. Atrens, M. J., Morris, R. G., and Wyllie, A. H. (1990) *Am. J. Pathol.* **136**, 593–608
3. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) *Cell* **89**, 175–184
4. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) *Nature* **391**, 43–50
5. Sakahira, H., Enari, M., and Nagata, S. (1998) *Nature* **391**, 96–98
6. Halenbeck, R., MacDonald, H., Roulston, A., Chen, T. T., Contoy, L., and Williams, L. T. (1998) *Curr. Biol.* **8**, 537–540
7. Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W., and Wang, X. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8461–8466
8. Zhang, J., Liu, X., Scherer, D. C., Boivin, G. P., Van Kaer, L., Wang, X., and Xu, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12480–12485
9. Cryns, V., and Yuan, J. (1998) *Genes Dev.* **12**, 1551–1570
10. Toh, S. Y., Wang, X., and Li, P. (1998) *Biochem. Biophys. Res. Commun.* **250**, 598–601
11. Bustin, M., and Reeves, R. (1996) *Prog. Nucleic Acid Res. Mol. Biol.* **54**, 35–100
12. Van Holde, K. E. (1989) *Chromatin*, pp. 198–288, Springer-Verlag New York Inc., New York
13. Janicke, R. U., Ng, P., Sprengart, M. L., and Porter, A. G. (1998) *J. Biol. Chem.* **273**, 15540–15545
14. Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A., Senaldi, G., Howard, T., Lowe, S. W., and Mak, T. W. (1998) *Genes Dev.* **12**, 806–819
15. Samejima, K., and Earnshaw, W. C. (1998) *Exp. Cell Res.* **243**, 453–459
16. Kunitz, M. (1950) *J. Gen. Physiol.* **33**, 349–362
17. Campbell, V. W., and Jackson, D. A. (1980) *J. Biol. Chem.* **255**, 3726–3735
18. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997) *J. Biol. Chem.* **272**, 9677–9682
19. Thornberry, N., Rano, T., Peterson, E., Rasper, D., Timkey, T., Garcia-Calvo, M., Houtzager, V., Nordsreom, P., Roy, S., Vaillancourt, J., Chapman, K., and Nicholson, D. (1997) *J. Biol. Chem.* **272**, 17907–17911