Cleavage Preferences of the Apoptotic Endonuclease DFF40 (Caspase-activated DNase or Nuclease) on Naked DNA and Chromatin Substrates*

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Here we report the co-factor requirements for DNA fragmentation factor (DFF) endonuclease and characterize its cleavage sites on naked DNA and chromatin substrates. The endonuclease exhibits a pH optimum of 7.5, requires Mg\(^{2+}\), not Ca\(^{2+}\), and is inhibited by Zn\(^{2+}\). The enzyme generates blunt ends or ends with 1-base 5'-overhangs possessing 5'-phosphate and 3'-hydroxyl groups and is specific for double- and not single-stranded DNA or RNA. DFF endonuclease has a moderately greater sequence preference than micrococcAL nuclease or DNase I, and the sites attacked possess a dyad axis of symmetry with respect to purine and pyrimidine content. Using HeLa cell nuclei or chromatin reconstituted on a 5 S rRNA gene tandem array, we prove that the enzyme attacks chromatin in the internucleosomal linker, generating oligonucleosomal DNA ladders sharper than those created by micrococcAL nuclease. Histone H1, high mobility group-1, and topoisomerase II activate DFF endonuclease activity on naked DNA substrates but much less so on chromatin substrates. We conclude that DFF is a useful reagent for chromatin research.

Apoptosis, or programmed cell death, plays an important role in the development of an organism and in the maintenance of tissue homeostasis (reviewed in Refs. 1 and 2). Hallmarks of the terminal stages of apoptosis are nucleosomal DNA fragmentation (also termed "DNA laddering") and chromatin condensation (3–5). The endonuclease primarily responsible for mediating DNA laddering is activated by caspase-3 treatment of DFF. In its inactive form, DFF is a heterodimer composed of a 45-kDa chaperone and inhibitor subunit (DFF45/ICAD) and a 40-kDa latent endonuclease subunit (DFF40/CAD/CPAN) (6–10). This protein complex resides in the cell nucleus (7, 11). Caspase-3 cleavage of DFF specifically cuts only DFF45, which results in the dissociation of cleaved DFF45 from DFF40 (6–10). Interestingly, the released endonuclease forms homo-oligomers that are the enzymatically active form of DFF40 (12). In addition, its activity on naked DNA substrates can be further activated by specific chromosomal proteins, such as histone H1 or HMG-1/2 (7, 12, 13), and topoisomerase II (this report). Furthermore, chromatin condensation can be initiated in isolated nuclei by the addition of recombinant activated DFF40, purified free of caspase-3 and DFF45 breakdown products (7).

It should be noted that the physiological significance of DFF in triggering DNA laddering and chromatin condensation during apoptosis has been unequivocally proven. A homozygous deletion of the single copy gene encoding DFF45 has been created in the mouse germ line (14). Significantly, thymocytes and splenocytes from these knockout mice exhibit greatly reduced DNA laddering or chromatin condensation when exposed to apoptotic stimuli, both in vivo and in vitro, proving that DFF is a principal player for the induction of these events (14, 15). Furthermore, such mice still express DFF40, indicating that the chaperone function of DFF45 is required in vivo for creating a potentially functional DFF40 endonuclease. Other gene products also appear to participate in the DFF pathway. Two proteins encoded by genes called cell death-inducing DFF45-like effectors, or CIDEs, which exhibit homology to the N-terminal domain of DFF45, can activate apoptosis in a DFF45-inhibitable fashion, but their precise mechanism of action remains to be elucidated (16). In addition, an isoform of DFF45, termed DFF35 (also termed ICAD-S (9)), is incapable of acting as a chaperone and acts as an inhibitor of the latent endonuclease (17, 18). In conclusion, it is well established experimentally that DFF plays a major and regulated role in apoptotic DNA fragmentation and chromatin condensation, although other pathways have also been identified (19, 20).

Nucleases have proven to be valuable reagents for the analysis of chromatin structure (21). Because apoptotic nucleases are known to efficiently create DNA ladders composed of total genomic sequences, if DFF40 is the major player in such laddering, then its sequence specificity for DNA cleavage would appear to be broad enough to be a useful additional tool for chromatin research. Here we report the cleavage site preferences for DFF40 on naked DNA and chromatin substrates and demonstrate that DFF40 is indeed a useful reagent for generating sharp internucleosomal DNA cleavage.

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1 The abbreviations used are: DFF, DNA fragmentation factor; CAD, caspase-activated deoxyribonuclease (also termed DFF40); CPAN, caspase-activated nuclease (also termed DFF40 and CAD); DFF45, 45-kDa subunit of DFF; DFF40, 40-kDa subunit of DFF; HMG, high mobility group; ICAD, inhibitor of CAD (also termed DFF45); MNase, micrococcAL nuclease; LTR, long terminal repeat; bp, base pair(s); kb, kilobase pair(s).

2 M. Xu, personal communication.
**Experimental Procedures**

**Nucleosome Chromatin Substrates**—HeLa cell nuclei were purified as described elsewhere (6). Nuclei were successively washed at 4 °C in 10 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 20 mM Tris, pH 7.6, and 0.2 M sucrose (buffer A) plus 1% Triton X-100 and then in buffer A alone, suspended at 1 µg/µl as DNA in buffer A plus 20% glycerol and aliquots were stored at −80 °C. Chromatin was reconstituted on a tandem 18-mg array of the *Lytechinus variegatus* 5 S rRNA gene (22), reconstituted into the vector pGEM3A to yield the recombinant plasmid pH2-207-18 (gift of Dr. Joe Gatewood, Los Alamos National Laboratory). The 18-mer was excised from pH2-207-18 by digestion with *Hind* I and purified from agarose gels after electrophoresis. Chromatin was reconstituted using a salt step dialysis procedure (23, 24). Core histones were purified from HeLa nuclei. Briefly, nuclei were incubated with MNase (Worthington) and washed with buffer containing 1 mM EDTA, and chromatin was eluted with 2 mM EDTA. Chromatin was equilibrated with 0.4 M NaCl and loaded onto a hydroxyapatite column. After washing the column extensively with 0.4 M NaCl, histone H1 was eluted with 0.6 M NaCl, and loaded onto a hydroxyapatite column. After washing the column extensively with 0.4 M NaCl, histone H1 was eluted with 0.6 M NaCl, and core histones were subsequently eluted with 2 M NaCl (25); no other protein bands were visible on overloaded SDS-polyacrylamide gels. Core histones were mixed with DNA at a histone:DNA weight ratio of 1.4 in 2.5 mM NaCl at 20 °C, pH 8.5, and 0.01 M phenylmethylsulfonyl fluoride, pH 7.6. The mixture was incubated for 15 min at 37 °C, added to a dialysis tube (SpectraPor, M cut-off of 6000–8000) and dialyzed at 4 °C as follows: 2 h with 1 mM NaCl, 3 h with 0.55 M NaCl, 4 h with 0.25 M NaCl, and finally 12 h with buffer without NaCl. If histone H1 was also to be assembled into chromatin, histone H1 purified from HeLa cell nuclei as described above was added at the 0.55 M NaCl step at a concentration of 100 µg of DNA/ml. This chromatin was concentrated using a Millipore Ultrafree-MC spin filters to about 0.25 µg of DNA/µl and stored at −80 °C after glycerol was added to a final concentration of 10%.

**Assay for Endonuclease Activity**—Recombinant caspase-3 was prepared as described (12). DFF was either purified from HeLa cells or from an *Escherichia coli* expression system as reported previously (6, 12). Caspase-3 substrates were obtained with the assistance of Zymed Laboratories. However, the recombinant protein had a significantly lower specific activity, possibly due to a lack of the appropriate post-translational modifications. One µg of naked DNA, nuclei, or chromatin (as DNA) was incubated at 37 °C with 1 µl of caspase-3 (0.2 µg) and 1 µl of DFF (1 unit) in buffer consisting of 10 mM NaCl, 3 mM MgCl₂, 0.5 mM dithiothreitol, 10 mM Hepes, pH 7.5 (final volume 15 µl) for varying times, in the absence or presence of supplemented cations as indicated in the legends to Figs. 3, 4, and 6. When comparisons were made between DFF and MNase, unless otherwise indicated, 3 mM CaCl₂ was added to the above buffer for the MNase reactions. As controls, we routinely preincubated caspase-3 with 10 µM Ac-DEAD-CHO (a tetrapeptide aldehyde inhibitor of caspase-3) to demonstrate caspase-3 dependence on DFF activation or similarly postincubated with the inhibitor to block any further digestion of the protein before the addition of active DFF or any of the above substrates. Although we have found that digestion of naked DNA by DFF was markedly more active under conditions of higher monovalent cations (e.g. 50–100 mM), this is not true for digestion of chromatin substrates; thus, to reduce potential protein exchange and redistribution during digestion of chromatin, we chose to employ buffers containing 10–25 mM monovalent cations. Aliquots of the endonuclease reaction were mixed with 1/2 volume of stop solution (0.6% SDS, 50 mM EDTA, and 6 mg/ml proteinase K) and incubated for 1 h at 50 °C. Gel loading dye buffer was added, and samples were then run on 1.5% SeaKem agarose gels using 1X TAE as the running buffer. After electrophoresis, DNA was stained with ethidium bromide, and gels were scanned with a FluorImager (Molecular Dynamics Inc., Sunnyvale, CA). Images were analyzed using ImageQuant software (Molecular Dynamics) and have been represented as negatives. When radioactive DNA was used as the substrate, gels were dried and imaged with a PhosphorImager (Molecular Dynamics), and images were analyzed as above.

**Analysis of Cleavage Sites**—For a relative comparison of DNA cleavage site sequence preferences between different endonucleases, pUC19 DNA digested with either *Bgl* II or *Ava* I was labeled with T4 polynucleotide kinase after dephosphorylation, and then DNA circles were cleaved with 0.25 units of MNase (Worthington) or with 0.0025 units of *Dra* I (Worthington) for 1, 2, 4, and 6 min, or with 0.5 units of DFF with 0.2 µg of caspase-3 for 3, 9, 30, and 60 min at 37 °C. Reaction mixtures were 10 µl each and contained MgCl₂ and CaCl₂ at 1.5 mM each. DNA was purified, digested with *Ava* I, and separated on 5% polyacrylamide sequencing gels. For detailed analyses of sequences at cleavage sites, a 177-bp fragment of the HIV-1 5'-LTR DNA was excised from plasmid pWLLTR11 (26) with *Bgl* II and *Ava* I, purified from an agarose gel after electrophoresis, and 5'-end labeled with T4 polynucleotide kinase. Labeled DNA was incubated with caspase-3 and DFF for 10 min at 37 °C, then purified by phenol/chloroform extraction and ethanol precipitation. To analyze cleavage sites on the coding strand, DNA was digested with *Bgl* I (releasing a 12-bp fragment labeled at the *Bgl* II site and allowing direct analysis from the *Ava* I site). To analyze cleavage sites on the noncoding strand, DNA was digested with *Scfl* (releasing a 20-bp fragment labeled at the *Scfl* site and allowing analysis from the *Ava* I site). Digestion products were resolved on 6% polyacrylamide sequencing gels together with the appropriate Sanger sequencing reactions (DNA was sequenced with the Amersham T7 Sequenase version 2.0 sequencing kit according to the vendor’s protocol).

**Analysis of DNA Ends**—Reconstituted chromatin was incubated with caspase-3 and DFF, and then DNA was purified. Mononucleosomal DNA was isolated after electrophoresis on a low melting agarose gel, 5'-end-dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals), and 32P-5'-end-labeled with T4 polynucleotide kinase. Aliquots of labeled DNA were digested with EcoRI or 3'-end-modified with either T4 DNA polymerase (U. S. Biochemical Corp.) or terminal deoxynucleotidyl transferase (U. S. Biochemical Corp.) according to the vendor’s protocols. DNA samples were then resolved on 6% polyacrylamide sequencing gels.

**Results**

Caspase-3-activated DFF Requires Mg²⁺, not Ca²⁺, and is Inhibited by Zn²⁺—A variety of endonucleases have been implicated in apoptotic DNA laddering, including non-metal ion-dependent (27–29), Ca²⁺- and Mg²⁺-dependent (30–37), Mg²⁺-dependent (38, 39), and either Ca²⁺- or Mg²⁺-dependent endonucleases (40, 41). To rigorously establish the divalent ion requirements for DFF, we first diazyl the protein against a buffer containing EDTA. We found that caspase-3-activated DFF endonuclease had a pH optimum of 7.5 and only required Mg²⁺ and not Ca²⁺ as its divalent cation, even in the presence of EGTA, thereby eliminating the possibility that traces of contaminating Ca²⁺ in Mg²⁺-containing solutions may be required for its activity (data not shown) (Ref. 42). Under the optimal reaction conditions, the enzyme does not digest either single-stranded DNA or RNA (data not shown). Because Zn²⁺ has been reported to block apoptotic DNA laddering in certain systems (43, 44), we also tested this ion and found it to be a strong inhibitor of DFF endonuclease activity (data not shown).

Caspase-3-activated DFF Possesses Moderately Greater Sequence Preferences Than MNase and DNase I—DFF potentially may be a useful reagent for chromatin structural analyses. We therefore evaluated DFF’s sequence preferences for naked DNA cleavage in comparison with those of MNase and DNase I, which have both been well characterized previously (45–48). Separation of the corresponding cleavage products of 3²P-5'-labeled pUC19 DNA on a sequencing gel reveals that caspase-3-activated DFF endonuclease possesses moderately more sequence selectivity than either MNase or DNase I (Fig. 1). Nevertheless, DNA products are eventually processed to fragments ≤20 bp after more extensive digestion (data not shown), indicating a quite broad sequence specificity, consistent with the fact that DFF endonuclease is capable of converting purified HeLa cell DNA quantitatively into very small DNA fragments (see below).

Caspase-3-activated DFF Generates Blunt Ends or Ends with a 1-Bp 5'-Overhang—We determined the nucleotide sequences at various cleavage sites by using 3²P-5'-labeled DNA restriction fragments as substrates and resolution of the Watson or Crick strands on sequencing gels. As shown in Fig. 2, the endonuclease generates blunt ends or ends with 1-base 5'-overhangs. We analyzed 57 cleavage sites at the nucleotide level within the HIV-1 LTR, the mouse immunoglobulin α gene, and pUC19 DNA. While these analyses revealed no simple
I, and caspase-3-activated DFF on naked DNA.

Circular, site-

4–7

lanes 14–16

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to mononucleosomes by DFF after extensive digestion (Fig. 3 revealed that 70% of the total genomic DNA could be processed previous reports (21). Quantitation by PhosphorImager analysis was approximately 180 bp, in reasonable agreement with pre-

DNA repeat length estimated after digestion by either enzyme

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-R (72%), R (74%), R (66%), Y (61%) ↓ R (65%), Y (67%), Y (75%), Y (75%)-3’. It is significant that this purine/pyrimidine preference exhibits a rotational (dyad) symmetry. This observation, together with the double-stranded cleavage activity of this enzyme, is consistent with our observation that only homo-oligomeric forms of DFF40 are enzymatically active (12).

Oligonucleosomal Ladders Generated by Caspase-3-activated DFF Are Sharper than Those Created by MNase—To determine the utility of DFF as a reagent for chromatin research, we compared its action with that of MNase for generating oligo-

nucleosomal DNA ladders. As shown in Fig. 3, digestion of chromatin in isolated HeLa cell nuclei was dependent on the presence of either enzyme (compare lanes 1 and 2 with lanes 4–7 and 9–12) and for DFF required caspase-3 cleavage (compare lanes 14–16 with lanes 9–12). The average nucleosomal DNA repeat length estimated after digestion by either enzyme was approximately 180 bp, in reasonable agreement with previous reports (21). Quantitation by PhosphorImager analysis revealed that 70% of the total genomic DNA could be processed to mononucleosomes by DFF after extensive digestion (Fig. 3A, lane 9). This incompleteness was not due to DFF-resistant sequences in the human genome, because high molecular weight naked HeLa cell DNA could be quantitatively converted to very small DNA fragments upon digestion by the enzyme (Fig. 3B, lanes 2–6). The incomplete conversion to mononucleosomes may be due to a subfraction of the genome being organized into DFF-resistant chromatin structures, and/or to nuclear heterogeneity in DFF permeability; DFF forms very large oligonucleosomal multimers upon activation (12), and its nuclear access may be partially limited. Consistent with this view is that the addition of 1 mM GTP to reaction mixtures stimulated the digestion kinetics about 2-fold on the nuclear substrate but had no effect on naked DNA digestion kinetics (data not shown), presumably by enhancing the nuclear import and/or retention of DFF by phosphorylation. Digestion of nuclei with caspase-

3-activated DFF resulted in oligonucleosomal DNA ladders somewhat sharper than those generated by MNase, as judged by oligonucleosomal multimer band sharpness and the ability to visualize bands of longer oligomers. This is because the interband background between successive oligonucleosomal multimers was higher for MNase digestion products; unlike MNase, DFF lacks exonuclease activity. Furthermore, cleavage within nucleosome core particles was undetectable for DFF digestion products, in contrast to MNase digestion products, which exhibited subnucleosomal DNA fragments (Fig. 3A, compare lanes 6 and 7 with lanes 9 and 10; Fig. 3C, compare lanes 1 and 3). We conclude that DFF is another reagent useful for chromatin research.

In order to establish an in vitro system to study further the action of DFF endonuclease on chromatin, we chose as a model substrate for in vitro chromatin assembly an 18-mer of a 207-bp 5 S rRNA gene sequence, which has been previously shown by Simpson and co-workers (22) to position nucleosomes on each tandem repeat. We then used a salt step gradient procedure with purified HeLa cell core histones to reconstitute nucleosomes onto this 5 S gene tandem array (see “Experimental Procedures”). As shown in Fig. 4, both DFF endonuclease and MNase generated nucleosomal DNA ladders upon digestion of the reconstituted chromatin. However, just as in the nuclear chromatin digests (Fig. 3), it is significant that the nucleosomal DNA ladders generated by DFF endonuclease are sharper than those generated by MNase. Therefore, DFF may be better than MNase as a reagent for some types of chromatin structural
Action and Requirements of Caspase-3-activated DFF

DNA cleavage accessibility, is compensated by an activation of DFF endonuclease by nucleosomal DNA wrapping (see below). In summary, we can generate nucleosomal DNA ladders by digestion of isolated nuclei or in vitro assembled chromatin with activated DFF very much like those generated in vivo during the terminal stages of apoptosis.

Caspase-3-activated DFF Cleaves Chromatin in the Internucleosomal Linkers Leaving 5’-Phosphate and 3’-Hydroxyl Groups—To prove that DFF cuts in the linker region between nucleosomes, we isolated mononucleosomal-length DNA fragments generated after digestion of the in vitro reconstituted chromatin with DFF endonuclease and mapped the positions of DNA cleavage with respect to the 5 S rRNA gene repeat. For this purpose, we cut the mononucleosomal DNA with EcoRI, whose pair of closely spaced recognition sequences are known to be located in the internucleosomal linker region after nucleosomal assembly (22). We found that the majority of the DNA fragments were reduced in length by ≤20 bp (Fig. 5, compare lanes 1 and 3), proving that the cutting sites for DFF and EcoRI are close together in the internucleosomal linker region (Fig. 5, bottom diagram). Quantitation by PhosphorImager analysis revealed that 80% of these DNA molecules were between 200 and 150 bp in length. The 20% below 150 bp in length may be in part accounted for by a background of non-positioned and/or partially assembled nucleosomes generated under the conditions of chromatin reconstitution that we employed. In addition, we found that the DNA ends generated by DFF endonuclease possessed 3’-hydroxyl groups, because they could be extended by terminal deoxynucleotidyl transferase (Fig. 5, compare lanes 3 and 5), and radioactive nucleotides could be incorporated by either terminal deoxynucleotidyl transferase, T4 or T7 DNA polymerase (data not shown). In addition, the 5’-ends of DFF-generated mononucleosomal DNA could not be phosphorylated by T4 polynucleotide kinase unless they were first dephosphorylated by treatment with alkaline phosphatase (Fig. 5, compare lanes 1 and 3), indicating the initial presence of 5’-phosphate groups. Finally, an extension reaction with T4 DNA polymerase barely shifted the DNA lengths of the products (Fig. 5, compare lanes 3 and 4), indi-
cating the nearly blunt-end nature of the cleavage products. Controls with restriction fragments bearing 5'-overhangs proved that the polymerase reaction was functional (data not shown).

**DISCUSSION**

It should be appreciated that the *in vitro* properties of DFF endonuclease cleavage fit the phenotype of the DNA products generated by apoptosis *in vivo*, namely nucleosomal DNA ladders, with fragments bearing 3'-hydroxyl groups; these features have been routinely used in bioassays for cells undergoing apoptosis. It is interesting that DFF endonuclease cleavage of a naked DNA substrate is markedly stimulated by topoisomerase II, a protein recently shown to be responsible for the creation of 50–100-kb DNA cleavage products during the initial stages of oxidative stress-induced apoptosis (55). This suggests a coordination or linkage between topoisomerase II and DFF in triggering efficient DNA breakdown during the terminal stages of apoptosis. Interestingly, the kinetics of DNA fragmentation by DFF endonuclease on naked DNA and chromatin substrates are nearly equivalent, in marked contrast to those of MNase or DNase I, which attack naked DNA orders of magnitude more rapidly than chromatin. Cleavage of naked DNA by DFF is activated by histone H1, HMG-1/2, and topoisomerase II, proteins that trap supercoils and DNA crossovers, which
induce DNA conformations that partially mimic nucleosomal DNA wrapping. DFF activation for chromatin cleavage by these proteins, however, is much less than for naked DNA digestion. Thus, the assembly of DNA into nucleosome core particles, which would be expected to reduce DNA cleavage accessibility, is apparently compensated by an activation of DFF endonuclease by the resulting nucleosomal DNA wrapping. The observed activation of DFF endonuclease for naked DNA cleavage may also in part be due to direct interactions with these chromosomal proteins, which may recruit the enzyme to the DNA substrates (12).

DFF has a place as a reagent for chromatin research. In the current study, we have proven that the endonuclease cuts in the internucleosomal linker regions of chromatin. Although the possibility was remote, it was formally possible that the presumptive nucleosomal DNA ladders observed upon apoptotic cell death by researchers in the field could have been a reflection of cleavage of chromatin at, for example, the dyad axis of symmetry of the nucleosomal repeat. The addition of caspase-3 alone or DFF alone to isolated nuclei did not result in detectable DNA breakdown (Fig. 3, lanes 14 and 15). Although DFF exists in the nucleus of living cells (7, 11), upon cellular fractionation during nuclei isolation, the protein is found in the cytoplasmic fraction (6). This nuclear leakage of DFF upon cell fractionation means that internucleosomal DNA laddering in isolated nuclei is dependent on the addition of exogenous caspase-3-activated DFF (6). This feature is ideal for experimentally controlling the extent of endonuclease digestion. The DNA sequence preferences for DFF are more selective than even MNase, however, indicating that the apoptotic endonuclease may not be the reagent of choice for precise mapping of in vivo nucleosome positions by first hit kinetics footprinting procedures. However, the lack of exonuclease activity, the marked preference for cleavage in the internucleosomal linker region, and the lack of intranucleosomal DNA cleavage after production of mononucleosomes make DFF an attractive choice for the generation and analysis of mononucleosomes. The extent of DFF endonuclease recognition of “nucleosome-free” regions known as DNase I-hypersensitive sites in chromatin, the nature of putative DFF-resistant chromatin structures, and how DFF attacks nucleosomal arrays in transcriptionally repressed and active genes are interesting questions that remain to be addressed in the future.

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