

DFF40/CAD HYPERSENSITIVE SITES ARE POTENTIALLY INVOLVED IN HIGH MOLECULAR WEIGHT DNA FRAGMENTATION DURING APOPTOSIS

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Abstract: Sequential cleavage of genomic DNA into large-scale DNA fragments of 50-300-kb, followed by formation of mono- and oligonucleosomal DNA fragments, is a biochemical hallmark of programmed cell death (apoptosis). The endonuclease DFF40/CAD mediates regulated internucleosomal DNA fragmentation and chromatin condensation in cells undergoing apoptosis. DFF40 hypersensitive sites were detected in purified HeLa cell nuclei, and excision of 50-kb DNA fragments preceded formation of oligonucleosomal DNA ladders in nuclei treated with the nuclease. Topoisomerase II, but not topoisomerase I, stimulates DFF40 activity on plasmid DNA substrates. This suggests that interactions of DFF with the nuclear matrix-bound topoisomerase II may be involved in formation of DFF40 hypersensitive sites.

Key Words: Apoptosis, CAD, DNA Fragmentation Factor, Topoisomerase II, Large-Scale DNA Fragmentation, Nuclease Hypersensitive Sites.

INTRODUCTION

Fragmentation of DNA and destruction of chromosomes is one of the biochemical hallmarks of apoptosis [1]. The sequential generation of high molecular weight (HMW) DNA fragments of 300-kb, and then 50-kb, is followed by advanced chromatin cleavage to mono- and oligonucleosomal DNA [2]. Initial breakdown of chromatin into large-scale fragments precedes the formation of apoptotic morphology, and characteristic morphological changes may occur in the absence of detectable nucleosomal DNA fragmentation [3]. The genome breakdown into HMW DNA fragments is presumably determined by chromatin topology. It has been postulated that

appearance of 50-kb fragments reflects chromatin loops, while formation of 300-kb fragments possibly reflects hexameric loop structures known as rosettes [4, 5]. Several different nucleases have been proposed to be involved in large scale DNA fragmentation during apoptosis [2, 6, 7]. However, neither were purified and identified, nor was mechanism of their action established. The pattern of DNA cleavage into HMW fragments during apoptosis is similar to that induced by topoisomerase II inhibitors [5], and it has been postulated that formation of irreversible DNA-topoisomerase II cleavable complexes is involved in DNA degradation during apoptosis [8].

One of the nucleases primarily responsible for DNA cleavage during apoptosis is called DNA fragmentation factor (DFF), or caspase-activated DNase (CAD) [9-11]. In inactive form, DFF is a heterodimer composed of a catalytic subunit of 40-kDa (also called CAD/CPAN) and a regulatory subunit of 45-kDa (also called ICAD). Upon caspase-3 cleavage of DFF, DFF45 is cut, releasing active form of the nuclease DFF40. The enzyme attacks chromatin preferentially in the internucleosomal linker, generating oligonucleosomal DNA ladders [12]. Transformed Jurkat cells expressing caspase-resistant form of DFF45/ICAD showed neither HMW nor oligonucleosomal DNA fragmentation upon apoptotic stimuli. This suggests involvement of DFF40/CAD also in large-scale chromosomal DNA breakdown [13]. On the other hand, oligonucleosomal DNA fragmentation but not HMW fragmentation is blocked in human neuronal NT2 cells that do not correctly process DFF45 with caspases [14].

Specific chromatin structures may promote accessibility of proteins to DNA fragments and such chromatin regions are known as nuclease hypersensitive sites [15]. Hypersensitive sites (HS) are potentially involved in HMW DNA fragmentation during apoptosis, and two mechanisms could be postulated: 1) preexisting HS are recognized by nucleases activated during apoptosis, and/or 2) apoptosis-specific changes in chromatin structure induce formation of HS. In the present work, the DFF-hypersensitive sites were detected, and DNA fragments of about 50-kb were preferentially excised from nuclei of HeLa cells.

MATERIALS AND METHODS

Materials

Recombinant human DFF (DFF40/DFF45 heterodimers) and hamster caspase-3 were prepared from *E. coli* expression systems as reported previously [11]. To activate the nuclease, DFF was pre-incubated with caspase-3 (in an approximate molar ratio of 2:1) for 15 minutes at 22°C, then caspase activity was inhibited by addition of the tetrapeptide Ac-DEAD-CHO. Nuclei from cultured HeLa cells were purified as described elsewhere [9]. Human recombinant topoisomerase II (p170) was purchased from TopoGEN. Calf thymus topoisomerase I was purchased from GibcoBRL.

Treatment of nuclei

Purified nuclei (10^5) were incubated with activated DFF (1 unit) at 34°C, in buffer consisting of 3 mM MgCl₂, 1 mM DTT, 1 mM ATP, 100 mM KCl, 20 mM Tris-HCl (pH 7.5), final volume 50 µl. After different time periods of incubation, the nuclease reaction was terminated by adding aurintricarboxylic acid (ATA) to a final concentration of 0.1 mM. Alternatively, nuclei were incubated for 30 minutes in the presence of ATA and 0.1 mM VM-26. One-half of the reaction mixtures was combined with a mixture of proteinase K, EDTA and SDS, and DNA was analyzed by electrophoresis in 1.5% SeaKem agarose gels as described elsewhere [12]. The other-half of the reaction mixtures was embedded in low-temperature melting agarose plugs, and plugs were then incubated for 3 hours at 37°C in lysing solution (0.5% SDS, 10 mM EDTA, 0.2 mg/ml proteinase K). After washing with TE buffer, the plugs were analyzed in a CHEF Mapper pulsed-field gel electrophoresis system (BioRad). Gels were stained with ethidium bromide.

Nuclease assay

One µg of plasmid pUC19 DNA was incubated with either caspase-3, DFF40/DFF45 inactive heterodimer or activated DFF (0.5 unit) for 30 minutes at 37°C in a buffer consisting of 3 mM MgCl₂, 1 mM DTT, 10 mM KCl, 20 mM Tris-HCl (pH 7.5), in a final volume of 15 µl. The nuclease activity was assayed in the presence of either BSA (0.2 µg) or topoisomerase (2 units). The nuclease reaction was terminated with the SDS/EDTA/Proteinase K mixture, and DNA was analyzed as described above.

RESULTS AND DISCUSSION

DNA fragments generated in HeLa cell nuclei upon treatment with either DFF or topoisomerase II inhibitor VM-26 were analyzed by agarose gel electrophoresis (Figure 1). Such an analysis on standard 1.5% agarose gel reveals that treatment with DFF for 30 and 90 minutes generated mono- and oligonucleosomal size DNA fragments (lanes 3 & 4 on Figure 1A).

Large-scale DNA fragments remained at the start of the gel or were observed as an artificial compression band of about 20-30-kb. To resolve these large-scale fragments the same DNA samples were analyzed by pulsed-field gel electrophoresis (Figure 1B). DNA from control nuclei (lane 1) remained within the plug at the start of the gel or formed compression band of about 700-kb, and no further DNA degradation was detected. In contrast, when nuclei were treated with either DFF or VM-26 shorter DNA fragments were also detected, which showed discontinuity in their size distribution. 5 minutes incubation of purified nuclei with DFF resulted in excision of fragments of about 50-kb, and no further DNA degradation was detected (lane 2). Longer incubation with DFF

induced gradual degradation of this 50-kb fragment, and shorter oligonucleosomal DNA fragments appeared (lanes 3 & 4 on Figure 1A & B). Treatment of nuclei with VM-26 induced excision of DNA fragments found in the 50-600-kb size range, and no discrete band pattern within this range was observed (lane 5).

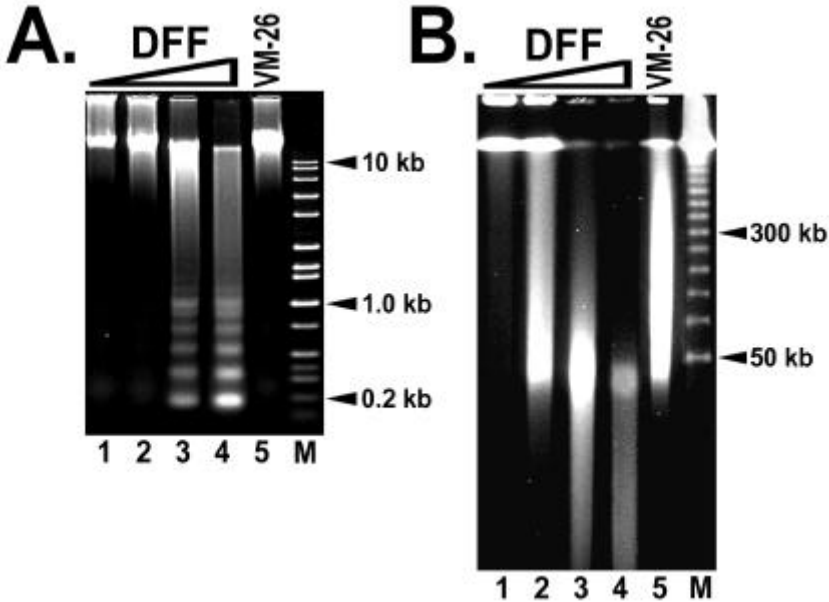


Fig. 1. Analysis of distribution of DNA fragments generated in purified HeLa cell nuclei upon treatment with DFF or VM-26. DNA was separated by either standard agarose gel electrophoresis (panel A) or pulsed-field gel electrophoresis (panel B). Nuclei were mixed with activated DFF and reaction was terminated after 0, 5, 30 and 90 minutes of incubation (lanes 1-4). Lanes M - appropriate molecular size standards (panel B - concatemers of phage λ DNA).

Data presented in Figure 1 shows that treatment of purified nuclei with DFF results in preferential excision from chromatin fragments of about 50-kb. In agreement with literature data, similar DNA fragments have been also excised from chromatin upon treatment of nuclei with topoisomerase II inhibitor VM-26. It has been reported that formation of cleavable complexes of topoisomerase II associated with high-salt insoluble nuclear matrix is responsible for excision of 50-kb DNA fragments, and such fragments reflect chromatin loops [5, 16]. Preferential excision of 50-kb DNA fragments by DFF suggests existence of hypersensitive sites recognized by this nuclease, and such sites might be localized at bases of chromatin loops.

It has been reported that several chromatin proteins stimulate DFF-catalyzed cleavage of linear naked DNA and oligonucleosomes [12]. Search for HeLa cell proteins that stimulate DFF activity identified HMG-2 [17]. However, another protein, whose tryptic peptides sequence revealed homology to topoisomerase II, was also detected (Li P., personal communication). Figure 2 shows results of the experiment where effects of recombinant human p170 topoisomerase II was studied. It stimulated several fold DFF-catalyzed cleavage of supercoiled plasmid DNA, and extent of such stimulation was dose-dependent. The stimulation of DFF activity by topoisomerase II was ATP-independent (not shown). In contrast, topoisomerase I did not stimulate DFF activity upon plasmid DNA (not shown).

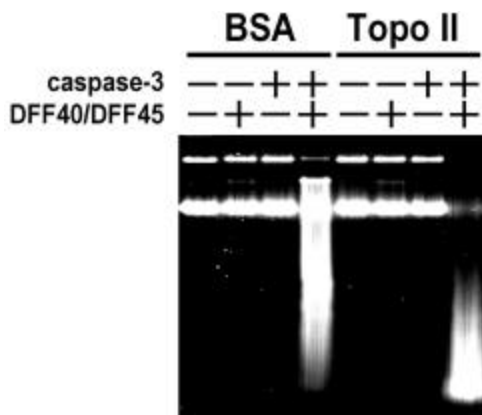


Fig. 2. The stimulation of DFF activity by topoisomerase II. Naked plasmid DNA was incubated with caspase-3, inactive or activated DFF in the presence of either BSA or topoisomerase II.

DNA regions known as MARs/SARs, that are presumably localized at bases of chromatin loops and mediate interactions with nuclear skeleton, contain sequences preferentially recognized by topoisomerase II. Topoisomerase II is believed to be an integral component of residual nucleoprotein structure defined as nuclear matrix, and also to be involved in formation of chromatin loops [18]. The finding that topoisomerase II stimulates activity of DFF makes attractive the speculation that DFF-hypersensitive sites are localized at bases of chromatin loops and depend on interactions of the enzyme with the nucleoskeleton-associated topoisomerase II. Alternatively, one may not exclude the possibility that DFF40 itself or caspase-cleavage products of DFF45 stimulate another enzyme (e.g. topoisomerase II) to excise chromatin loops of 50-kb. It seems clear that DFF is not the only protein responsible for a large-scale fragmentation of DNA during apoptosis [14]. However, the mechanism of DFF involvement in

this stage of chromosome break-down needs to be elucidated in further experiments.

Acknowledgements. I would like to express thanks to Prof. W. T. Garrard for helpful discussions and to Dr. A. Sochanik for help in preparation of the manuscript. This work was supported by the Committee for Scientific Research (KBN), grant 6P04A01317.

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