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Minireview

The DFF40/CAD endonuclease and its role in apoptosis✪

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The sequential generation of large-scale DNA fragments followed by internucleosomal chromatin fragmentation is a biochemical hallmark of apoptosis. One of the nucleases primarily responsible for genomic DNA fragmentation during apoptosis is called DNA Fragmentation Factor 40 (DFF40) or Caspase-activated DNase (CAD). DFF40/CAD is a magnesium-dependent endonuclease specific for double stranded DNA that generates double strand breaks with 3′-hydroxyl ends. DFF40/CAD is activated by caspase-3 that cuts the nuclease's inhibitor DFF45/ICAD. The nuclease preferentially attacks chromatin in the internucleosomal linker DNA. However, the nuclease hypersensitive sites can be detected and DFF40/CAD is potentially involved in large-scale DNA fragmentation as well. DFF40/CAD-mediated DNA fragmentation triggers chromatin condensation that is another hallmark of apoptosis.

Programmed cell death, or apoptosis, plays an important role in the development of organs and in the maintenance of tissue homeostasis. The process depends on actively controlled degradation of intracellular structures, and allows to remove unwanted, incorrect or damaged cells from multicellular organisms. Cells undergoing apoptosis exhibit specific morphological changes, which include membrane blebbing, cytoplasmic and chromatin condensation, nuclear breakdown, assembly of membrane-enclosed vesicles termed apoptotic bodies eventually subjected to phagocytosis. A variety of apoptotic signals trig-

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Abbreviations: AIF, apoptosis inducing factor; Apaf-1, apoptotic protease activating factor-1; Bid, BH3 domain-containing protein; bp, base pair; CAD, caspase-activated DNase; CIDE, cell death inducing DFF45-like effector; CPAN, caspase-activated nuclease; DFF, DNA fragmentation factor; DNA-PK, DNA-dependent protein kinase; FAS, cell membrane receptor from TNF family; ICAD, inhibitor of CAD; kb, thousand of base pairs; MDM2, inhibitor of p53; MNase, micrococcal nuclease; PARP, poly(ADP-ribose) polymerase; R, purine; TNF, tumor necrosis factor; Y, pyrimidine.

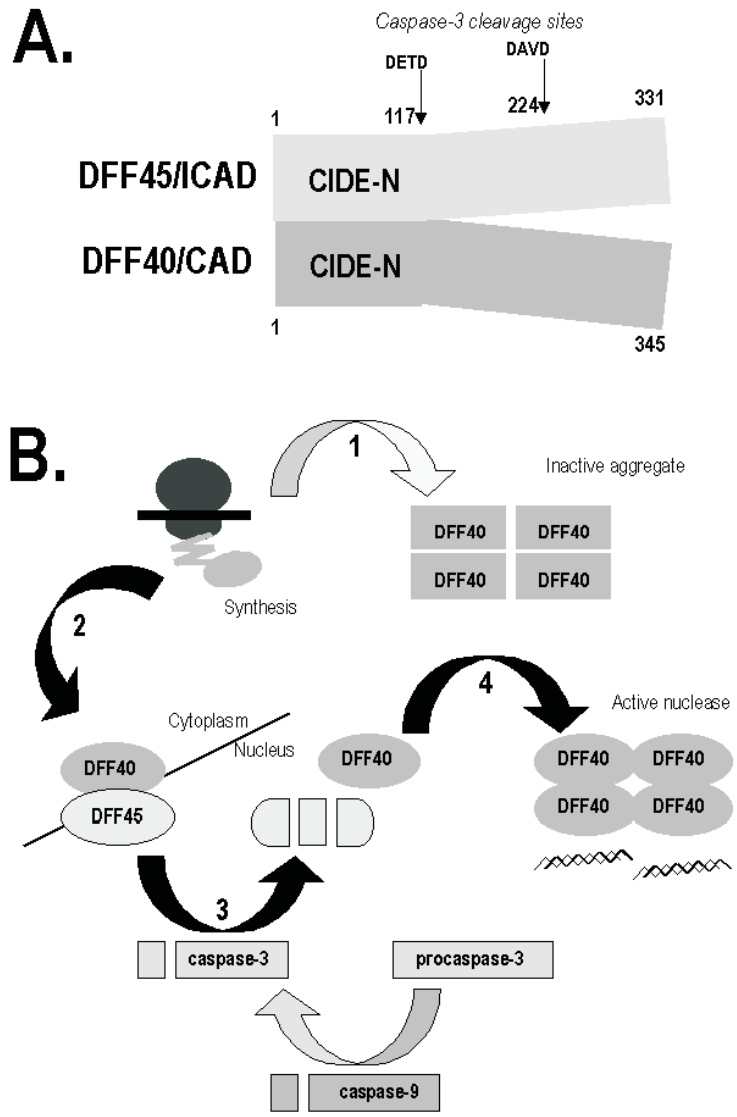

Figure 1. Structure and mechanisms of activation of DFF.

Panel A. Scheme of the DFF40/DFF45 heterodimer. The positions of aspartate residues of DFF45 recognized by caspase-3 are marked with arrows. Panel B. Diagram showing mechanisms of DFF activation. DFF40 synthesized in the absence of DFF45 form inactive aggregates (1) or binding of DFF45 promotes proper folding of DFF40 and DFF40/DFF45 heterodimers transfer to nucleus (2), then under apoptotic stimuli DFF45 are cut with caspase-3 (3) and released DFF40 form active homodimers (4).

ger the initiation of the process, which include FAS or TNF receptor engagement, growth factor deprivation, cell cycle perturbations or DNA damage. Defects in apoptosis have been associated with a number of disease states, including neoplasia, AIDS, ischemic strokes and neurodegeneration (rev. in: Wyllie *et al.*, 1980; Jacobson *et al.*, 1997; Nagata, 1997).

Apoptotic signals activate a cascade of cysteine proteases called caspases. The first caspases activated are called initiators, which include caspase-8. Among caspase-8 substrates is BID protein, whose cleaved C-terminal fragment triggers cytochrome *c* release from mitochondria. The released cytochrome *c* binds to Apaf-1 (Ced-4 homologous), which induces auto-activation of caspase-9, which in turn activates caspase-3. Alternatively, caspase-8 may directly activate caspase-3. Caspase-3, major executioner caspase, has numerous cytoplasmic and nuclear targets (e.g. PARP, DNA-PK, MDM2, actin, phospholipase A2). Caspase-3 also activates caspase-6, which cleaves nuclear lamins, and DNA fragmentation factor, which is the "heroes" of the review (rev. in: Villa *et al.*, 1997; Budihardjo *et al.*, 1999; Hengartner, 2000).

One of the biochemical hallmarks of apoptosis is genomic DNA fragmentation (Arends *et al.*, 1990). First the genome is cleaved into 50–300 kb DNA fragments,

which possibly reflects structure of chromatin loops and hexameric loop structures known as rosettes (Filipski *et al.*, 1990; Zhivotovsky *et al.*, 1994). Generation of high molecular mass DNA fragments can be triggered *in vitro* by addition of mitochondrial AIF to isolated nuclei (Susin *et al.*, 1999) and under certain conditions can be mediated by topoisomerase II (Lagarkova *et al.*, 1995; Li *et al.*, 1999). This large-scale DNA fragmentation is followed by internucleosomal cleavage that generates mono- and oligonucleosomal DNA fragments, and is termed "DNA laddering" (Wyllie, 1980). Several different endonucleases have been proposed to be responsible for apoptotic DNA fragmentation. Such nucleases have been characterized as Mg-dependent, Mg/Ca-dependent and cation-independent, and include NUC18/cyclophilins, DNase I, DNase γ or DNase II (rev. in: Zhivotovsky *et al.*, 1994; Montague & Cidlowski, 1996; Khodarev *et al.*, 1998). One of the major nucleases responsible for the internucleosomal DNA fragmentation step has been elucidated by several groups in 1997/1998, and was called DNA fragmentation factor (Liu *et al.*, 1997), caspase-activated DNase (Enari *et al.*, 1998) or caspase-activated nuclease (Halenbeck *et al.*, 1998).

Figure 1A schematically shows the structure of DFF. In its inactive form, DFF is a heterodimer composed of 40 and 45 kDa protein subunits, termed DFF40/CAD/CPAN and DFF45/ICAD. Human DFF40 is a basic protein with pI of 9.3 composed of 345 amino acids, while DFF45 is an acidic protein with pI of 4.5 composed of 331 amino acids. Human DFF40 and mouse CAD (as well as DFF45/ICAD, respectively) are about 75% identical. A short form of DFF45, called DFF35 (or ICAD-S), that lacks C-terminus and consists of 265 amino acids has been also detected in human and mouse. DFF45 carries two caspase-3-recognition sites – aspartate residues 117 and 224 (Liu *et al.*, 1997; Enari *et al.*, 1998; Liu *et al.*, 1998; Halenbeck *et al.*, 1998). DFF40 and DFF45 have no apparent overall homology, but carry a conserved domain of 80 amino acids at their N-terminus. This domain has been found in several proteins called CIDEs, and has been termed CIDE-N domain (Inohara *et al.*, 1998). C-terminus of DFF40 functions as a nuclear localization signal. DFF40 is a catalytic subunit while DFF45 is a regulatory subunit of the DFF. CIDE-N domains are involved in DFF40/DFF45 interactions (Lugovskoy *et al.*, 1999; Otomo *et al.*, 2000). Deletion of the C-terminal residues 290–345 of DFF40 resulted in complete loss in enzyme activity, and it has been suggested that C-terminus contains catalytic domain of the nuclease (Inohara *et al.*, 1999).

DFF45 bound to DFF40 inhibits activity of the nuclease. Upon caspase-3 cleavage of DFF, DFF45 is cut releasing active DFF40. DFF45 can be also cut by caspase-7 and granzyme B (yet with lower efficiency as compared to caspase-3), but not caspase-1, -2, -4, -6, or -8. DFF45 plays a dual role of inhibitor and chaperone of DFF40. The expression of DFF40 in various systems in the absence of co-expressed DFF45 results in generation of DFF40 inactive aggregates. This suggests that DFF45 is required for the proper folding of the active DFF40 during its synthesis (Liu *et al.*, 1997; Enari *et al.*, 1998; Liu *et al.*, 1998). DFF35 (a splicing variant of DFF45) tightly binds to and inhibits DFF40, and can dissociate when cut with caspase-3, but lacks the chaperone function (Gu *et al.*, 1999; Sakahira *et al.*, 1999a). DFF45 mutants carrying point mutations at either or both caspase-recognition sites are full inhibitors and cannot be removed upon caspase-3 cleavage to activate the nuclease (Sakahira *et al.*, 1999b). Purified DFF40 released from the complex with DFF45 forms homo-oligomers that are the enzymatically active form of the nuclease. Low molecular mass form of released DFF40 lack the activity, while atomic force microscopy revealed that active DFF40 is a heterologous series of multimers possibly composed of a unit-sized smaller oligomer. However, it is not clear whether such multimers form in cells *in vivo* (Liu *et al.*, 1999). Activity of the nuclease on

naked DNA substrates can be further enhanced by several chromatin proteins: histone H1, HMG-1/2 or topoisomerase II. On chromatin substrates such stimulatory proteins are much less effective. It has been proposed that these proteins trap DNA supercoils or crossovers that create a DNA conformation resembling nucleosomal DNA wrapping, which may be preferentially recognized by DFF40 (Widłak *et al.*, 2000). On the other hand, both histone H1 (Liu *et al.*, 1999) and topoisomerase II (Durrieu *et al.*, 2000) directly bind to DFF40, and may recruit the enzyme to DNA. DFF had been originally purified from a cytoplasmic fraction (Liu *et al.*, 1997), and it was postulated that DFF40 undergoes translocation to the nucleus upon caspase-3 cleavage of DFF45, by analogy to NFκB/IκB. However, it has been shown that either active DFF40 or inactive heterodimer DFF40/DFF45 reside in nuclei (Liu *et al.*, 1998). The specific activity of DFF40 is comparable to that of DNase I (Enari *et al.*, 1998; Halenbeck *et al.*, 1998). However, the recombinant protein produced in bacteria had a significantly lower specific activity, suggesting that additional post-translational modifications may be involved in its activation (Widłak *et al.*, 2000). The mechanisms of regulation of DFF are summarized on Fig. 1B. When DFF40 is synthesized in cytoplasm DFF45 binds to its nascent chain and promotes its correct folding. Catalytically inactive complex DFF40/DFF45 is then transferred to nuclei. When apoptotic stimuli trigger activation of caspase-3 it cuts DFF45 releasing DFF40 from the complex. Released DFF40 forms catalytically active homo-oligomers that degrade chromosomal DNA.

DFF40 requires Mg^{2+}, exhibits a trace of activity in the presence of Mn^{2+}, is not co-stimulated by Ca^{2+}, and is inhibited by Zn^{2+} or Cu^{2+}. The enzyme has its optimum at neutral pH. DFF40 is an endonuclease specific for double stranded DNA, but not single stranded DNA or RNA. This introduces double-strand breaks but not single-strand nicks during its DNA cleavage reaction in normal conditions (Liu *et al.*, 1999; Widłak *et al.*, 2000). However, the enzyme may cleave each strand stepwise and at high ionic strength or low digestion temperature it may dissociate before the adjacent second strand can occur (Widłak & Garrard, 2001). DFF40 generates blunt ends (or nearly blunt-ends) possessing 5′-phosphate and 3′-hydroxyl groups, which can be substrates for the terminal transferase that is used to detect apoptotic cells in TUNEL assay. The enzyme possesses some sequence preference with respect to purines and pyrimidines that show rotational symmetry (5′-R-R- R-Y/ R-Y-Y-Y-3′) (Widłak *et al.*, 2000). The activity of DFF40 is markedly affected by ionic strength. At the optimal K^+ concentration of 50–125 mM, which is in the range of the cytoplasmic K^+ concentration for cells undergoing apoptosis, the activity of DFF40 on naked DNA is about 100-fold higher than at 0 or 200 mM salt (Widłak & Garrard, 2001).

DFF40 attacks chromatin in the internucleosomal linker DNA, generating mono- and oligonucleosomal fragments. The enzyme lacks exonuclease activity and a cleavage within nucleosome core particles is undetectable, thus DFF-generated DNA ladders are sharper than those created by MNase (Widłak *et al.*, 2000). When activated DFF was incubated with purified nuclei, excision of DNA fragments of about 50 kb preceded oligonucleosomal laddering (Widłak, 2000; Fig. 2A, B). This suggests existence of DFF hypersensitive sites, and such sites may be eventually involved in large-scale DNA fragmentation. Consistent with this idea, cells expressing caspase-resistant mutated DFF45 form showed neither oligonucleosomal laddering nor large-scale DNA fragmentation upon apoptotic stimuli (Sakahira *et al.*, 1999b). Since DFF40 binds to (Durrieu *et al.*, 2000) and is activated by topisomerase II (Widłak *et al.*, 2000), interactions between DFF40 and nucleoskeleton-associated topoisomerase II molecules may be involved in formation of the nuclease hypersensitive sites.

Another hallmark of the terminal stages of apoptosis is chromatin condensation. Although internucleosomal DNA breakdown is often temporally correlated with chromatin condensation, it is not absolutely required to trigger this event. At least three pathways have been identified that mediate chromatin condensation: 1) A caspase-3 independent pathway of chromatin condensation triggered by AIF, which leads to an accompanying large-scale DNA fragmentation without internucleosomal DNA cleavage (Susin *et al.*, 1999); 2) A caspase-3 dependent pathway of chromatin condensation triggered by a protein called *"acinus"*, which occurs without inducing any DNA fragmentation (Sahara *et al.*, 1999); and 3) A caspase-3 dependent pathway of chromatin condensation mediated by DFF40 (Liu *et al.*, 1998; Fig. 2C). DFF-mediated chromatin condensation seems to be caused by DNA breakdown *per se* under the appropriate ionic conditions (physiological Mg^{2+} and K^{+} concentration) and does not require ATP. It is not DFF40 specific, and MNase, DNase I or restriction enzyme treatment of purified nuclei leads to similar chromatin condensations (Widłak & Garrard, unpublished). However, pre-incubation of nuclei with inhibitors of topoisomerase II suppress chromatin condensation induced by DFF40 (Durrieu *et al.*, 2000).

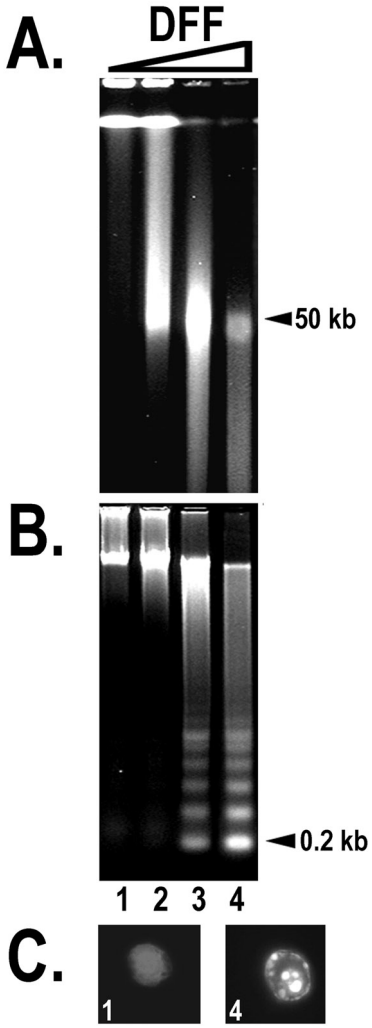

Figure 2. DNA fragmentation and chromatin condensation induced by DFF.

Nuclei purified from HeLa cells were incubated with caspase-3-activated DFF for 0, 5, 30 and 90 min (lanes **1**, **2**, **3** and **4**, respectively). Nuclear DNA fragmentation was analyzed by either pulsed-field (panel **A**) or standard gel electrophoresis (panel **B**). The position of molecular standards is marked with arrowheads. Alternatively, nuclei were stained with 4′,6-diamidinono-2-phenylindole and chromatin condensation was observed microscopically (panel **C**).

The physiological significance of DFF in triggering DNA laddering during apoptosis has been unequivocally proven. DFF40 and DFF45 mRNAs and proteins are expressed in most tissues and cell lines. Cell lines showing high level of DFF40 and DFF45 expression quickly undergo DNA fragmentation under apoptotic stimuli (Mukae *et al.*, 1998). Thymocytes and splenocytes from mice that lack functional DFF45 gene exhibit neither DNA laddering nor chromatin condensation when exposed to apoptotic stimuli (Zhang *et al.*, 1998). Although such mice appeared normal, thymocytes from these mice are more resistant to apoptosis than wild-type animals are (Zhang *et al.*, 1999). Nevertheless, alternative DFF-independent pathways of internucleosomal DNA breakdown can be induced in embryonic fibroblasts from mice lacking DFF45 (Li & Wang, unpublished).

The degradation of genomic DNA into nucleosomal units is one of the best characterized biochemical hallmarks of programmed cell death (Arends *et al.*, 1990). The phenome-

non serves as the basis for commonly used techniques to detect apoptotic cells (e.g. TUNEL assay). One of the physiological roles of apoptosis is to remove harmful cell (i.e. cancer or virally infected) from organisms. It can be hypothesized that mechanisms of DNA breakdown during apoptosis were developed to prevent transfer of potentially incorrect DNA (e.g., activated oncogenes or viral genes) to another cells or to reduce the autoimmune response. It has been shown that cells can die as a result of apoptotic stimuli without internucleosomal DNA cleavage (Oberhammer *et al.*, 1993; Cohen *et al.*, 1994). However, although apoptotic cell death can be disconnected from DNA breakdown, this event is probably beneficial for the efficient removal of toxic cell debris from the organism.

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