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Discovery, Regulation, and Action of the Major Apoptotic Nucleases DFF40/CAD and Endonuclease G

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Abstract Toward the end of the 20th and beginning of the 21st centuries, clever in vitro biochemical complementation experiments and genetic screens from the laboratories of Xiaodong Wang, Shigekazu Nagata, and Ding Xue led to the discovery of two major apoptotic nucleases, termed DNA fragmentation factor (DFF) or caspase-activated DNase (CAD) and endonuclease G (Endo G). Both endonucleases attack chromatin to yield 3′-hydroxyl groups and 5′-phosphate residues, first at the level of 50–300 kb cleavage products and next at the level of internucleosomal DNA fragmentation, but these nucleases possess completely different cellular locations in normal cells and are regulated in vastly different ways. In non-apoptotic cells, DFF exists in the nucleus as a heterodimer, composed of a 45 kD chaperone and inhibitor subunit (DFF45) [also called inhibitor of CAD (ICAD-L)] and a 40 kD latent nuclease subunit (DFF40/CAD). Apoptotic activation of caspase-3 or -7 results in the cleavage of DFF45/ICAD and release of active DFF40/CAD nuclease. DFF40's nuclease activity is further activated by specific chromosomal proteins, such as histone H1, HMGB1/2, and topoisomerase II. DFF is regulated by multiple pre- and post-activation fail-safe steps, which include the requirements for DFF45/ICAD, Hsp70, and Hsp40 proteins to mediate appropriate folding during translation to generate a potentially activatable nuclease, and the synthesis in stoichiometric excess of the inhibitors (DFF45/35; ICAD-S/L). By contrast, Endo G resides in the mitochondrial intermembrane space in normal cells, and is released into the nucleus upon apoptotic disruption of mitochondrial membrane permeability in association with co-activators such as apoptosis-inducing factor (AIF). Understanding further regulatory check-points involved in safeguarding non-apoptotic cells against accidental activation of these nucleases remain as future challenges, as well as designing ways to selectively activate these nucleases in tumor cells. J. Cell. Biochem. 94: 1078–1087, 2005. © 2005 Wiley-Liss, Inc.

Key words: apoptotic nuclease mechanisms; DNA degradation; higher-order chromatin cleavage; nucleosomes; apoptotic chromosome condensation; regulation of apoptotic nucleases

Apoptosis, or programmed cell death, is a fundamental process essential for both development and maintenance of tissue homeostasis [reviewed in Jacobson et al., 1997; Nagata, 1997]. This process was first recognized by Kerr et al. [1972] in a pioneering publication with profound insight, for as stated in the first sentence of the summary of this article, "the term apoptosis is proposed for a hitherto little recognized mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations." Cells undergoing apoptosis exhibit membrane blebbing, cytoplasmic morphological changes, chromatin condensation, DNA fragmentation, nuclear breakdown, assembly of membrane-enclosed vesicles termed apoptotic bodies, and eventual subjection to phagocytosis [reviewed in Wyllie et al., 1980]. Defects in apoptosis have been associated with a number of disease states, including neoplasia, AIDS, ischemic strokes, autoimmunity, and neurodegeneration [reviewed in Thompson, 1995].

Two major apoptotic pathways exist: the death-receptor pathway and the mitochondrial pathway [reviewed in Green, 2000; Wang, 2001]. Multiple apoptotic stimuli, which include activation of Fas receptors, serum starvation,

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ionizing radiation, or various drugs that target DNA, trigger the activation of proteases called caspases, which in turn initiate and execute the apoptotic program [reviewed in Reidl and Shi, 2004]. The genetics of programmed cell death and several of the key molecules involved were largely worked out in *Caenorhabditis elegans* by Brenner, Horvitz, and Sulston, who were awarded the Nobel Prize in Physiology and Medicine in 2002 [Marx, 2002].

One of the hallmarks of the terminal stages of apoptosis is internucleosomal DNA breakdown, which was first recognized by Wyllie [1980]. Interestingly, a very earlier report of such DNA breakdown in dying cells appeared in the literature 4 years prior to the realization that the eukaryotic genome is packaged into nucleosomes [Williamson, 1970]. From an evolutionary point of view, nucleosome structure serves both crucial regulatory and packaging roles for genomes in living cells, but also prepares the genomes of dying cells to be processed into "bite-size" mononucleosomal pieces by apoptotic endonucleases for the efficient clearance of DNA by phagocytosis. DNA fragmentation during apoptosis has further functional significance [reviewed in Zhang and Xu, 2002; Nagata et al., 2003]. Although cell death can occur without significant DNA degradation, cell autonomous DNA breakdown of tumor, or virally-infected cells functionally prepares the resulting apoptotic corpses for engulfment by phagocytes, and as such eliminates the transforming potential of any hazardous oncogenes. Interestingly, patients or an animal model with defective apoptotic DNA processing have a predisposition to auto-immune disease, which is known to be associated with the appearance of anti-DNA and anti-nucleosomal antibodies [Napirei et al., 2000; Yasutomo et al., 2001]. Finally, the execution of apoptotic DNA fragmentation and the cell death program appears to be reversible at a low frequency, and under such conditions chromosomal translocations mediated by non-homologous end joining of proto-oncogenes are thought to be one mechanism of cellular transformation [Betti et al., 2003]. In particular, it is well documented that treatment of leukemias with pro-apoptotic drugs, which target DNA, can lead to secondary tumors that exhibit new chromosomal translocations [Roulston et al., 1998]. Taken together, it seems clear that understanding the regulation of proteins that are involved in DNA processing events during apoptosis is of fundamental importance, as well as how these processes are safeguarded from accidental activation in non-apoptotic cells. In the current review, we address these issues for two major apoptotic nucleases, termed DNA fragmentation factor (DFF) or caspase-activated DNase (CAD) and endonuclease G (Endo G).

DISCOVERY OF DFF/CAD

The discovery of DFF was made possible through the successful establishment of an in vitro system in which DNA fragmentation in normal cell nuclei could be triggered by the addition of specific components. Wang et al. found that addition to normal cell nuclei of activated recombinant caspase-3 together with a cytoplasmic extract from normal HeLa cells resulted in nucleosomal DNA cleavage, but that the addition of either component alone did not [Liu et al., 1997]. This assay allowed for the purification of DFF, which proved to be a heterodimer composed of 40- and 45-kD subunits. The cDNA encoding the 45-kD subunit was cloned and sequenced, and it was demonstrated that the 45-kD subunit possessed two caspase-3 cleavage sites. The 40-kD subunit was not cut by caspase-3. Questions remaining from this study included whether either the 40-kD or caspase-3-processed 45-kD subunits of DFF encoded the apoptotic endonuclease, or whether one or both of these proteins triggered the activation of an endonuclease present in normal cell nuclei [Liu et al., 1997]. This and other early studies also lead to the incorrect assumption that DFF was a cytoplasmic protein because subsequent immunocytochemistry demonstrated clearly that DFF is a nuclear protein in living or non-disrupted cells [Liu et al., 1998; Samejima and Earnshaw, 1998, 2000; Lechardeur et al., 2000]. It is to be appreciated that many bona fide nuclear proteins leak out of nuclei upon cell lysis.

In 1998, Nagata and co-workers used a similar in vitro biochemical complementation assay to purify the mouse homologs of human DFF40 and DFF45, which they renamed CAD and ICAD, respectively, for CAD and inhibitor of CAD (ICAD-L) because their results showed clearly that the DFF40/CAD subunit was a latent endonuclease that was inhibited by the DFF45/ICAD subunit [Enari et al., 1998; Sakahira et al., 1998]. Besides making the significant

steps forward of identifying the subunits specifying the nuclease and its inhibitor, they cloned and sequenced the mouse cDNAs encoding DFF45/ICAD and DFF40/CAD, albeit they got the CAD sequence wrong because of a deletion of three nucleotides, resulting in a 30 amino acid reading error, which was later corrected by the same group [Mukae et al., 1998]. Subsequently human DFF40 was cloned by three groups later that year and was also demonstrated to encode the nuclease [Halenbeck et al., 1998; Liu et al., 1998; Mukae et al., 1998]. A significant aspect of the results of all these studies was the demonstration that in order to generate a caspase-3 activatable nuclease, DFF40/CAD must be co-expressed with its inhibitor subunit, DFF45/ICAD, which also act as a chaperone to correctly fold DFF40/CAD. In other words, expression of DFF40/CAD per se leads only to inactive enzyme.

DISCOVERY OF ENDONUCLEASE G

Knockout mice defective in DFF/CAD activation do not exhibit significant phenotypes [Zhang et al., 1998; McIlory et al., 2000]. It is known in these animals that the DNA of apoptotic corpses is digested after engulfment by DNase II released from phagocytes' lysozomes [McIlory et al., 2000], and that under many conditions of apoptotic stimuli, cell autonomous DNA fragmentation is severely comprised in cells derived from these animals [Zhang et al., 1999]. However, under certain conditions apoptotic stimuli can trigger cell autonomous nucleosomal DNA cleavage in such DFF/CAD defective knockout cells [Li et al., 2001]. These observations lead to the discovery of another apoptotic nuclease activation pathway.

The discovery of Endo G was made possible through the successful establishment of an in vitro system in which DNA fragmentation in normal HeLa cell nuclei could be triggered by the addition of specific components [Li et al., 2001], and independently through a genetic screen in *C. elegans* [Parrish et al., 2001]. Wang et al. found that addition to nuclei of purified mitochondria, recombinant Bid, and recombinant caspase-8, resulted in nucleosomal DNA cleavage, but that the addition of either component alone or in different pairwise combinations did not [Li et al., 2001]. This assay allowed for the purification of Endo G, which was shown to be released from the mitochondrial intermembrane space in response to tBid. Recombinant Endo G added to normal cell nuclei mimicked the nucleosomal DNA cleavage events seen in the crude system [Li et al., 2001; Widlak et al., 2001]. Xue and co-workers found that a mutation in the *C. elegans cps-6* gene, which encodes the homolog of human Endo G, resulted in the delayed appearance of cell corpses during *C. elegans* development [Parrish et al., 2001]. Significantly, this was the first indication of any role for mitochondria in apoptotic pathways in this organism. Interestingly, a knockout of the *Endo G* gene in *Saccharomyces cerevisae* exhibits no significant phenotype [Zassenhaus et al., 1988], yet a knockout of the mouse *Endo G* gene results in embryonic lethality by day 3 of development [Zhang et al., 2003].

REGULATION OF DFF/CAD

Figure 1 shows a cartoon that schematically represents the state of DFF and other molecules in normal and apoptotic cells. In non-apoptotic cells, DFF exists as a heterodimer, composed of a 45 kD chaperone and inhibitor subunit (DFF45) [also called ICAD-L], and a 40 kD latent nuclease subunit (DFF40/CAD/CPAN) [Liu et al., 1997, 1998; Enari et al., 1998; Halenbeck et al., 1998; Sakahira et al., 1998; Widlak et al., 2003]. During translation, Hsp70 and Hsp40 also participate in chaperoning the functional assembly of the heterodimer [Sakahira and Nagata, 2002]. Both DFF45/ICAD-L and DFF40/CAD reside in the cell nucleus [Liu et al., 1998; Samejima and Earnshaw, 1998, 2000; Lechardeur et al., 2000], and each subunit possesses its own nuclear localization sequence (NLS) [Samejima and Earnshaw, 1998, 2000; Lechardeur et al., 2000]. A 35 kD splicing variant of DFF45/ICAD-L (DFF35/ICAD-S) also exists, which resides in the cytoplasm because of a spliced-out NLS in its C-terminus [Enari et al., 1998; Widlak et al., 2003]. Apoptotic activation of caspase-3 or -7 results in the cleavage of DFF45/ICAD-L and DFF35/ICAD-S and release of active DFF40/CAD nuclease, which forms homo-oligomers [Liu et al., 1999; Widlak et al., 2003; Woo et al., 2004]. DFF40/CAD's nuclease activity is further activated by specific chromosomal proteins, such as histone H1, HMGB1/2, and topoisomerase II [Liu et al., 1998, 1999; Widlak et al., 2000]. Other gene products also appear to participate in the DFF pathway. Two

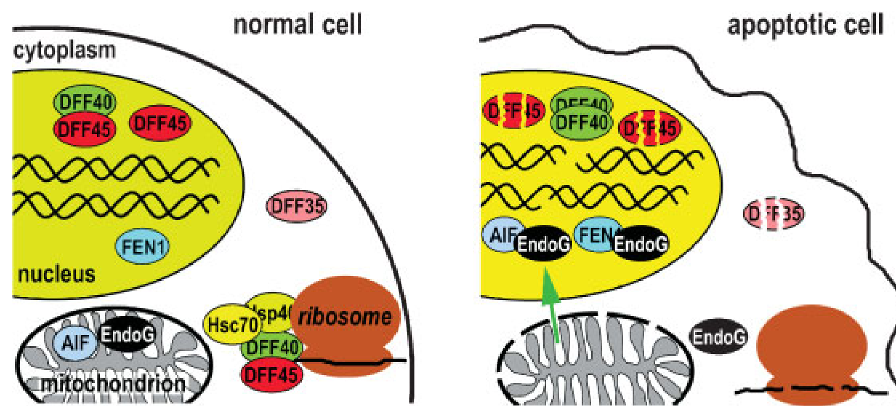

Fig. 1. Intracellular localization of nucleases and other factors involved in apoptotic DNA fragmentation in a normal healthy cell and in a cell undergoing apoptosis.

proteins encoded by genes called CIDEs that exhibit homology to the N-terminal domain of DFF45/ICAD can activate apoptosis in a DFF45/ICAD-inhibitable fashion, but their precise functions and mechanisms of action remain to be elucidated [Inohara et al., 1998].

The DFF/CAD activation pathway, subunit stoichiometries, and inhibition mechanisms suggest that multiple fail-safe steps exist to protect non-apoptotic cells from corresponding catastrophic accidents. DFF45/ICAD must be co-expressed with DFF40/CAD because it serves as a molecular chaperone to allow for the appropriate folding of DFF40/CAD to become a potentially activatable nuclease [Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1998]. The chaperone associates with DFF40/CAD to form a heterodimer and in turn serves as an inhibitor to block the homo-oligomerization of DFF40/CAD, which is known to accompany its nuclease activation [Liu et al., 1999; Widlak et al., 2003; Woo et al., 2004]. Several rare events could lead to the inappropriate activation of DFF40/CAD nuclease in non-apoptotic cells, which might include accidental trace activation of caspases-3 or -7, infrequent spontaneous misfolding of DFF40/CAD to form auto-catalytic species, or the low frequency dissociation of DFF45/ICAD inhibitor from DFF40/CAD. As schematically depicted in Figure 1, we have found a vast stoichiometric excess of free DFF45/ICAD-L subunits in the nucleus and free DFF35/ICAD-S subunits in the cytoplasm in a variety of cell lines; significantly, we have also found that these free proteins have the ability to inhibit activated DFF40/CAD homo-oligomers [Widlak et al., 2003]. These observations suggest that these species serve as fail-safe nuclear and cytoplasmic guardians for reversing the hypothetical accidental DFF40/CAD nuclease activation pathways discussed above, and protecting cells from its consequences. The questions remain open as to whether the binding of other inhibitory proteins to active DFF40/CAD homo-oligomers or the formation of excessively giant homo-oligomers of DFF40/CAD with reduced activity happen at the very late stages of apoptotic cell nuclear breakdown to protect neighboring normal cells against possible damage from released nuclease.

DFF40/CAD is known to exhibit an extraordinary preference for cleaving the internucleosomal linker regions in chromatin [Widlak et al., 2000]. This preference stems from the scissors-like structure of the DFF40/CAD active site [Woo et al., 2004]. We propose this preference for linker DNA, is significantly enhanced by binding of histone H1 to the enzyme [Liu et al., 1999]. We further propose that the binding and activation of DFF40/CAD by topoisomerase II may be responsible for targeting the 50- to 300-kb cleavage events of chromosomal loop domains that occur during initial apototic chromosomal fragmentation [Cockerill and Garrard, 1986; Durrieu et al., 2000; Widlak et al., 2000].

REGULATION OF ENDONUCLEASE G

Compartmentalization of Endo G in the mitochondrial intermembrane space prevents genomic DNA from degradation in normal cells, and release from mitochondria in response to ap-

optotic signals is a major mechanism for its activation (Fig. 1). It has been shown that mitochondrial outer-membrane permeabilization induced by pro-apoptotic Bcl-2 family members such as Bid and Bim plays a pivotal role in the release of Endo G [Li et al., 2001]. More recently it has been suggested that mitochondrial release of apoptogenic factors is regulated in a hierarchical order: release of cytochrome c would activate caspase-3, which in turn would be required for release of Endo G [Arnoult et al., 2003]. Thus, it is not clear whether Endo G is associated with "caspase-dependent" or "caspase-independent" apoptotic pathways.

A number of early observations suggested to us that other proteins may facilitate Endo G's ability to fragment DNA during apoptosis [Widlak et al., 2001]. The activity of the enzyme is quite low at physiological ionic strengths and elevated markedly on single-stranded nucleic acids. There is a high internucleosomal DNA background upon Endo G digestion of isolated nuclei, not completely characteristic of the DNA laddering pattern seen during apoptosis in DFF-knockout cells [Li et al., 2001]. Finally, DNase I knockout cells fail to ladder chromatin under apoptotic conditions that block DFF-activation and the ladder generated by DNase I digestion of chromatin also exhibits a high internucleosomal DNA background, which is not nearly as sharp as the reported DNase I-dependent apoptotic ladder [Oliveri et al., 2001]. Taken together, these observations prompted us to test whether nicks generated by DNase I would be targets for Endo G action because of their single-stranded character, and whether exonuclease gapping of nicks generated by Endo G or DNase I would also stimulate DNA processing under physiological ionic strengths. Indeed, we found that digestion of chromatin substrates leads to more than additive DNA processing events and much sharper ladders of nucleosomal fragments when either ExoIII, DNase I, or both are combined with Endo G [Widlak et al., 2001]. These results led to our prediction that Endo G must participate with DNase I-like enzymes along with exonucleases in vivo for apoptotic DNA processing, and that another important in vivo function for Endo G is RNA breakdown during apoptosis. As described below, these predictions have been largely fulfilled from the results of experiments from Xue et al. in *C. elegans*.

Biochemical and genetic evidence has shown that in *C. elegans* CPS-6/Endo G interacts with and is stimulated by WAH-1 protein, the worm homolog of apoptosis-inducing factor (AIF) [Wang et al., 2002]. AIF is a mitochondrial flavoprotein, which upon apoptotic stimuli translocates to the nucleus together with Endo G (Fig. 1), and first induces large-scale DNA fragmentation, and the initial stages of chromatin condensation [Susin et al., 1999]. It has been shown that the DNA-binding activity but not the oxidoreductase activity of AIF is required for its apoptogenic role [Ye et al., 2002]. A RNAi-based genetic screen resulted in the identification of several proteins involved in apoptotic DNA degradation in *C. elegans*. Four nucleases were among these (cell death-related nucleases, *crn* genes): CRN-1, -4, -5, and CYP-13, which potentially co-operates with CPS-6/Endo G. It has been suggested that all five nucleases and the WAH-1 protein form a DNA degradation complex termed a "degradosome" [Parrish and Xue, 2003]. CRN-4 and CRN-5 are exonucleases, CYP-13 is an endonuclease while CRN-1 is both a structure-specific endonuclease and an exonuclease homologous to the mammalian flap endonuclease 1 (FEN1), which is involved in DNA replication and repair [reviewed in Henneke et al., 2003]. CRN-1 interacts and enhances CPS-6 nuclease activity in vitro. The loss of either the endonuclease or exonuclease activity of CRN-1 reduces its ability to stimulate the activity of CPS-6. However, a nuclease-defective form of CRN-1 is still capable of enhancing the activity of CPS-6, suggesting that both nuclease-dependent and nuclease-independent mechanisms are involved in CRN-1 mediated activation of CPS-6 [Parrish et al., 2003].

ACTIONS OF DFF/CAD AND ENDONUCLEASE G

We and others have performed a series of in vitro biochemical experiments with purified recombinant enzymes on either naked DNA or chromatin substrates to establish the catalytic properties and substrate cleavage preferences for both DFF40/CAD and Endo G, which are summarized in Table I [Widlak et al., 2000, 2001; Widlak and Garrard, 2001]. DFF40/CAD is specific for double-stranded DNA while Endo G is sugar non-specific and can cleave either double-stranded DNA, single-stranded DNA, or

TABLE I. Comparison of the Properties of the Major Apoptotic Nucleases, DFF40/CAD and Endonuclease G*

	DFF40/CAD	Endonuclease G
Localization in healthy cells	Nucleus, in the latent complex with specific inhibitor	Intermembrane space of mitochondria
Inhibitors	DFF45/ICAD, DFF35/ICAD-S	
Major mechanism of activation	Cleavage of specific inhibitor by caspases	Translocation from mitochondria to nucleus
Active form	Homodimer/homo-oligomers	Monomer/homodimer
Co-activators	Histone H1, HMGB, TOPOII	FEN1, AIF
Metal co-factors	Magnesium, zinc	Magnesium, manganese
pH optimum	Neutral	Neutral
Salt requirements	Optimum at physiological $[K^+]$	Inhibited at physiological $[K^+]$
Substrates	dsDNA	RNA and ssDNA >>dsDNA
Mechanism of DNA cleavage	Blunt-end double strand breaks with 5′-P and 3′OH	Single strand nicks with 5′-P and 3′OH
Sequence preferences	Purine/pyrimidine blocks with rotational symmetry	5′ of G > C/A residues
Preferred cleavage sites in chromatin	Internucleosomal linker DNA, borders of chromatin loops	Internucleosomal linker DNA, borders of chromatin loops
Intranucleosomal cleavage	None	With 10.4 base periodicity

*Results adapted from [Liu et al., 1998, 1999; Widlak et al., 2000, 2001, 2003; Widlak and Garrard, 2001].

RNA. Both DFF40/CAD and Endo G are endonucleases dependent on magnesium cations (although Endo G also accepts manganese). Though both nucleases are inhibited by milimolar concentrations of zinc, DFF40/CAD requires trace amounts of zinc cations for stabilization of active homodimers [Woo et al., 2004]. Both nucleases are active at neutral pH. However, they are differently affected by ionic strength. DFF40/CAD is highly active at a broad range of monovalent ions (up to 150 mM). In a marked contrast, cleavage of duplex DNA by purified Endo G is inhibited at a $[K^+]$ higher then 50 mM. Importantly, even at higher salt concentrations duplex DNA is effectively cleaved by Endo G in the presence of nuclear co-activators [P.W. and W.T.G., unpublished results]. More interestingly, cleavage of RNA by Endo G is not inhibited at physiological ionic strength, which indicates that Endo G is a possible apoptotic RNase. Both endonucleases cleave substrates to generate fragments possessing ends with 5′-phosphate and 3′-hydroxyl groups that are substrates for terminal deoxynucleotide transferase (which is exploited in the TUNEL assay). However, DFF40/CAD generates exclusively double-strand breaks (prmarily blunt ends), while Endo G generates single-strand nicks. Like many other nucleases, both DFF40/CAD and Endo G have some sequence preferences on naked DNA substrates: DFF40/CAD prefers purine/pyrimidine blocks with rotational symmetry while Endo G preferentially attacks DNA 5′ of G residues.

Apoptotic cell genomic DNA cleavage is known to occur in at least two stages: initial cleavage at ≥50 kb intervals, a size consistent with chromatin loop domains, followed by a second stage of internucleosomal DNA cleavage [Oberhammer et al., 1993]. As shown by the pulsed-field gel DNA pattern in Figure 2A, addition of human recombinant activated DFF40/CAD or human recombinant Endo G to normal HeLa cell nuclei results in such higher-order initial DNA cleavage, just like that initially triggered in apoptotic cells. These higher-order cleavage events may represent an attack of pre-formed hypersensitive sites demarcating the boundaries of chromatin domains, such as those associated with domain insulators or locus control regions. More extensive nuclear digestion with either of these nucleases results in internucleosomal DNA cleavage, which we have analyzed in detail by two-dimensional gel electrophoresis; cleavage by DFF40/CAD results exclusively in double-stranded breaks between nucleosomes in the DNA linkers, whereas cleavage by Endo G often results in single-stranded nicks between nucleosomes in the DNA linkers (Fig. 2B). For example, Endo G processed chromatin migrating as trinucleosomal-sized fragments during native DNA gel electrophorsis in the first dimension migrate as mono-, di-, and trinucleosomal-sized DNA fragments after denaturation (Fig. 2B). By analysis of DNA cleavage products on sequencing gels, we can further show that Endo G, but not activated DFF40/CAD, cuts DNA within nucleosomes with an

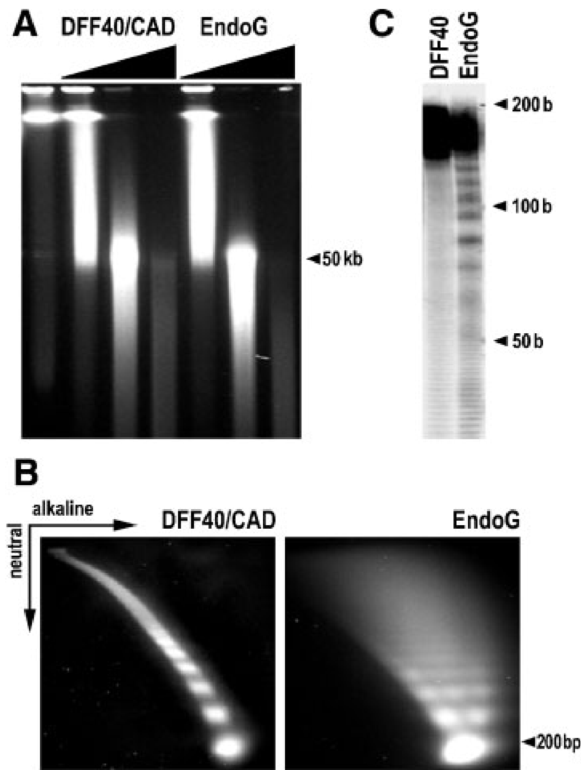

Fig. 2. In vitro chromatin cleavage by the major apoptotic nucleases DFF40/CAD and endonuclease G (Endo G). [Results adapted from Widlak et al., 2001.] Nuclei isolated from normal HeLa cells were incubated with recombinant enzymes: caspase-activated DFF40/CAD and Endo G. **Panel A**: Reaction mixtures after 5-, 15-, and 45-min incubation were analyzed by pulsed-field gel electrophoresis. **Panel B**: Isolated DNA was separated on 1.5% agarose gels in a two-dimension system: after electrophoresis in 1× TAE buffer (neutral electrophoresis), gels were turned 90°, soaked in denaturing buffer (50 mM NaOH, 1 mM EDTA), and run in the same buffer for a second dimension (alkaline). **Panel C**: Gel-purified mononucleosomal DNA was 5′ end-labeled with polynucleotide kinase and separated on an 8% sequencing gel.

approximate 10.4 b periodicity (Fig. 2C), much like DNase I [van Holde, 1988].

MODELING CHROMATIN CONDENSATION DURING APOPTOSIS

Another hallmark of the terminal stages of apoptosis is chromatin condensation [reviewed in Wyllie et al., 1980]. Three pathways have been identified that mediate apoptotic chromatin condensation: (i) a caspase-3 independent pathway triggered by mitochondrial AIF, which leads to an accompanying large-scale DNA fragmentation without internucleosomal DNA cleavage [Susin et al., 1999]; (ii) a caspase-3 dependent pathway triggered by the protein acinus, which occurs without inducing any DNA fragmentation [Sahara et al., 1999]; and (iii) a caspase-3 dependent pathway that leads to internucleosomal DNA cleavage mediated by activated DFF40/CAD [Liu et al., 1998]. We chose to study this last pathway using the well-established model of isolated HeLa cell nuclei for in vitro reconstitution experiments of apoptotic events [Liu et al., 1997].

As shown in Figure 3A,B, we have found that DNA fragmentation per se of isolated nuclei from non-apoptotic cells induces chromatin condensation that closely resembles the morphology seen in apoptotic cells, independent of ATP utilization, under physiological ionic strengths. This observed ionic strength dependency on chromatin condensation is consistent with a role for histone H1 in this process because H1-containing nucleosomes are insoluble at physiological ionic strengths [Olins et al., 1976]. Interestingly, we have found that chromatin condensation is accompanied by release of nuclear actin, and both condensation and actin release can be blocked by reversibly stabilizing internal nuclear components by various pre-treatments [Widlak et al., 2002]. For example, as shown in Figure 3C,D nuclei pre-treated with Cu^{2+} no longer exhibit chromatin condensation after internucleosomal DNA cleavage. Moreover, specific inhibition of nuclear F-actin depolymerization or promoting its formation also reduces chromatin condensation [Widlak et al., 2002]. Chromatin condensation can also be inhibited by exposing nuclei to reagents that bind to the DNA minor groove, such as ethidium bromide (Fig. 3C,D), which disrupt native nucleosomal DNA wrapping. In addition, we have also found that in cultured cells undergoing apoptosis, drugs that inhibit depolymerization of actin or that bind to the minor groove also reduce chromatin condensation but not DNA fragmentation [Widlak et al., 2002]. Therefore, the ability of chromatin fragments with intact nucleosomes to form large clumps of condensed chromatin during apoptosis requires the apparent disassembly of internal nuclear structures that may normally constrain chromosome subdomains in non-apoptotic cells. This view is consistent with the results of Ruchaud et al. [2002], who found that caspase-6-mediated proteolysis of nuclear proteins is required for chromatin condensation and nuclear breakdown in apoptotic cells in vivo.

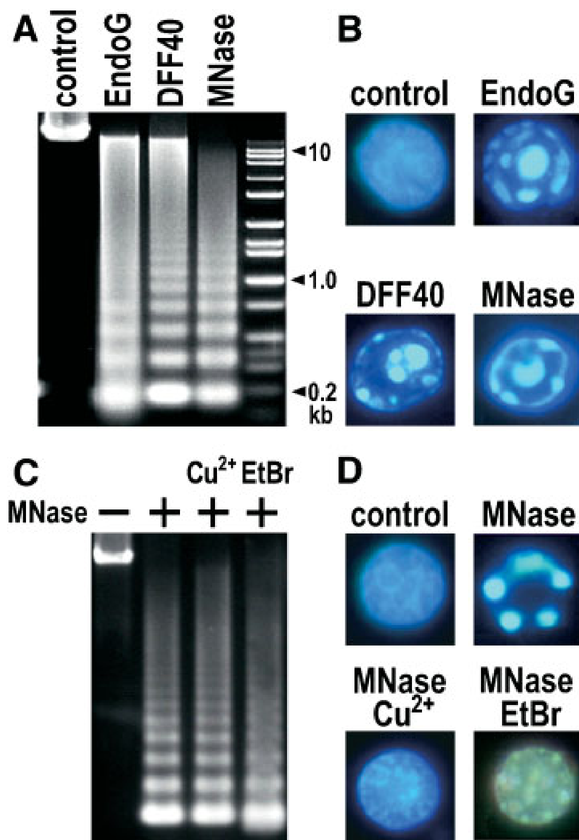

Fig. 3. Nuclease-induced chromatin condensation in vitro depends on DNA fragmentation per se and can be prevented by stabilizing intranuclear components or by dye binding to the DNA minor groove. [Results adapted from Widlak et al., 2002.] **Panel A**: Nuclei isolated from normal HeLa cells were incubated with either Endo G, caspase-activated DFF40/CAD, or micrococcal nuclease (MNase). Isolated DNA samples were analyzed by agarose gel electrophoresis. **Panel B**: Nuclei of panel A were fixed with formaldehyde, stained with DAPI, and analyzed microscopically. **Panel C**: Isolated nuclei were pre-incubated with either 1 mM $CuCl_2$ or 50 mM ethidium bromide (EtBr), washed, and then incubated with MNase. Isolated DNA samples were analyzed by agarose gel electrophoresis. **Panel D**: Nuclei of panel C were fixed with formaldehyde, stained with DAPI, and analyzed microscopically.

CONCLUSIONS

During the past 6 years, considerable progress has been made on the discovery and elucidation of the roles of the major apoptotic nucleases, DFF40/CAD and Endo G. Using normal cell nuclei with recombinant forms of these enzymes, we have been able to successfully reconstitute the DNA cleavage events in vitro normally seen in vivo in cells undergoing apoptosis. Investigations by others and ourselves of the actions of these proteins and their stoichiometries in normal cells reveals the existence of several co-activators as well as inhibitors that safeguard healthy cells against accidental nuclease activation. Clearly, a more complete understanding of all the molecules involved in regulating these nucleases will be important in the future for reducing chromosome instability and the spontaneous generation of tumor cells during aging, as well as for the selective targeting of tumor cells with ligands that activate these nucleases for antitumor therapies. We have also been able to model many aspects of one of the pathways of apoptotic chromatin condensation using normal cell nuclei in our in vitro system. Our results reveal that nuclei are pre-programmed to trigger condensation in response to internucleosomal DNA cleavage without energy at physiological ionic strengths. It is interesting to reflect from an evolutionary view that nucleosome structure not only fulfills crucial regulatory and packaging roles in living cells, but also prepares apoptotic cells for the efficient clearance of DNA by phagocytosis during cell death. Although not known at present, we favor the notion that the condensation process that we observe represents a specific form(s) of stacking of nucleosomes into supramolecular structures, rather than non-specific aggregation phenomena. Interestingly, chromatin condensation is accompanied by release of nuclear actin, and both condensation and actin release can be blocked by stabilizing internal nuclear components. We therefore propose that the ability of chromatin fragments with intact nucleosomes to form large clumps of condensed chromatin during apoptosis requires the apparent disassembly of internal nuclear structures that may normally constrain chromosome subdomains in non-apoptotic cells.

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