The Contribution of Apoptosis-inducing Factor, Caspase-activated DNase, and Inhibitor of Caspase-activated DNase to the Nuclear Phenotype and DNA Degradation during Apoptosis*

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DNA degradation is considered to be one of the defining hallmarks of apoptosis and is one of the first biochemical characteristics described for this type of cell death (1). DNA fragmentation is usually a two-step process in which the DNA is first cleaved into 50- to 300-kb fragments, termed high molecular weight (HMW) DNA fragmentation. Subsequently, DNA is degraded into smaller fragments of oligonucleosomal size, known as low molecular weight (LMW) DNA degradation or DNA ladder (2). The early HMW degradation is associated with initial nuclear morphological changes characterized by a condensation of the chromatin around the nuclear membrane (stage I chromatin condensation or nuclear morphology). The later, LMW degradation coincides with a more advanced nuclear chromatin condensation into highly packed round masses (stage II chromatin condensation) (2). Although HMW DNA fragmentation usually precedes LMW DNA fragmentation, there are evidences indicating that apoptosis can proceed without LMW DNA degradation while still displaying the HMW (3–9).

The execution of apoptotic cell death is governed by caspases, a family of cysteine proteases that, after activation by different pro-apoptotic stimuli, cleave target cell substrates (10–11). Caspases induce DNA degradation through the activation of the specific nuclease, caspase-activated DNase (CAD) (12), also known as caspase-activated nuclease (13) or DNA fragmentation factor 40 kDa (DFF40) (14). CAD is sufficient to induce both stage II chromatin condensation and LMW DNA degradation in isolated nuclei (14, 15). In growing cells, CAD remains inactive in the cytoplasm associated to the inhibitor of CAD (ICAD) (16), also known as DNA fragmentation factor 45 kDa (DDF45) (17). ICAD is encoded by alternatively spliced mRNAs that generate long (ICAD-L) and short (ICAD-S) forms of ICAD. ICAD has dual functions, acting both as a CAD inhibitor and as a chaperone for CAD synthesis. Therefore, the expression of CAD in the absence of co-expressed ICAD results in the generation of inactive aggregates of CAD (18). ICAD harbors two caspase recognition sites at Asp117 and Asp224, CAD release from ICAD inhibition is achieved by cleavage of ICAD at these Asp residues by caspase-3 (14, 17). These authors contributed equally to this work.

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7. The abbreviations used are: HMW, high molecular weight; LMW, low molecular weight; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline; AIF, apoptosis-inducing factor; CAD, caspase-activated DNase; ICAD, inhibitor of CAD; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; STP, staurosporine; dsDNA, double-stranded RNA; Z, benzylloxycarbonyl; FMK, fluoromethyl ketone; CHEF, clamped homogeneous electric fields electrophoresis; Q-VD-OPh, N-(2-quinolinyl)valyl-aspartyl-2,6-difluorophenyl/methyl ketone.
CAD synthesis, ICAD knock-out mice also lack a functional CAD. From these studies, the functional consequences of ICAD deficiency (20, 21) have been attributed to the lack of active CAD, and do not show HMW or LMW DNA fragmentation, a result comparable to that obtained with a CAD−/− mouse (22). Additionally, human lymphoma cell lines overexpressing caspase-resistant ICAD also lack both LMW and HMW DNA fragmentation (23). Altogether, these results suggest that CAD is the enzyme also responsible for both HMW and LMW DNA fragmentation. However, it cannot be discarded that ICAD might have additional functions independent of CAD, and therefore, the phenotype of ICAD−/− cells could be different from that of those expressing caspase-resistant ICAD. In that sense, other reports have shown that CAD is dispensable for apoptotic HMW DNA fragmentation. Samejima et al. (15) have demonstrated that the activity causing nuclear morphological apoptotic changes could be inhibited by caspase inhibitors but not by ICAD. More significantly, CAD-deficient chicken DT40 cells failed to undergo LMW degradation and stage II chromatin condensation but displayed normal HMW DNA degradation and stage I morphology. These data suggest that factors other than CAD might be involved (24).

In summary, these results provide evidence that CAD triggers LMW DNA fragmentation, although the role of CAD and ICAD in HMW fragmentation remains controversial (25). It has been suggested that endonucleases, other than CAD, could be responsible for HMW DNA fragmentation (24, 28–33).

In the search for additional endonucleases that could be involved in DNA fragmentation, several molecules have been proposed. Among them, apoptosis-inducing factor (AIF) has been demonstrated to play a role in HMW DNA fragmentation (26). AIF promotes caspase-independent HMW fragmentation and type I nuclear condensation when released from mitochondria by pro-apoptotic stimuli (27). This protein lacks intrinsic nuclease activity, suggesting that AIF activates an unidentified nuclease responsible for DNA fragmentation (26). Seven additional cell death-related nucleases (crn-1 to -6 and cyp-13) have been identified in *Caenorhabditis elegans* through functional genomic analysis, which, along with two known nucleases (CPS-6 and NUC-1), comprise at least two independent pathways that contribute to cell killing by degrading chromosomal DNA. Several of these proteins are components of important cellular processes such as RNA processing, protein folding, and DNA replication and repair, and appear to be important for the survival and proper development of the nematode (28). One of the genes found, *CYP-13*, belongs to the family of cyclophilins, which have been previously involved in chromosomal DNA degradation in vertebrates. Cyclophilins show a nuclease activity distinct from the *cis-trans* isomerase one, and their ionic requirements are similar to that
described for apoptotic nucleases (29, 30). Cyp C has been shown to
induce 50-kbp DNA fragmentation on isolated nuclei (29), whereas Cyp
A has been demonstrated to interact and cooperate with AIF in apop-
tosis-associated chromatinolysis (31). In addition, it has been shown that
Xenopus egg extracts have a HMW DNA degradation activity activated
by Caspase 3, which is different than the Xenopus CAD homolog, whose

FIGURE 2. Mutagenesis strategy of ICAD and behavior of stably transfected SH-SY5Y cells. A, scheme representing wild type (ICAD) or mutant forms (D117E and D224E) of ICAD tagged with a FLAG epitope at the N terminus. Mutated nucleotides replacing the processed Asp sites by Glu residues are indicated in bold, and the new restriction sites generated for cloning are underlined. Full bars show the predicted fragments generated by caspase processing of each form of ICAD. B, Western blot showing the protein levels of FLAG-ICAD, ICAD, CAD, or α-tubulin in total cell extracts from SH-SY5Y cells transfected with the empty vector (pcDNA3), wild type (ICAD), or the single mutant forms (D117E or D224E) of ICAD. C, Western blot against FLAG epitope showing the different mutant forms of FLAG-ICAD (FLAG, upper panel) or against total ICAD (ICAD, middle panel) after 6 h of 1 μM STP treatment (STP) or in non-treated cells (C). An anti-ERK antibody was used as a loading control (ERK-2, lower panel). Detection with the anti-FLAG monoclonal antibody reveals high levels of the ectopically expressed protein in the transfected cells and, as expected, the incomplete processing fragment corresponding to the first 224 residues of the D117E ICAD in cells treated with STP (FLAG-p30 (N-term.), upper panel). Detection with an anti-ICAD antibody reveals the correct processing of endogenous ICAD in all transfected cells when treated with STP (p11 (C-term.), middle panel). Note also that in the D224E ICAD a band of 30 kDa (p30 (C-term.)) is accumulated.
molecular identity remains uncharacterized (32). The relationship of this activity with AIF has not been analyzed. Other poorly defined endonuclease activities capable of processing the DNA into 50-kb fragments have also been reported in spermatozoa (33).

We have previously demonstrated that IMR-5 neuroblastoma cells failed to display LMW DNA fragmentation due to a defect in the ICAD/CAD system that results in a non-functional CAD (9). In the current report, we show that IMR-5 cells display HMW DNA fragmentation with undetectable TUNEL reactivity after staurosporine (STP) treatment. To ascertain the importance of ICAD in the nuclear apoptotic changes, we analyzed the contribution of the two caspase-target aspartic residues of ICAD (Asp177 or Asp224) in SH-SY5Y cells. We demonstrate that overexpression of the ICAD-D117E single mutant prevents LMW DNA fragmentation, stage II chromatin condensation, and detection of TUNEL staining, whereas HMW DNA degradation and stage I nuclear morphology remain unaltered. In cells overexpressing wild type ICAD or the ICAD-D224E single mutant no alterations to DNA fragmentation and nuclear morphology were observed. Overexpression of D117E-ICAD does not affect the release of AIF or cytochrome c from mitochondria to cytosol. We show for the first time that dsRNA knock-down of endogenous AIF expression in SH-SY5Y cells abolishes stage I nuclear condensation but not HMW or LMW DNA degradation. Similar results are obtained by intracellular delivery of blocking anti-AIF antibodies. We conclude that CAD is not responsible for HMW DNA degradation and stage I chromatin condensation, and this depends instead on the activity of other endonucleases that generate TUNEL-negative ends on DNA fragments. Our results, together with those reported using the ICAD−/− mice (21), further suggest a role for ICAD in HMW DNA degradation.

MATERIALS AND METHODS

Chemical Reagents—STP and Me2SO were purchased from Sigma. Z-VD(OMe)VA(Ome)-FMK (caspase inhibitor-2), Z-D(Ome)E-(Ome)VD(Ome)-FMK (caspase inhibitor-3/7), Z-VE(Ome)ID(Ome)-FMK (caspase inhibitor-6), Z-IE(Ome)TD(Ome)-FMK (caspase inhibitor-8), Z-LE(Ome)HD(Ome)-FMK (caspase inhibitor-9), and Q-VD(non-Ome)-OPh (pan caspase inhibitor) were from Calbiochem (Barcelona, Spain).

Plasmid Construction—The pCRII vector containing the full open reading frame of the long form of human ICAD (1.3 kb) was kindly provided by Dr. X. Wang (17). Mutated forms of hICAD, hICAD-D117E and hICAD-D224E, were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The primers used for introducing the D117E mutation, primers were, forward 5'-ATT TCC CAA GAG TCC TTT GAC GTC GAT GAA ACA GAG AGC GGG GCA GGG TTG AAG-3' and reverse 5'-CTT CAA CCC TGC CCC GCT CTC TGT TTC ATC GAT GCA GTA GAG ACG GAT ATC AGA GAG ACC TCC-3' and reverse GGA GGT TCT GCT GAT ATC CGT CTG TAC TGC ATC CAC CTC CTC C (changed positions are indicated in bold). In these primers we also introduced a silent mutation that generates a unique restriction site for AatII (underlined). For the D224E mutation, primers were, forward 5'-G GAG GAG GGT GTG GAT GCA GTA GAG ACG GAT ATC AGA GAG ACC TCC-3' and reverse GGA GGT TCT GCT GAT ATC CGT CTG TAC TGC ATC CAC CTC CTC C (changed positions are highlighted in bold). These primers also introduced a silent mutation that generates a restriction site for EcoRV (underlined). A FLAG epitope was introduced at 5' position in the wild type and mutant ICAD by PCR using the primers HindIII-FLAG-hICAD (forward) 5'-CCC AAC GCT ATG GAC TAT AAG GAT GAC GAT GAC AAG GAG GTG ACC GGG GAC GCC GGG GTA-3' and EcoRl-DFF45-R (reverse) 5'-CGG AAT TCT ATG TGG GAT CCT GTC TG GCT-3'. The PCR products were subcloned into the pcDNA3 expression vector (Invitrogen). All constructs were confirmed by DNA sequencing. Final constructs referred to as pcDNA3-hICAD-5'FLAG, pcDNA3-hICAD-D117E-5'FLAG, and pcDNA3-hICAD-D224E-5'FLAG. Empty vector (pcDNA3) was used as control.

Cell Culture and Constitutive Transfection—Human SH-SY5Y and IMR-5 cell lines were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen). Cells were maintained at 37°C in a saturating humidity atmosphere containing 95% air and 5% CO2. Cells were transfected with 3 µg of empty pcDNA3, pcDNA3-hICAD-5'FLAG, pcDNA3-hICAD-D117E-5'FLAG, or pcDNA3-hICAD-D224E-5'FLAG. Transfections were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Stably transfected cells were obtained by selection with 500 µg/ml Geneticin (Invitrogen) and several clones, as well as the pool of transfected cells, were propagated and tested for ICAD expression and processing by Western blotting. Most of the experiments were carried out using pools of transfected cells. Clones gave comparable results.

MTT Reduction and Trypan Blue Exclusion Cell Viability Assays and Chromatin Staining with Hoechst 33258—MTT is a water-soluble tet-

![FIGURE 1](https://example.com/figure1.png)

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| Chemical Reagents: STP and Me2SO were purchased from Sigma. Z-VD(Ome)VA(Ome)-FMK (caspase inhibitor-2), Z-D(Ome)E-(Ome)VD(Ome)-FMK (caspase inhibitor-3/7), Z-VE(Ome)ID(Ome)-FMK (caspase inhibitor-6), Z-IE(Ome)TD(Ome)-FMK (caspase inhibitor-8), Z-LE(Ome)HD(Ome)-FMK (caspase inhibitor-9), and Q-VD(non-Ome)-OPh (pan caspase inhibitor) were from Calbiochem (Barcelona, Spain). | Plasmid Construction: The pCRII vector containing the full open reading frame of the long form of human ICAD (1.3 kb) was kindly provided by Dr. X. Wang (17). Mutated forms of hICAD, hICAD-D117E and hICAD-D224E, were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The primers used for introducing the D117E mutation, primers were, forward 5'-ATT TCC CAA GAG TCC TTT GAC GTC GAT GAA ACA GAG AGC GGG GCA GGG TTG AAG-3' and reverse 5'-CTT CAA CCC TGC CCC GCT CTC TGT TTC ATC GAT GCA GTA GAG ACG GAT ATC AGA GAG ACC TCC-3' and reverse GGA GGT TCT GCT GAT ATC CGT CTG TAC TGC ATC CAC CTC CTC C (changed positions are indicated in bold). In these primers we also introduced a silent mutation that generates a unique restriction site for AatII (underlined). For the D224E mutation, primers were, forward 5'-G GAG GAG GGT GTG GAT GCA GTA GAG ACG GAT ATC AGA GAG ACC TCC-3' and reverse GGA GGT TCT GCT GAT ATC CGT CTG TAC TGC ATC CAC CTC CTC C (changed positions are highlighted in bold). These primers also introduced a silent mutation that generates a restriction site for EcoRV (underlined). A FLAG epitope was introduced at 5' position in the wild type and mutant ICAD by PCR using the primers HindIII-FLAG-hICAD (forward) 5'-CCC AAC GCT ATG GAC TAT AAG GAT GAC GAT GAC AAG GAG GTG ACC GGG GAC GCC GGG GTA-3' and EcoRl-DFF45-R (reverse) 5'-CGG AAT TCT ATG TGG GAT CCT GTC TG GCT-3'. The PCR products were subcloned into the pcDNA3 expression vector (Invitrogen). All constructs were confirmed by DNA sequencing. Final constructs referred to as pcDNA3-hICAD-5'FLAG, pcDNA3-hICAD-D117E-5'FLAG, and pcDNA3-hICAD-D224E-5'FLAG. Empty vector (pcDNA3) was used as control. |
razolium salt that is reduced by metabolically viable cells to a colored, water-insoluble formazan salt. The procedure employed for this assay was the same as that described by Boix et al. (5). For trypan blue staining, cells were seeded in 24-multiwell plates at 5–3 × 10^4 cells/well. After 24 h of seeding, cells were treated with STP at the adequate doses and times. Then cells were gently dissociated with a blue tip in their own culture medium, and a sample (100 μl) was taken and mixed with 20 μl of trypan blue solution (0.4%) (Sigma). Ten microliters of the resulting cell suspension were counted with a hemocytometer. The results were expressed as percentages of trypan blue-stained cells over the total

FIGURE 4. D117E SH-SY5Y cells treated with STP present stage I of apoptotic DNA condensation. Cells transfected with empty vector (pcDNA3), wild type (ICAD), or single mutant forms (D117E or D224E) of ICAD were treated for 6 h with 1 μM of STP (STP) or left untreated (Control). Apoptotic nuclear morphology was analyzed by Hoechst 33258 nuclear staining (A) and by electron microscopy (B). A high chromatin condensation and fragmentation in rounded masses (stage II) is observed in the empty vector, wild type ICAD, and D224E ICAD transfected cells when treated with STP. On the contrary, forced expression of single mutant D117E of ICAD prevents stage II nuclear morphology and the chromatin appears homogeneously condensed by Hoechst staining. Ultrastructural analysis shows that chromatin is margined in the periphery of the nucleus (stage I). All photographs are representative fields for each condition studied (scale bar, 20 μm). In A, white squares correspond to magnifications of selected STP-treated nuclei.
D117E ICAD-expressing SH-SY5Y cells treated with STP present HMW but not LMW DNA cleavage nor TUNEL staining. Cells transfected with empty vector (pc or pcDNA3), wild type ICAD (I or ICAD), or single mutants forms (117 or D117E and 224 or D224E) of ICAD were treated for 6 h with 1 μM STP (S or STP) or left untreated (C or Control) and were analyzed for DNA degradation. A, the CHEF-agarose gel shows DNA degradation into high molecular weight fragments in all the transfected cells when treated with STP (st, Sigma’s 1000-kb DNA size marker). B, ladder analysis reveals that overexpression of the D117E ICAD form in cells treated with increasing concentrations of STP (0, 125, 250, and 1000 nM, respectively) prevents LMW DNA fragmentation. Overexpression of the wild type or D224E ICAD mutant does not have any effect on DNA laddering. C, quantification of cell death with Hoechst and TUNEL staining after 24 h of 1 μM STP treatment. Type I and II mean nuclear morphology of apoptotic cells. The mean ± S.E. of three independent experiments are shown. D, representative low and high magnification photomicrographs are shown (arrowheads indicate the selected cells illustrated in the insets). Scale bar, 20 μm.
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number of cells. Nuclear morphology was assessed by staining cells with the Hoechst 33258, which is also known as bisbenzimide (2′- (4-hydroxyphenyl)-5′-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride) as established in our laboratory (5). The normal or apoptotic cell nuclei were visualized with an Olympus microscope equipped with epifluorescence optics under UV illumination.

dsRNA Preparation and Transfection—Two different dsRNA oligonucleotides against human AIF (R1, CCGGCUCCCAGGCAACUUUG-dTdT and R2, GCCAUGCUCUAAGAUAAAdTdT) and one scrambled sequence were bioinformatically designed and synthesized (Promega, Parc, France, France). Transfections of 5 × 105 cells with 10 μg of the indicated dsRNA were performed with Nucleofector (Amara, Cologne, Germany) using the G-04 program and Cell Line Nucleofector Solution V, following manufacturer’s instructions. After 2 days, cells were replated in adequate culture dishes, depending on the experiment, and 72 h post transfection, the treatment and the corresponding protocol were performed.

Protein Extractions and Western Blotting—When whole protein extracts were used, ~1 × 106 cells per condition were detached from 35-mm culture dishes, gently pelleted by centrifugation and washed twice in phosphate-buffered saline (PBS). Cells were lysed in total extraction buffer containing 125 mM Tris-HCl, pH 6.8, and 2% SDS pre-warmed at 95°C. Mitochondria-free cytosolic extraction of AIF and cytochrome c was performed with 2 × 106 cells seeded on 60-mm plates and treated with STP for the times indicated. After treatment, cells were detached, harvested in PFS, and resuspended in ten volumes of extraction buffer containing 220 mM mannitol, 70 mM sucrose, 50 mM Heps-KOH (pH 7.2), 10 mM KCl, 5 mM EGTA, 2 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride, and kept on ice for 15 min. Cells were centrifuged in a Microfuge at 16,000 × g for 15 min at 4°C, and the supernatant was retained. Nuclear and mitochondrial subfractionation extracts were performed with NE-PER® Nuclear and Cytosolic Extraction Reagents (Pierce Biotechnology, Perbio Science France, Brebières, France) following the manufacturer’s instructions. In all cases, protein content was quantified by a modified Lowry assay (Bio-Rad Dc protein assay, Bio-Rad).

Around 5–25 μg of protein per condition were electrophoresed in 15% (FLAG, ICAD, CAD, caspase-3, and cytochrome c detection), 10% (AIF detection), or 6% (α-fodrin and poly(ADP-ribose) polymerase detection) SDS-polyacrylamide gels that were electrotransferred to Immobilon-polyvinylidene difluoride membranes (Millipore, Bedford, MA) with a semidry apparatus (Hoefer, Amersham Biosciences). Filters were probed with the indicated primary antibodies and incubated with secondary antibodies conjugated with peroxidase (Sigma). As substrates for immunodetection we used either ECL (Amersham Biosciences) or SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology). Antibodies used in this study were anti-FLAG M2, anti-Hsp60, anti-actin, anti-cytochrome c, and anti-AIF (Sigma), anti-DFF45/ICAD (Stressgen Biotechnologies Corp., Victoria, BC, Canada), anti-α-spectrin/fodrin, anti-CAD (Chemicon International, Inc., Temecula, CA), anti-poly(ADP-ribose) polymerase C2.10 (Enzyme System Products, Livermore, CA), anti-caspase 3, anti-cleaved caspase-3, anti-panERK, anti-SOS-2 (BD Biosciences), and anti-fibrillarin (Abcam Ltd., Cambridge, UK). When required, the membranes were stained with Naftol Blue to assess comparable loading of lanes.

TUNEL Assay and Nuclei Staining—SH-SY5Y and IMR-5 cells were treated with staurosporine as indicated in figure legends. Detection of blunt double-stranded fragments carrying a 5′-phosphate and 3′-hydroxyl group was carried out by fixing cells in freshly prepared 1% paraformaldehyde for 30 min at 4°C and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 10 min at 4°C. After washing in PBS containing 0.1% Triton X-100, they were incubated with 50 μl of a reaction mixture containing 0.025 nmol of Fluorescein-12-dUTP, 0.25 nmol of dATP, 2.5 mM CoCl2, 40 units of recombinant terminal deoxynucleotidyl transferase (TdT), and TdT reaction buffer from Roche Applied Science for 1 h at 37°C. The reaction was stopped by adding 20 μl EGTA. Cells were washed twice with PBS, stained with 0.05 μg/ml Hoechst 33258, mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA), and photographed under fluorescence microscope (Olympus IX70-S8F2) coupled to a camera (model OM-4 Ti; Olympus).

Electron Microscopy—Approximately 3 × 106 cells per condition were detached from 60-mm culture dish, gently pelleted by centrifugation, and washed with PBS. Pellets were fixed at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, rinsed three times with 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide (OsO4), dehydrated in graded acetone, and embedded in Durcupan ACM Epoxy resin (Fluka, Buchs, Switzerland). Ultrathin sections of selected areas were obtained, mounted in copper grids, counterstained with uranyl acetate and lead citrate, and observed with a Zeiss EM910 electron microscope (Carl Zeiss Microscope Systems, Oberkochen, Germany).

DNA Degradation Analysis—High molecular DNA fragmentation was assayed by clamped homogeneous electric fields (CHEF) electro-
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FIGURE 7. HMW and LMW DNA fragmentation can be inhibited by different caspase inhibitors. A, mock transfected SH-SY5Y cells were left untreated (C) or treated with 1 μM STP plus 100 μM Z-VD(Ome)YAD(Ome)-FMK (caspase inhibitor-2), Z-D(Ome)E(Ome)VD(Ome)-FMK (caspase inhibitor-3/7), Z-VE(Ome)ID(Ome)-FMK (caspase inhibitor-6), Z-IE(Ome)TD(Ome)-FMK (caspase inhibitor-9), Z-E(Ome)TD(Ome)-FMK (caspase inhibitor-8), 10 μM Q-VD(non-OMe)-OPh (pan caspase inhibitor, Q), or vehicle (w/o) for 6 h. HMW (CHEF electrophoresis) and LMW (conventional agarose electrophoresis) DNA degradation. B, Western blot analysis of the same cell extracts obtained in A to demonstrate the effectiveness of the different specific caspase inhibitors. Detection of ICAD was studied by CHEF-agarose gel (HMW) or by conventional agarose gels (LMW). C, Western blot analysis of the same cell extracts obtained in A to demonstrate the effectiveness of the different specific caspase inhibitors. Detection of ICAD was studied by Western blot analysis of the same cell extracts obtained in C to demonstrate the inhibition of ICAD processing and caspase-3 activation with the different dose of QVD used.

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phoresis using a CHEF-DR (Bio-Rad) apparatus. Approximately 2 × 10⁶ cells per condition were suspended in 40 μl of PBS, warmed at 60 °C for 5 min, mixed with an equal volume of warm 1% low melting point agarose in 0.5 M plus 10, 5, 1, and 0.5 M Q-VD(non-OMe)-OPh (QVD) or vehicle (w/o) for 6 h. HMW (CHEF electrophoresis) and LMW (conventional agarose electrophoresis) DNA degradation. D, Western blot analysis, carried out in the same samples used in C, demonstrate the inhibition of ICAD processing and caspase-3 activation with the different dose of QVD used.

For LMW DNA degradation, equal volumes of NDS supernatants and cold 100% ethanol were mixed, and the mixture was incubated at −20 °C overnight and centrifuged at maximum speed for 10 min at 4 °C. Pellets were washed once in 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 9.0, 1 mM EDTA) containing 200 μg/ml proteinase K with gentle shaking. The NDS supernatants were further processed for internucleosomal DNA degradation (LMW DNA degradation). Next, the blocks were further incubated for 24 h with the same buffer containing 10 μg/ml RNase A. After two 1-h washes at room temperature in 0.5 × TBE buffer, the blocks were inserted into wells of a 1% CHEF agarose gel in 0.5 × TBE. Electrophoresis was carried out at 6 V/cm for 14 h at 14 °C with a switch time of 5-50 s.

For LMW DNA degradation, equal volumes of NDS supernatants and cold 100% ethanol were mixed, and the mixture was incubated at −20 °C overnight and centrifuged at maximum speed for 10 min at 4 °C. Pellets were washed once in 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 9.0, 1 mM EDTA) containing 200 μg/ml RNase 15 min at 60 °C. DNA was analyzed in 1.5% agarose gel in TAE buffer (1 mM EDTA, 40 mM Tris acetate, pH 7.6) stained with ethidium bromide.

Cell cycle analysis and sub-G₁ peak of DNA fragmentation were performed using propidium iodide staining. Briefly, after treatments, cells were recovered, rinsed with PBS, and resuspended with PBS containing 0.5 μg/ml propidium iodide, 0.1% Igepal CA-630, and 50 μg/ml DNase-free RNase. After 20 min of incubation, DNA content was determined using a FACSCalibur (BD Biosciences, Oxford, UK) flow cytometer. Data were analyzed by CellQuest software.

Intracellular Antibody Delivery Approach into Living Cells—To inhibit AIF function, the BioPORTER® Protein Delivery system (Gene Therapy Systems, Inc., San Diego, CA) was used following the manufacturer’s instructions. In brief, the BioPORTER reagent was dissolved in chloroform, aliquoted, and let to evaporate under laminar flow hood. 20 μg of a purified rabbit polyclonal anti-AIF antibody (raised against an AIF peptide (109TLLRFKQWNGKERSYFQQPSFYSVSAQD216) or a secondary rabbit anti-mouse-fluorescein isothiocyanate (irrelevant IgG) used as a control, were diluted in Tris buffer (10 mM Tris, pH 7.0, and 150 mM NaCl). The BioPORTER®-protein complexes were prepared by adding the antibody solution into the tube containing the dry film of lipidic reagent, incubating for 5 min, and vortexing gently. Finally, the lipid-antibody complexes were directly added to cells previously washed and resuspended in serum-free medium. After 4 h of incubation, DNA degradation analysis was performed. In parallel, to demonstrate that antibodies were delivered to the cells, cytosolic extracts were incubated with protein G-agarose (Amersham Biosciences) and immunoprecipitated. AIF depletion from the cytosol by the specific antibody was tested by Western blot.

S. Susin, unpublished results.
RESULTS

Context and Biochemical Features of Our Experimental Model—Previous results published by our group have demonstrated that IMR-5 cells lack oligonucleosomal fragmentation (9) (Fig. 1A). We have demonstrated that these cells have a defect in the CAD endonuclease that results from its precipitation from the soluble fraction after STP treatment, thereby preventing its enzymatic action. Nonetheless, these cells display stage I of nuclear condensation. Fig. 1A shows that STP is able to induce HMW fragmentation in both IMR-5 and SH-SY5Y cells but, DNA fragments generated in IMR-5 cells do not show free 3′-OH, because they are undetectable for TUNEL staining (Fig. 1B). In contrast, SH-SY5Y cells display LMW DNA fragmentation and DNA fragments that are TUNEL-positive (Fig. 1B). These results suggest that TUNEL staining is only observed when oligonucleosomal DNA cleavage occurs (Fig. 1, B and C). In our previous work, we were able to transform the nuclear apoptotic feature of IMR-5 cells into that of a SH-SY5Y one by overexpressing CAD endonuclease. Here, we aimed to perform the opposite by reversing the SH-SY5Y nuclear apoptotic phenotype to an IMR-5 one. This experimental paradigm should allow us to understand the molecular mechanisms regulating DNA fragmentation, karyorrhexis (nuclear breakage), and pyknosis (chromatin condensation and nuclear shrinkage) observed during apoptosis more precisely. Because the ICAD/CAD system is considered the core of the regulation of the nuclear apoptotic outcome, we assessed if overexpression of caspase-resistant forms of ICAD convert SH-SY5Y cells into IMR-5-like cells. To this end, we generated ICAD mutants in which the Asp224 were changed to Glu, which is not cleaved by caspases (Fig. 2A). SH-SY5Y cells were transfected with plasmids coding for either FLAG-tagged wild-type ICAD, FLAG-D117E ICAD, or FLAG-D224E ICAD and stable pools of cells were obtained. The levels of FLAG-tagged ICAD and total CAD were visualized by Western blotting using specific antibodies and found to be comparable among the different populations of cells (Fig. 2B).

To assess the processing properties of the ICAD mutants we performed a Western blot using an anti-FLAG antibody on transfected pools of cells treated with 1 μM STP for 6 h (Fig. 2C). Cells overexpressing wild-type ICAD, D117E, or D224E mutants, but not the mock transfected ones, showed a band at the expected size of FLAG-tagged ICAD. The intensity of this band decreased upon STP treatment suggesting that ICAD was being processed. Because ICAD was FLAG-tagged at the N terminus, no band corresponding to the processed fragments could be observed in the D224 mutants. In contrast, in the D117E mutants, the STP-induced processing of ICAD resulted in the accumulation of a band of 30 kDa corresponding to the amino-terminal fragment up to amino acid residue 224 (FLAG-p30 N-term in Fig. 2C, upper panel). When the membrane was reprobed with an ICAD antibody directed against the C terminus of ICAD, bands corresponding to the processing of endogenous plus ectopic ICAD could be observed. No major differences between the different conditions were observed except for the higher amounts of ICAD in the transfected cells. Noticeably, a band of 30 kDa accumulated in D224E mutants after STP treatment (p30 C-term in Fig. 2C, middle panel). All ICAD mutants gener-
ated correctly bound to endogenous CAD, because this it could be co-immunoprecipitated with the anti-FLAG antibody (data not shown).

To ascertain whether overexpression of wild-type ICAD or the D117E and D224E mutants affected cell death induced by STP in the SH-SY5Y cell line, we carried out the MTT reduction and Trypan Blue exclusion viability assays. As shown in Fig. 3, STP-induced cell death, irrespectively of the tested doses, does not substantially differ between wild-type ICAD and ICAD mutant-overexpressing cells. Therefore, ICAD mutant overexpression does not affect the extent of cell death.

Cells Overexpressing D117E-mutated ICAD Do Not Reach Stage II of the Nuclear Apoptotic Morphology—To evaluate the effect of each of the mutants on the nuclear apoptotic phenotype, STP-treated cells were analyzed by Hoechst nuclear staining and electron microscopy. As shown in Fig. 4A, the STP-treated cells transfected with either the empty vector, wild type ICAD, or D224E ICAD exhibited a clear stage II apoptotic pattern, characterized by highly condensed chromatin and fragmentation of the nuclei into rounded masses of chromatin. These chromatin changes were further confirmed by electron microscopy (Fig. 4B). Expression of D117E ICAD resulted in the complete absence of stage II nuclear morphology. Nevertheless, these apoptotic cells were different from their controls, because they showed a diffuse chromatin condensation throughout the nucleus, with a higher accumulation of the staining in the nuclear periphery (Fig. 4A). Electron microscopy analysis demonstrated a minor chromatin condensation in the marginal zone of the nucleus that was never fragmented (Fig. 4B). This morphological pattern has been described as stage I of nuclear apoptosis. In conclusion, ectopic expression of D117E ICAD induced an arrest in the early phases of chromatin condensation.

D117E ICAD Overexpression in SH-SY5Y Cells Prevents LMW DNA Fragmentation and Detection of TUNEL Staining, but Not HMW DNA Cleavage—Because we have demonstrated that apoptosis proceeds without accomplishing stage II in the SH-SY5Y cells overexpressing D117E ICAD, we were further interested in correlating this observation with the DNA degradation pattern. The progressive chromatin condensation that occurs during apoptosis has been associated with an early DNA degradation into HMW fragments that is followed by DNA degradation into oligonucleosomal fragments. The different SH-SY5Y-transfected cells were treated with STP, and both HMW and LMW DNA fragmentation were assessed by electrophoresis. As shown in Fig. 5A, cells transfected with the different constructs exhibited a pattern of DNA degradation into HMW fragments of ~50 kb when treated with STP. Of note is the sharp limit in the DNA smear below the 50-kb marker band that is only observed in the lane corresponding to the D117E-transfected cells. This absence of DNA degradation could reflect the absence of oligonucleosomal DNA fragmentation. We further confirmed this assumption by performing conventional agarose DNA electrophoresis. The LMW DNA degradation analysis shown in Fig. 5B clearly shows that DNA laddering occurs only in cells transfected with

![FIGURE 9. Delivery of an AIF blocking antibody in living cells does not prevent HMW DNA fragmentation.](http://www.jbc.org/)

A purified AIF antibody (α-AIF) or an irrelevant antibody (IgG) was delivered in SH-SY5Y cells expressing D117E ICAD using the BioPORTER Protein Transfection Reagent. 4 h later, cells were treated with 1 μM STP for 6 h or were left untreated (control). A, demonstration of the efficiency of the antibody delivery and binding activity as assessed by the complete immunodepletion of AIF from the cytosol of STP-treated cells using protein G coupled to agarose beads. AIF is released to the cytosol in STP-treated cells (input, upper panel) and appears in the flow-through of the precipitates in the control condition (IgG, middle panel) and bound to the agarose beads in the anti-AIF condition (arrowhead, lower panel). B, DNA fragmentation analysis reveals that blocking AIF function did not prevent HMW DNA fragmentation. C, nuclear morphology was analyzed by Hoechst 33258 staining. Selected pictures of representative fields are shown (scale bar, 10 μm).
FIGURE 10. Reduction of AIF expression avoids the appearance of stage I nuclear apoptotic morphology. SH-SY5Y cells stably transfected with empty vector (pcDNA3) or with D117E ICAD (D117E) were transiently transfected with the different dsRNA against AIF (R1 and R2) or with the scrambled sequence (Scr). After 3 days, cells were treated for 6 h with 1 μM STP (STP) or left untreated (Control). A, Hoechst nuclear staining reveals that AIF-knockdown in D117E ICAD-expressing cells completely prevents the stage I nuclear condensation that is present in cells transfected with the scrambled sequence (insets are high magnifications of the cells indicated by the arrowhead). Scale bar, 20 μm. Also note that the presence of the dsRNA against AIF does not prevent stage II morphology in mock transfected cells (upper images). B, percentage distribution between the three different nuclear apoptotic morphologies found among all the dead nuclei. C, electron microscopy of D117E ICAD-expressing cells transiently transfected with the dsRNA against AIF (R2) or with the scrambled sequence (Scr) after 6 h of 1 μM STP treatment (STP) or left untreated (Control). D, cell cycle and apoptotic sub-G1 peak analysis by flow cytometry of empty vector (Neo) or D117E mutant-overexpressing SH-SY5Y cells transfected for 72 h with scrambled (Scr) or AIF (R2) RNA interference and further treated with 1 μM STP for 6 h or left untreated.
the empty vector, wild type ICAD or D224E ICAD, but not in cells overexpressing the D117E construct. Our results indicate that mutations in either 117 or 224 Asp residues do not affect HMW processing, suggesting that the processing of ICAD is not necessary for this type of DNA fragmentation. Upon STP treatment, the appearance of stage II chromatin condensation nuclei correlated with detection of TUNEL staining as quantified in cells transfected with the empty vector, wild type ICAD, or D224E ICAD (Fig. 5C). In contrast, cells expressing D117E ICAD did not show any TUNEL reactivity (Fig. 5, C and D). These results demonstrate that the early stages of chromatin condensation are not mediated by endonucleases that generate 3'-OH ends on DNA fragments, because stage I chromatin condensation proceeded without positive TUNEL staining. Therefore, we can infer that CAD is not the apoptotic endonuclease responsible for large scale chromatin fragmentation, because it only generates 3'-OH DNA ends. These results further suggest that overexpression of D117E ICAD is enough to induce an IMR-5-like nuclear apoptotic phenotype.

Expression of D117E ICAD in SH-SY5Y Cells Does Not Alter the Activation of Caspase-3—It has been previously demonstrated that the lack of caspase-3 activity results in a nuclear apoptotic phenotype similar to that observed here in SH-SY5Y cells transfected with D117E ICAD (6, 7). Although the partial processing of D117E ICAD suggests an STP-induced activation of caspases, we further studied the implication of caspase-3 in the apoptotic death induced by STP in these cells. We first analyzed the release of cytochrome c and AIF from mitochondria to cytosol and observed that expression of D117E ICAD did not affect the release of either protein (Fig. 6, A and B). We also observed that AIF adequately translocates from mitochondria to the nucleus upon STP induction of apoptosis in both cell types (Fig. 6C). When we analyzed the processing of procaspase-3 to its active p17 form by Western blotting, no differences were observed between control and D117E ICAD-expressing cells (Fig. 6D). We also analyzed the pattern of processing of α-fodrin and poly(ADP-ribose) polymerase, two well established substrates of caspase-3. STP treatment induced cleavage of both substrates into their respective 120- and 85-kDa fragments in all the cells analyzed (Fig. 6E, upper and middle panels). Therefore, overexpression of D117E ICAD resulted in a caspase-3 mutant-like apoptotic phenotype without altering the adequate activation of this caspase or the release of cytochrome c or AIF from mitochondria.

Implication of Executor Caspases in HMW and LMW DNA Degradation—Because caspases are the main proteases regulating apoptosis, we wanted to know if their specific inhibitors could control the extent of both HMW and LMW DNA degradation in SH-SY5Y cells. As shown in Fig. 7A, inhibitors of caspase-3, -7, or -9, as well as the general inhibitor Q-VD-OPh (34), but not the specific inhibitors of caspase-2, -6, or -8, were able to prevent both types of DNA degradation. The DNA degradation correlated with the processing of ICAD to its inactive forms (p11 C-terminal fragment), as well as with the appearance of the active p17 fragment of caspase-3 (Fig. 7B).

Among the caspase inhibitors used, the most powerful peptide was Q-VD-OPh. This compound completely abolished HMW and LMW DNA fragmentation as well as caspase-3 activation and ICAD processing, even at concentrations as low as 10 μM (Fig. 7B). These results suggest that caspases play a central role in the regulation of the DNA degradation. We wanted to test if there was a dose of caspase inhibitor (Q-VD-OPh) that allowed HMW DNA fragmentation without the appearance of the DNA ladder. As shown in Fig. 7C, 5 μM Q-VD-OPh efficiently suppressed LMW DNA degradation without affecting HMW DNA fragmentation. Moreover, this dose induced a nearly complete inhibition of caspase-3 cleavage and significantly reduced ICAD processing as depicted in Fig. 7D. In contrast, doses of Q-VD-OPh higher than 5 μM completely abolished all apoptotic DNA degradation. Concentrations lower than 5 μM were sufficient neither to inhibit caspases nor to prevent LMW or HMW degradation (Fig. 7, C and D).

Knockdown of AIF Does Not Prevent HMW DNA Fragmentation in Either Empty-vector or D117E ICAD-transfected Cells—To ascertain the role of endogenous AIF in HMW DNA degradation, we designed specific dsRNAs targeting AIF. As shown in Fig. 8A, two different dsRNA (R1 and R2) efficiently reduced AIF protein levels but had no effect on HMW DNA fragmentation, in either control (pcDNA3) or D117E ICAD-expressing cells (Fig. 8B, upper panel). DNA laddering was used to confirm the effectiveness of the STP treatment (Fig. 8B, lower panel). pcDNA3-transfected cells showed clear oligonucleosomal degradation of DNA, whereas, as expected, cells expressing the D177E mutant did not show LMW DNA fragmentation. We additionally treated cells transfected with R1, R2, or scrambled with 5 μM Q-VD-OPh. In the scrambled dsRNA-transfected cells, Q-VD-OPh did not inhibit HMW DNA degradation induced by STP treatment (Fig. 8C, upper panel). Knock-down of AIF with the two dsRNA failed to modify DNA degradation in response to STP. Of note is that when cells were treated with a low dose of Q-VD-OPh (2.5 μM) the magnitude of the HMW DNA degradation was higher than in the cells without inhibitor. Additionally, AIF knock-down seemed to significantly increase the oligonucleosomal DNA degradation (compare Scr with R1 or R2 in STP-treated conditions in the lower panel of Fig. 8C).

To further explore the implication of AIF in HMW DNA fragmentation, we tried to directly inhibit the AIF function intracellular delivery of a blocking antibody. As shown in Fig. 9A, AIF was released from the mitochondria to the cytosol when the cells were treated with STP. The anti-AIF antibody introduced into the cells was able to interact with the released AIF from the mitochondria, because precipitation of the immune complexes with protein G-agarose completely depleted AIF from the cytosol, thus implying a complete blockade of AIF function (Fig. 9A). In this context, we found that inhibition of apoptotic AIF did not alter the extent of HMW DNA fragments upon STP induction (Fig. 9B). Nonetheless, nuclear morphology was drastically affected by the presence of anti-AIF antibody (Fig. 9C). Treatment of cells with the anti-AIF antibody completely prevented the appearance of the stage I nuclear morphology when apoptosis was induced. All these experiments were carried out in D117E ICAD-overexpressing cells (Fig. 9, A–C) or control cells (data not shown), with comparable results.

AIF Is Necessary for the Achievement of Stage I of Nuclear Apoptotic Morphology—To further confirm the involvement of AIF in the induction of stage I nuclear apoptotic morphology, we used the D177E ICAD-overexpressing cells, which do not show stage II nuclear morphology. Thus, we transfected AIF dsRNAs in control or D177E ICAD-expressing cells. As shown in Fig. 10 (A and B), none of the dsRNA modified the stage II nuclear morphology (type II) after STP treatment in cells transfected with the empty vector. In the D117E ICAD-overexpressing cells, scrambled dsRNA transfection did not impede achieving stage I nuclear apoptotic morphology (type I). However, cells transfected with dsRNA against AIF (either R1 or R2) did not display any of the classic nuclear morphologies associated with apoptosis. In these cases, nuclei shrunk and remained rounded with a highly compacted chromatin (Fig. 10C), described as type III in the graph (Fig. 10B). In addition, cells overexpressing the mutated ICAD avoided DNA ladder formation (Fig. 8B) and sub-G1 peak in flow cytometry (Fig. 10D) after STP treatment. Intriguingly, knockdown of AIF in empty vector-transfected cells renders them 2-fold more susceptible to DNA fragmentation induced by an apoptotic stimuli such as STP (Fig. 10D). In conclusion, all these data
prove that AIF is responsible for stage I chromatin condensation but is not involved in the HMW DNA fragmentation.

**DISCUSSION**

During apoptosis, DNA is cleaved into blunt-end double-stranded fragments. The ICAD-CAD complex is primarily responsible for this process. ICAD has two caspase recognition sites at Asp\(^117\) and Asp\(^224\) (12, 17). After caspase cleavage ICAD is no longer able to inhibit CAD, whose endonuclease activity is therefore unleashed promoting DNA degradation. Contradictory results have been published on the relevance of ICAD processing at these Asp residues in the final nuclear apoptotic outcome. Some groups have reported that ICAD must be cleaved at both sites to lose its CAD-inhibitory activity (35). Here, we demonstrate that overexpression of the D117E ICAD mutant, but not the D224E one, is sufficient to abolish LMW DNA degradation.

The involvement of the ICAD-CAD complex in the HMW degradation of DNA and nuclear condensation is also controversial (25). Previous reports (23) have demonstrated that overexpression of the double ICAD mutant (Asp\(^117\) and Asp\(^224\)) in Jurkat cells resulted in type I chromatin condensation but lacked HMW DNA degradation. Accordingly, thymocytes and embryonic fibroblasts from ICAD-null mice do not show any obvious HMW DNA fragmentation upon treatment with various apoptotic agents (21). The same authors concluded that ICAD may be an integral part of a large nucleosome complex responsible for the chromatin degradation into 50- to 300-kbp size DNA fragments during apoptosis. Because CAD is no longer expressed in the absence of ICAD, it has been assumed that this endonuclease must be responsible for both HMW and LMW apoptotic DNA degradation (25). Results obtained with the CAD\(^−/−\) mice seem to confirm this hypothesis (22). However, some other reports are contradictory. For instance, DT40 chicken cells lacking CAD show HMW DNA fragmentation, but not LMW degradation, upon treatment with different apoptotic inducers (24). In the same line, our results demonstrate that the processing of ICAD at Asp\(^117\) is not necessary for HMW DNA degradation. Therefore, we suggest that ICAD could be involved in the regulation of endonucleases other than CAD, which contribute to the HMW degradation of the DNA during early phases of apoptosis.

During apoptotic DNA degradation, two different 3′ and 5′ DNA ends are generated. Most of the apoptotic nucleases produce 3′-OH/5′-P termini. Such DNA breaks can be recognized by TdT and can be labeled using the TUNEL assay. On the other hand, some reports have demonstrated DNA breaks with 3′P/5′OH ends. In this case, the fragments are undetectable by the TUNEL assay (2). The nature of the DNases concerned in these two types of terminal DNA fragmentation is very different. CAD belongs to the group of endonucleases that generate 3′-OH/5′-P ends (36). Therefore, TdT is able to recognize CAD-mediated DNA fragmentation. Taking into account that CAD has been related to HMW apoptotic DNA degradation, we can presume that this kind of fragmentation is TUNEL-positive. Nevertheless, our work does not agree with this assumption, because HMW DNA fragmentation and absence of detection of TUNEL staining coexist in our experimental paradigm (cells transfected with ICAD mutated at Asp\(^117\)). In agreement with our result, DT40 chicken CAD-deficient cells show HMW DNA degradation, whereas TUNEL staining remains negative (24). In this work, an AIF-associated nuclease is proposed to be the enzyme responsible for the large scale DNA degradation observed. Nonetheless, we cannot formally discard that the absence of TUNEL staining detection could be due to a much smaller number of 3′-OH ends in the HMW DNA fragmentation. Moreover, MEFs from both caspase-3\(^−/−\) or Apaf-1\(^−/−\) mice, which are unable to activate CAD, show HMW DNA degradation upon apoptotic stimuli (27). Other studies support the existence of other nucleases different from CAD that are involved in HMW fragmentation. These endonucleases would form a multienzyme complex to execute chromosomal fragmentation/degradation (28–33). Taken together these data could indicate that AIF might act in a parallel pathway leading to HMW fragmentation. In addition to this, recombinant AIF produces type I chromatin condensation when added to isolated nuclei (27). However, because AIF knock-down failed to prevent the HMW DNA fragmentation in our model, the endonuclease regulated by AIF may not be directly responsible for the initial apoptotic DNA degradation in cells in vivo. It should be noted that AIF translocates to the nucleus even in the presence of D177E ICAD. This observation suggests that AIF has a nuclear function different from DNA fragmentation during the early steps of the cell death program. Our work provides evidence that the knock-down of AIF completely abolished the appearance of type I of nuclear apoptotic morphology without affecting HMW DNA fragmentation. A combination of D177E ICAD overexpression and knock-down of AIF allowed us to separate the DNA degradation, karyorrhexis, and pyknosis processes. Whereas DNA fragmentation and karyorrhexis seem to be mainly controlled by the ICAD-CAD complex, AIF negatively affects the extent of nuclear pyknosis. Moreover, overexpression of D177E ICAD abolishes both generation of nuclear apoptotic bodies and LMW DNA fragmentation. Down-regulation of AIF in cells that constitutively overexpress D177E ICAD induces an extreme chromatin condensation and nuclear compaction upon STP treatment without displaying the initial type I nuclear morphology. Intriguingly, down-regulation of AIF in cells with a functional ICAD-CAD system increased the type II nuclear apoptotic morphology and HMW DNA degradation.

Taken together, our results support those previously obtained with the ICAD\(^−/−\) mice, and suggest that ICAD regulates endonucleases other than CAD. Moreover, AIF does not seem to be implicated in the regulation of HMW DNA fragmentation, although AIF is implicated as being primarily responsible for the stage I nuclear apoptotic morphology. In summary, we propose that 1) ICAD controls both HMW and LMW DNA fragmentation (and stage I and II of nuclear apoptotic phenotype), 2) CAD endonuclease is responsible for the DNA laddering that precedes karyorrhexis (stage II of apoptotic chromatin condensation), and 3) AIF regulates stage I chromatin condensation but not HMW DNA fragmentation.

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The Contribution of Apoptosis-inducing Factor, Caspase-activated DNase, and Inhibitor of Caspase-activated DNase to the Nuclear Phenotype and DNA Degradation during Apoptosis

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