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Apoptotic DNA Fragmentation May Be a Cooperative Activity between Caspase-activated Deoxyribonuclease and the Poly(ADP-ribose) Polymerase-regulated DNAS1L3, an Endoplasmic Reticulum-localized Endonuclease That Translocates to the Nucleus during Apoptosis*[S]

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Background: The mechanism by which apoptotic internucleosomal DNA fragmentation occurs remains unclear.
Results: CAD and DNAS1L3 cooperate to process chromatin degradation during apoptosis. DNAS1L3 achieves such function by translocating from the ER to the nucleus.
Conclusion: The results provide new insight on the mechanism by which chromatin degradation takes place during apoptosis.
Significance: Our results exemplify the complexity of chromatin degradation during apoptosis.

Caspase-activated DNase (CAD) is the most favorable candidate for chromatin degradation during apoptosis. Ca^{2+}-dependent endonucleases are equally important in internucleosomal DNA fragmentation (INDF), including the PARP-1-regulated DNAS1L3. Despite the elaborate work on these endonucleases, the question of whether these enzymes cooperate during INDF was not addressed. Here, we show a lack of correlation between INDF and CAD expression levels and inactivation by cleavage of its inhibitor (ICAD) during apoptosis. The cells that failed to induce INDF accumulated large amounts of 50-kb breaks, which is suggestive of incomplete chromatin processing. Similarly, INDF was blocked by Ca^{2+} chelation without a block in ICAD cleavage or caspase-3 activation, which is consistent with the involvement of CAD in 50-kb DNA fragmentation and its Ca^{2+} independence. However, DNAS1L3 expression in INDF-deficient cells promoted INDF during apoptosis and was blocked by Ca^{2+} chelation. Interestingly, expression of DNAS1L3 in ICAD-deficient cells failed to promote tumor necrosis factor α-induced INDF but required the coexpression of ICAD. These results suggest a cooperative activity between CAD and DNAS1L3 to accomplish INDF. In HT-29 cells, endogenous DNAS1L3 localized to the endoplasmic reticulum (ER) and translocated to the nucleus upon apoptosis induction but prior to INDF manifestation, making it the first reported Ca^{2+}-dependent endonuclease to migrate from the ER to the nucleus. The nuclear accumulation of DNAS1L3, but not its exit out of the ER, required the activity of cysteine and serine proteases. Interestingly, the endonuclease accumulated in the cytosol upon inhibition of serine, but not cysteine, proteases. These results exemplify the complexity of chromatin degradation during apoptosis.

Internucleosomal DNA fragmentation (INDF)[2] is the most common terminal point and predominant indicator of apoptosis. Such fragmentation is thought to be an important step for the disposal of genomic DNA from dying cells to avoid secondary undesirable side effects, such as development of autoimmune diseases and cell transformation. Genomic DNA is first degraded into large 50- to 300-kb fragments, which correspond to single (50-kb) or folded hexameric (300-kb) loop structures released from nuclear scaffold proteins (1). These large DNA breaks are then processed into repeats of 200-bp fragments, generating the DNA ladder apparent by agarose gel electrophoresis.

Although the mechanism by which DNA fragmentation takes place in apoptotic cells has received a great deal of atten-

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[2] The abbreviations used are: INDF, internucleosomal DNA fragmentation; DFF, DNA fragmentation factor; CAD, caspase-activated DNase; ICAD, inhibitor of CAD; PARP, poly(ADP-ribose) polymerase; ER, endoplasmic reticulum; MEF, mouse embryonic fibroblast; TNF, tumor necrosis factor; CHX, cyclohexamide; BAPTA-AM, 1,2-*Bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl-ester)-AM.

tion, a number of questions remain unanswered. Several apoptotic endonucleases have been identified, and a number of studies have focused on their individual roles during apoptotic chromatin degradation. However, the reality is that many of these endonucleases coexist within the same cells. The question becomes how do these endonucleases function during apoptosis? The DNA fragmentation factor (DFF) has hitherto been the most favorable candidate for the degradation of DNA in response to a variety of apoptosis stimuli in a variety of cells (2, 3). The DFF is composed of two subunits of 45- and 40-kDa polypeptides (*i.e.* DFF45/ICAD and DFF40/CAD). The endonuclease activity of this enzyme, which is intrinsic to CAD, is induced after cleavage of ICAD by caspases. ICAD expression is required for the correct folding of CAD after its synthesis (4). Several other endonucleases have been identified that equally contribute to INDF, including NUC70, a cytoplasmic hematopoietic endonuclease (5, 6); DNase X, a muscle cell endonuclease (7); DNase-γ, a divalent-dependent neutral pH and Ca^{2+}-Mg^{2+}-dependent endonuclease (8); Endo G, a mitochondrial endonuclease that is believed to translocate to the nuclei of apoptotic cells (9); as well as apoptosis-inducing factor, a mitochondrial and caspase-independent DNase (10). In a number of studies, we have characterized a chromatin-bound Ca^{2+}/Mg^{2+}-dependent endonuclease termed DNAS1L3, which is also suggested to play a role in apoptotic DNA fragmentation (11–13). The activity of this endonuclease is inhibited by poly-(ADP)ribosylation and thus requires cleavage and inactivation of PARP-1 by caspase-3 for its activity during apoptosis (12, 13).

This study attempted to answer how these endonucleases function during apoptosis, focusing primarily on CAD and DNAS1L3. We show that the INDF process, in our cell system, is mediated by the cooperative activities of two distinct endonucleases with differential requirements for Ca^{2+}, CAD, and DNAS1L3. We also show, for the first time, that DNAS1L3 translocates from the endoplasmic reticulum (ER) to the nucleus early during the apoptotic process.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatments, Transfection, and Immunofluorescence—The prostate cancer DU-145, histiocytic lymphoma U-937, promyelocytic leukemia HL-60, cervical cancer HeLa, Burkitt's lymphoma Daudi, and colon adenocarcinoma HT-29 cell lines (all obtained from the ATCC) as well as mouse embryonic fibroblasts (MEFs) were maintained in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin. MEFs and splenocytes were derived from wild-type or $ICAD^{-/-}$ mice using standard methods as described previously (14). Splenocytes were maintained in RPMI 1640 medium in addition to the supplements mentioned above. Cells were transfected with 5 μg of a vector encoding human DNAS1L3 (12, 13) or empty vector with the use of Mirus TransIT-100 reagent (Mirus Bio LLC, Madison, WI). DNAS1L3 cDNAs were cloned in a pcDNA3.1 mammalian expression vector (Invitrogen). Expression of DNAS1L3 was tested by RT-PCR with specific primers. Apoptosis was induced by exposing cells at 80% confluency to 50 μM VP-16 for varying time periods to achieve 50 or 75% cell death or for the time periods indicated in the figure legends. MEFs were treated with a combination of TNF-α (10 ng/ml) (Roche Diagnostics) and 1 μg/ml cycloheximide (TNF/CHX) for the time period indicated in the figure legends. In some experiments, cells were pretreated with 10 μM of the intracellular Ca^{2+}-chelator 1,2-*Bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl-ester)-AM, 100 μM of the extracellular Ca^{2+}-chelator EGTA, 10 μM of the pan-caspase inhibitor z-VAD-fmk (BD Biosciences), or 10 μM of the serine proteases inhibitor TPCK. Immunofluorescence was conducted essentially as described (15). Briefly, cells were fixed for 20 min in PBS containing 4% formaldehyde. After washing with PBS, cells were permeabilized for 5 min in PBS containing 0.05% Triton X-100. Cells were then incubated overnight at 4 °C with 20 μg/ml of antibodies to DNAS1L3. After a series of washes, cells were incubated with Alexa Fluor 594 secondary antibodies (Invitrogen), followed by an additional series of washes and incubation with ER-Tracker Red (BODIPY® TR glibenclamide) (Invitrogen). Antibody-antigen complexes and the ER were visualized using a Leica DMRA2 fluorescence microscope (Leica, Buffalo Grove, IL). All chemicals were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

RT-PCR and Genomic DNA Preparation and Analysis of DNA Fragmentation—RNA was extracted from cells using standard methods, and cDNA was generated using reverse transcriptase III (Invitrogen). Oligonucleotide primers to specifically amplify a fragment of ICAD, CAD, DNAS1L3, or β-actin were purchased from Integrated DNA Technologies. The specific primers were as follows: ICAD, 5′-TTC CCA AGA GTC CTT TGA GGC AGA-3′ (forward) and 5′-TTG CCC ACC TCC AAA TCC TGA CTA-3′ (reverse); CAD, 5′-ATG CTC CAG AAG CCC AAG-3′ (forward) and 5′-TCA CTG GCG TTT CCG CAC-3′ (reverse); DNAS1L3, 5′-GTT CCA ATC TCC CCA CAC TG-3′ (forward) and 5′-GTC CTC TAA GCA CAA TCC TG-3′ (reverse); and β-actin, 5′-ACG GTG AAA GAT GAC CCA GATC-3′ (forward) and 5′-TAG TTT CAT GGA TGC CAC AGG-3′ (reverse). The amplification program was as follows: 3 min at 95 °C, 30 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C. The cycle numbers were optimized for each primer pair. The PCR products were then incubated for 15 min at 72 °C. The resulting PCR products were subjected to electrophoresis in a 2%-agarose gel and stained with ethidium bromide. DNA was isolated from cells essentially as described (16). DNA samples were applied to a 1.5% agarose gel in Tris borate-EDTA buffer and subjected to electrophoresis at 4 V/cm. Pulse field electrophoresis was performed using the CHEFII system (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. DNA fragments were visualized by staining with ethidium bromide.

Preparation of Total, Cytosolic, Endoplasmic Reticulum (Membrane), and Nuclear Fractions and Immunoblot Analysis—After treatment with the indicated agents, cells were washed with ice-cold PBS followed by centrifugation. For total protein extracts, cell pellets were incubated for 15 min on ice in lysis buffer supplemented with proteases and phosphatase inhibitors as described previously (16). The preparation of cytosolic, membrane, and nuclear fractions was performed using a commercial kit (Thermo Scientific, Rockford, IL) according to the instructions of the manufacturer. A portion (20 μg of protein)

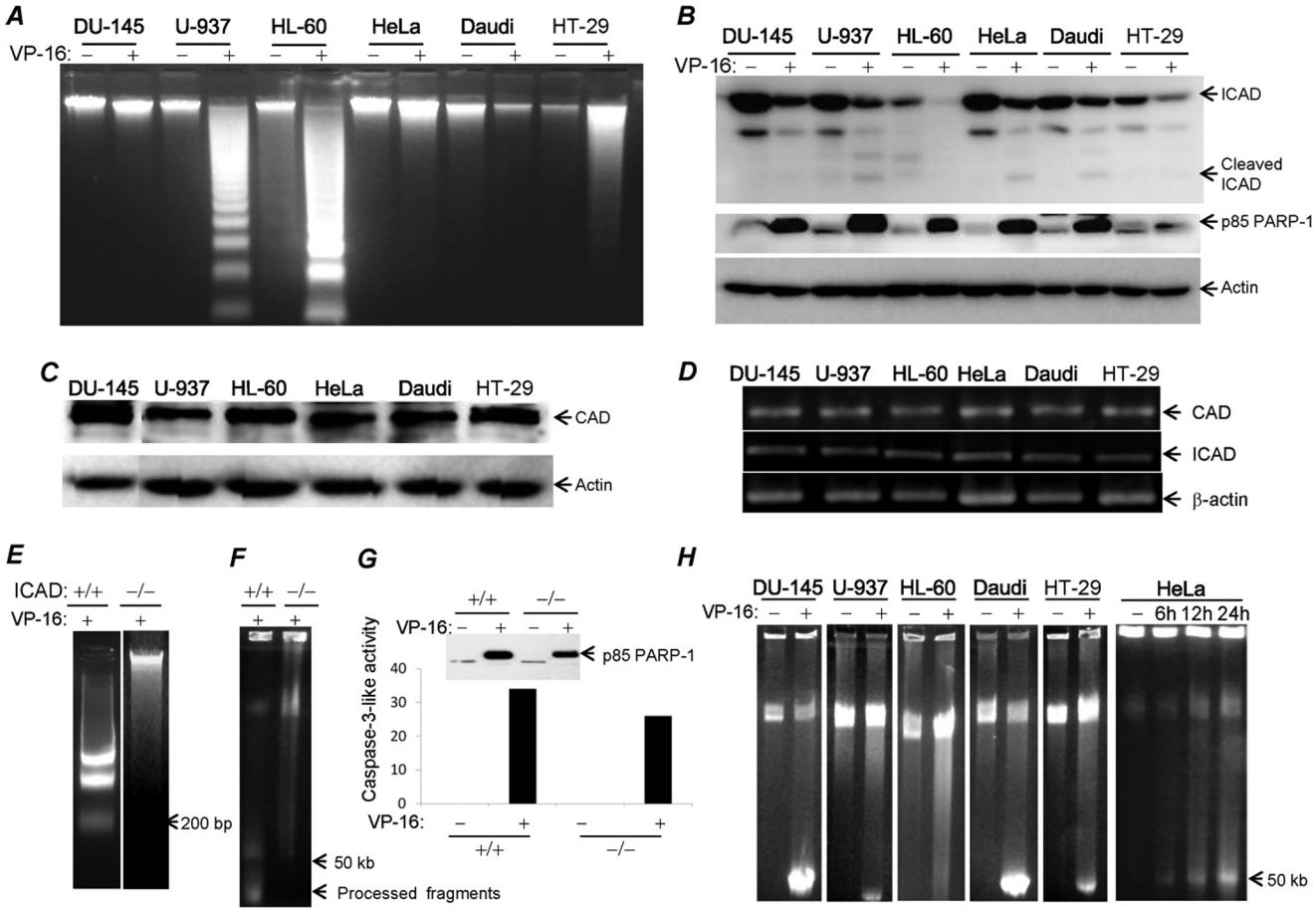

FIGURE 1. **Expression levels of CAD do not correlate with the proficiency of cells to undergo INDF during apoptosis.** *A*, the different cell lines were treated with 50 μM VP-16 for varying durations to achieve 75% cell killing as assessed by calcein-AM (data not shown). Cells were then collected and subjected to genomic DNA preparation. DNA was then subjected to agarose electrophoresis. *B*, cells were treated as in *A* but collected after 50% cell death and subjected to total protein extraction followed by immunoblot analysis with antibodies to human ICAD, cleaved PARP-1 (p85), or actin. *C*, protein extracts from untreated cells were subjected to immunoblot analysis with antibodies to human CAD or actin. *D*, RNA extracted from untreated cells was subjected to cDNA generation followed by PCR analysis with primers specific to human CAD, ICAD, or β-actin. *E–G*, wild-type and ICAD$^{-/-}$ splenocytes were treated with VP-16 for the indicated time period, after which cells were collected and subjected to DNA and protein extractions. DNA was analyzed by conventional (*E*) or pulse field (*F*) agarose electrophoresis. Total proteins (*G*) were assayed for caspase-3 activity or subjected to immunoblot analysis with antibodies to cleaved PARP-1 (*inset*). *H*, the different cell lines were treated for varying durations to achieve 75% cell killing, after which cells were collected for genomic DNA preparation. DNAs were then subjected to pulse field electrophoresis.

of each lysate was then fractionated by SDS-PAGE on a 4–20% gradient gel, and the separated proteins were transferred to a nitrocellulose filter. The filter was stained with Ponceau S to confirm equal loading and transfer of samples and was then probed with antibodies to ICAD (Santa Cruz Biotechnology, Santa Cruz, CA), CAD (Santa Cruz Biotechnology), PARP-1 (BD Biosciences, San Jose, CA), the p85 cleaved fragment of PARP-1 (Promega, Madison, WI), the active form of caspase-3 (Abcam), DNAS1L3 (Santa Cruz Biotechnology), KDEL (Abcam, Cambridge, MA), GAPDH (Abcam), or actin (Santa Cruz Biotechnology). Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (PerkinElmer Life Sciences).

Assay of Caspase-3 Activity—Caspase-3 activity was measured essentially as described (16). In brief, cell extracts (25 μg of protein) were incubated for 10 min at 37 °C with 40 μM DEVD-aminomethylcoumarin peptide substrate (Sigma-Aldrich) in a total volume of 200 μl. The fluorescence of free aminomethylcoumarin, generated as a result of cleavage of the aspartate-aminomethylcoumarin bond, was monitored continuously over 10 min with a CytoFluor 4000 fluorometer at excitation and emission wavelengths of 360 and 460 nm, respectively. The emission from each well was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

RESULTS AND DISCUSSION

Lack of Correlation between Internucleosomal DNA Fragmentation and Expression Levels of CAD and Cleavage of ICAD in a Variety Of Human Cell Lines in Response to Etoposide Treatment—We investigated the relationship between the induction of apoptotic INDF and CAD by examining whether the ability of a number of cell lines to undergo INDF correlated with the expression levels of the apoptotic DNase. To this end, cells were treated with the chemotherapeutic drug and apoptosis inducer etoposide (also termed VP-16) to achieve 75% cell death as assessed by calcein-AM staining (data not shown), after which genomic DNA was extracted and subjected to agarose electrophoresis. Fig. 1*A* shows that although INDF was observed in HL-60, U-937, and HT-29 cells in response to

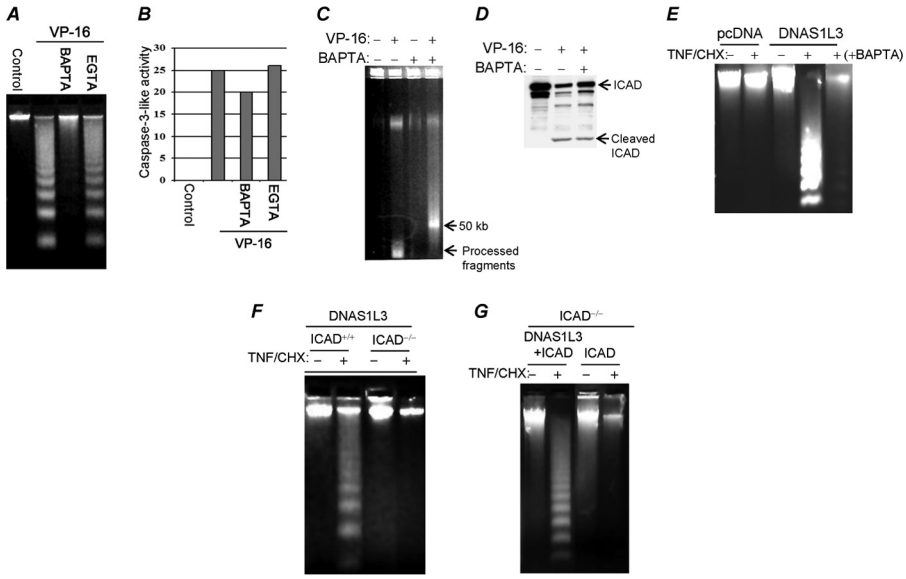

FIGURE 2. **Differential requirements for calcium between internucleosomal and 50-kb DNA fragmentation and cooperative activity between CAD and DNAS1L3 during apoptotic to process chromatin into internucleosomal fragments.** *A*, U-937 cells were treated with 50 μM VP-16 for 8 h in the presence of absence of 10 μM BAPTA-AM or 100 μM EGTA. Cells were collected and processed for genomic DNA preparation followed by agarose electrophoresis. *B*, a portion of the collected cells were subjected to protein extraction. Protein extracts were assayed for caspase-3-like activity using the fluorescent caspase-3 substrate DEVD-AMC. Data are expressed in arbitrary units and are means of duplicates from a representative experiment. *C*, U-937 cells were treated for 8 h with VP16 in the presence or absence of 10 μM BAPTA-AM. Cells were then collected and subjected to genomic DNA preparation for pulse field electrophoresis. A portion of the collected cells were subjected to protein extraction followed by immunoblot analysis with antibodies to ICAD (*D*). *E*, MEFs were transfected with the DNAS1L3-expressing vector or control (*pcDNA*) plasmid (supplemental Fig. S1). Cells were treated with the TNF/CHX combination for 12 h in the presence or absence of BAPTA-AM. Cells were collected for genomic DNA preparation. DNA was then subjected to conventional agarose electrophoresis. *F*, wild-type and ICAD$^{-/-}$ MEFs were transfected with the DNAS1L3 encoding plasmid. Expression of DNAS1L3 was assessed by RT-PCR as indicated above. Cells were then treated with the TNF/CHX combination for 12 h, after which genomic DNA was extracted and subjected to conventional agarose electrophoresis. *G*, ICAD$^{-/-}$ MEFs were transfected with the DNAS1L3-encoding plasmid and/or a plasmid encoding for the human ICAD. Expression of DNAS1L3 and ICAD was assessed by RT-PCR (supplemental Fig. S2). Cells were then treated with TNF/CHX for 12 h, after which genomic DNA was extracted and subjected to conventional agarose electrophoresis.

VP-16, little to no INDF was detected in HeLa, DU-145, or Daudi cells. INDF was most pronounced in HL-60 and U-937 cells and was rather moderate in HT-29 cells. These differences were not linked to major changes in inactivation of the inhibitory subunit ICAD by cleavage or to activation of caspases as assessed by PARP-1 cleavage (Fig. 1*B*). Interestingly, the levels of CAD at the protein (Fig. 1*C*) or mRNA (*D*) levels in these cell lines did not differ sufficiently to explain the differences in the differential display of INDF. Daudi and U-937 cells, for instance, expressed similar levels of CAD as well as cleavage of ICAD and PARP-1. However, although U-937 cells displayed extensive INDF, Daudi cells were incapable of inducing DNA fragmentation in response to the chemotherapeutic drug. Thus, these results revealed a lack of correlation between induction of INDF and the DNA fragmentation complex (CAD/ICAD) and its activation, exemplifying the complexity of chromatin fragmentation, which may involve more than the CAD/ICAD system.

The above results do not, however, suggest that the ICAD/CAD system is dispensable for INDF. Indeed, as reported previously by us and others (14, 17), ICAD expression is required for cleavage of chromatin in VP-16-treated splenocytes both at the internucleosomal level and at matrix-associated regions that generate 200-bp repeats (Fig. 1*E*) and large 50-kb fragments (*F*), respectively. The lack of INDF and large 50-kb chromatin breaks in VP-16-treated ICAD$^{-/-}$ splenocytes was not a result of a drastic reduction in caspase-3 activity and subsequent cleavage of PARP-1 (Fig. 1*G*). Overall, these results suggest that the ICAD/CAD system is required but not sufficient for the INDF process.

Given that the ICAD/CAD system appeared to be intact in all tested cell lines, we surmise that the cells that failed to display INDF may still induce cleavage of their chromatin into 50-kb breaks. Fig. 1*H* shows that VP-16 induced the generation of 50-kb breaks in all cell lines except HL-60. Interestingly, the cells that failed to induce INDF displayed large amounts of 50-kb breaks, which appear to accumulate over time as shown for VP-16-treated HeLa cells. The failure of HL-60 cells to display 50-kb DNA breaks may be associated with a more efficient processing of these breaks into internucleosomal 200-bp DNA repeats. Indeed, analysis of these cells after a shorter exposure to VP-16 revealed the generation of such breaks (data not shown). These results suggest that the 50-kb DNA breaks accumulate because of a failure in further processing of these fragments into 200-bp internucleosomal repeats.

Differential Requirements for Calcium between Internucleosomal and 50-kb DNA Fragmentation—In mammalian cells, INDF has always been regarded as an intracellular calcium-dependent process (3, 18). Indeed, VP-16-induced INDF in U-937 cells was severely reduced by the cell-permeable Ca^{2+} chelator BAPTA-AM but not by the cell-impermeable chelator EGTA (Fig. 2*A*). The marked blockade of INDF achieved by BAPTA-AM was not accompanied by a substantial reduction in caspase-3 activity (Fig. 2*B*) or cleavage of caspase-3 into its active form (data not shown), suggesting a direct inhibition of the process. The effects of BAPTA-AM on INDF and caspase activation are consistent with published reports (19). To further separate the INDF process from cleavage of chromatin into 50-kb fragments, we tested the effect of BAPTA on the latter

process in U-937 cells. U-937 cells were chosen for this experiment because of their ability to efficiently process the 50-kb breaks into internucleosomal fragments (Fig. 2*C*). Such efficient processing of chromatin was blocked by BAPTA-AM treatment, resulting in a drastic increase in the levels of 50-kb DNA fragments in VP-16-treated U-937 cells (Fig. 2*C*). Interestingly, BAPTA-AM had very little effect on the cleavage of ICAD (Fig. 2*D*), suggesting a fully functional CAD. These results are consistent with those reported by Zhang *et al.* (20), showing that BAPTA-AM only inhibited INDF but neither caspase-3 activation nor cleavage of ICAD. Altogether, these results clearly separate internucleosomal fragmentation from cleavage of chromatin into breaks during apoptosis.

Because CAD endonuclease activity is not dependent on Ca^{2+} (21) and its expression is required for the generation of large 50-kb breaks (17, 22), we hypothesize that CAD is solely responsible for the processing of genomic DNA into 50-kb breaks and that further processing of these breaks into 200-bp repeats is mediated, in a cooperative manner, by a different endonuclease that is dependent on intracellular Ca^{2+}. Furthermore, it appears that the cleavage of chromatin into 50-kb DNA breaks may be necessary for INDF. Recently, for instance, we characterized the role of the human chromatin-bound Ca^{2+}- and Mg^{2+}-dependent endonuclease, DNAS1L3, which is inhibited by the DNA repair enzyme PARP-1 and is differentially expressed in a number of cell lines and tissues (11–13). For example, althoughU-937 cells, used in the latter experiment, highly express DNAS1L3, osteosarcoma cells do not (12). Similarly, MEFs do not express the mouse homolog of the endonuclease (supplemental Fig. S1) and, as previously reported (14, 22), do not exhibit INDF in response to treatment with a combination of 10 ng/ml TNF-α and 1 μg/ml cyclohexamide (Fig. 2*E*). Transfection of MEFs with a plasmid encoding human DNAS1L3 conferred the capability to undergo Ca^{2+}-dependent INDF upon TNF/CHX treatment (Fig. 2*E*). The TNF/CHX combination was used to induce apoptosis because of the better sensitivity of MEFs to such a stimulus compared with VP-16. Intracellular Ca^{2+} chelation by BAPTA-AM treatment almost completely blocked TNF/CHX-induced INDF in DNAS1L3-expressing MEFs. These results are consistent with our earlier reports using HeLa cells and human osteosarcoma cells (12, 13, 23) and clearly suggest that DNAS1L3 may be one of the Ca^{2+}-dependent endonucleases responsible for the processing of chromatin into internucleosomal fragments.

Cooperative Activity between CAD and DNAS1L3 to Process Chromatin into Internucleosomal Fragments during Apoptosis—To test our hypothesis of the cooperative activities of both CAD and DNAS1L3 in the process of INDF, we employed MEFs derived from wild-type and $ICAD^{-/-}$ mice. As mentioned above, these cells do not exhibit INDF in response to TNF/CHX, which makes them an excellent model in which to test our hypothesis. Exposure of wild-type cells, transiently transfected with the DNAS1L3 plasmid, to TNF/CHX resulted in marked INDF (Fig. 2*F*). As shown above (Fig. 2*E*), this effect was blocked by inclusion of BAPTA-AM in the culture medium. To determine whether ICAD expression and the concomitant expression and functional integrity of CAD are required for DNAS1L3-mediated INDF, $ICAD^{-/-}$ fibroblasts were transiently transfected with the DNAS1L3-expressing plasmid prior to treatment with TNF/CHX. Fig. 2*F* shows that DNAS1L3 expression failed to confer the ability to induce INDF upon TNF/CHX treatment to $ICAD^{-/-}$ MEFs, suggesting a requirement for ICAD expression and CAD endonuclease activity for this process to take place. To confirm the requirement for the ICAD/CAD system, $ICAD^{-/-}$ MEFs were transfected with either both DNAS1L3 and ICAD expression plasmids or with ICAD plasmid alone. Although reestablishment of ICAD expression in $ICAD^{-/-}$ MEFs did not result in INDF, reestablishment of ICAD expression in conjunction with DNAS1L3 expression triggered INDF upon TNF/CHX treatment (Fig. 2*G*). Altogether, these results suggest, for the first time, a cooperative activity between CAD and DNAS1L3 during apoptotic chromatin fragmentation.

The fact that $ICAD^{-/-}$ cells do not display INDF in response to a variety of apoptotic inducers, including those that are known to cause Ca^{2+}-mediated degradation of DNA such as VP-16, TNF-α, or dexamethasone (14, 24), suggests three possibilities: 1) CAD is responsible for all DNA fragmentation processes in all cell types and tissues; 2) CAD is responsible for some but not all DNA fragmentation processes, whereas other endonucleases are responsible for such fragmentation independent of CAD; and 3) CAD, in collaboration with other endonucleases that might differ according to cell types and tissues, is responsible for the DNA fragmentation process. The first possibility is unlikely because it discounts the role of a variety of endonucleases that have been found to be involved in INDF. In addition, this option would not explain the sensitivity of INDF to Ca^{2+} chelators in a variety of cells in light of the fact that CAD does not require Ca^{2+} for activity (21). The second point is also unlikely because a variety of inducers of apoptosis have been shown to be unable to cause both 50-kb and internucleosomal DNA fragmentation in $ICAD^{-/-}$ cells (14, 17). The third possibility may constitute the most plausible hypothesis in that CAD is required for most DNA fragmentation processes but can collaborate with other endonucleases to complete this cell process. This hypothesis is corroborated by the evidence that Ca^{2+} is required only for INDF but promotes the accumulation of 50-kb DNA fragments. This notion is also supported by the fact that a number of cell types such as macrophages, thymocytes, and splenocytes express DNAS1L3 but fail to undergo a Ca^{2+}-dependent INDF. Given that the cooperation between CAD and DNAS1L3 or any other Ca^{2+}-dependent endonuclease may require caspase activation and cleavage of ICAD, INDF may also occur during caspase-independent cell death, suggesting that some endonucleases may function autonomously. Indeed, we have reported previously that upon traumatic brain injury, INDF took place despite a deletion in ICAD (24). Further, in an animal model of liver failure upon acetaminophen overdose, INDF was also observed in livers of $ICAD^{-/-}$ mice[3] despite the fact that, in isolated splenocytes, the ICAD/CAD system is required for acetaminophen-induced INDF *in vitro* (25). These results exemplify the complexity of chromatin

[3] Y. Errami, A. S. Naura, H. Kim, J. Ju, Y. Suzuki, A. H. El-Bahrawy, M. A. Ghonin, R. A. Hemeida, M. S. Mansy, J. Zhang, M. Xu, M. E. Smulson, H. Brim, and A. H. Boulares, unpublished observations.

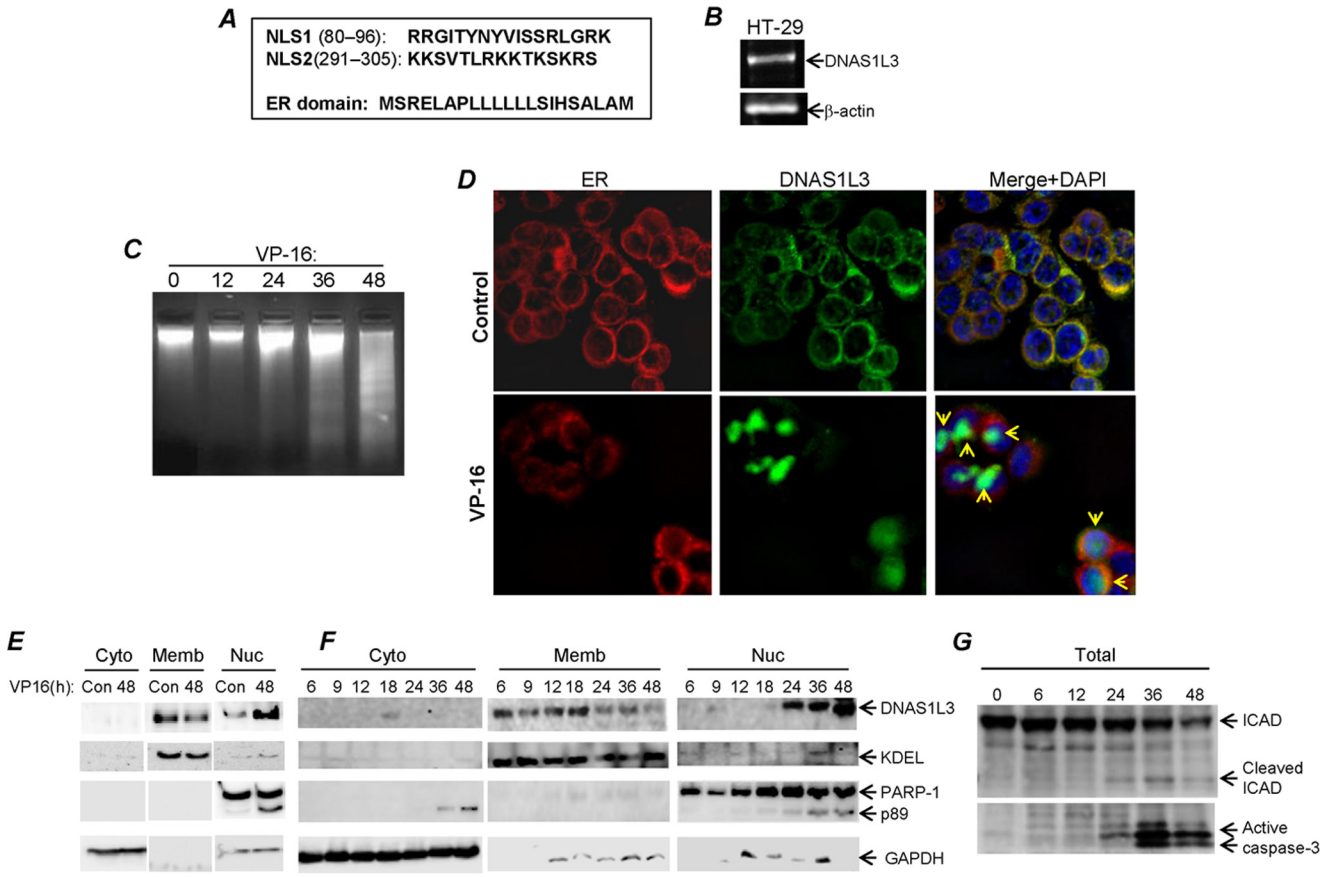

FIGURE 3. **Translocation of DNAS1L3 from the ER to the nuclei of HT-29 cells during apoptosis.** *A*, predicted nuclear localization signals (*NLS1* and *NLS2*) and ER-targeting peptide within the human DNAS1L3 protein sequence. *B*, untreated HT-29 cells were subjected to RNA extraction followed by RT-PCR with primers to human DNAS1L3 or β-actin. *C*, HT-29 cells were treated with 50 μM VP-16 for the indicated time intervals, after which genomic DNA was prepared and subjected to agarose electrophoresis. *D*, HT-29 cells were treated with VP-16 for 12 h, after which cells were fixed and subjected to immunofluorescence with antibodies to human DNAS1L3. Cells were also stained with ER tracker and DAPI. *E*, HT-29 cells were treated with VP-16 for 48 h, after which cytosolic (*Cyto*), membrane (*Memb*), and nuclear (*Nuc*) fractions were prepared. Proteins from these fractions were then subjected to immunoblot analysis with antibodies to DNAS1L3, full-length PARP-1, KDEL, or GAPDH. *F*, HT-29 cells were treated with VP-16 for the indicated time intervals, after which cell fractions were prepared and subjected to immunoblot analysis with the aforementioned antibodies. *G*, HT-29 cells were treated with VP-16 for the indicated time intervals, after which total protein extracts were prepared and subjected to immunoblot analysis with antibodies to ICAD or the active peptide of caspase-3.

degradation and that cells and tissues harbor a number of enzymes and systems that secure the efficient degradation of chromatin.

DNAS1L3 Is a Ca^{2+}-dependent Endonuclease That Localizes to the Endoplasmic Reticulum and Translocates to the Nucleus upon Induction of Apoptosis by VP-16—It is noteworthy that several additional Ca^{2+}-dependent endonucleases have been suggested to participate in apoptotic chromatin degradation (3). We focused on DNAS1L3 in this study, given its regulation by PARP-1 and our long interest in the DNA repair enzyme. The direct involvement of DNAS1L3 in apoptotic cell death remains unsettled primarily because of the discrepancies on its cellular localization and potential secretion, rendering it potentially irrelevant to apoptotic intracellular chromatin degradation. Indeed, DNAS1L3 has been reported by us and others to be exclusively localized in the nucleus on the basis of ectopic expression of the tagged protein (13, 26). This is supported by the fact that the protein harbors two putative nuclear localization signals (Fig. 3*A*). However, the endonuclease was also reported to localize to the ER (Fig. 3*A*) (27) or to be simply secreted, functioning in a manner similar to that of DNase 1 with no major intracellular function (28). Interestingly, DNase X with some homology to DNAS1L3 was reported to localize to the ER, but its activity was similar to that of DNase 1 (29). The discrepancies may be due to the differential expression and function of DNAS1L3 that is specific to different cell types and tissues. On such a basis, we found that the ectopic expression of DNAS1L3 in mouse skin fibroblasts renders the cells extremely sensitive to TNF/CHX, causing them to die rapidly (data not shown). Furthermore, human Jurkat T cells, which do not express DNAS1L3 (25), were equally sensitive to DNAS1L3 even without any treatment, as the mere expression of the endonuclease triggered cell death and abnormal aggregation of the cells (data not shown). These observations further suggest that the function of DNAS1L3 may be controlled in a cell type-specific manner. However, in the cell lines tested in this study, expression of DNAS1L3 correlated with the ability of the cells to undergo INDF in response to VP-16 treatment (supplemental Fig. S3).

To examine the subcellular localization of DNAS1L3 and its behavior during apoptosis, we utilized the colon cancer cell line HT-29, which expresses DNAS1L3 (Fig. 3*B*), because of its moderate sensitivity to VP-16 and relatively slow induction of INDF (*C*). Immunofluorescence analysis revealed that in

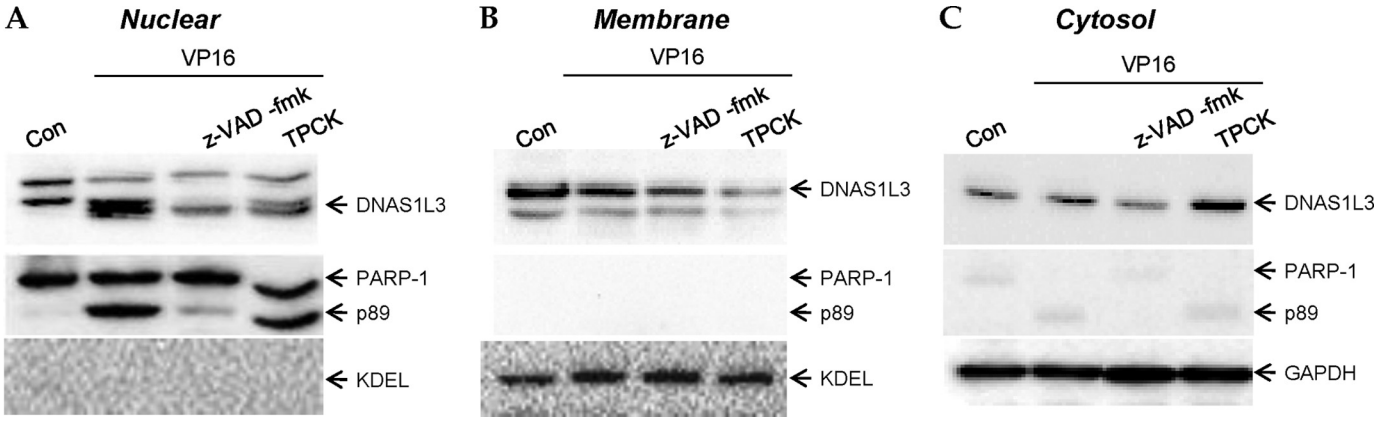

FIGURE 4. **Effect of inhibiting cysteine or serine proteases on translocation of DNAS1L3 from the ER to the nuclei of VP-16-treated HT-29 cells.** Cells were treated with 50 μM VP-16 for 48 h, after which cytosolic, membrane, and nuclear fractions were prepared. Nuclear (*A*), membrane (*B*), and cytosolic (*C*) fractions were then subjected to immunoblot analysis with the indicated antibodies.

HT-29 cells, DNAS1L3 primarily localized to the ER (Fig. 3*D*), which is consistent with the report by Napirei *et al.* using GFP-tagged DNAS1L3 (27). Remarkably, upon induction of apoptosis by VP-16, DNAS1L3 translocated to the nuclei of treated cells (Fig. 3*D*). The localization of DNAS1L3 to the ER in control cells and its translocation to the nucleus during VP-16-induced apoptosis was verified by cell fractionation followed by immunoblot analysis (Fig. 3*E*). The purity of the ER, nuclear, and cytosolic fractions was assessed by immunoblot analysis with antibodies to KDEL, PARP-1, and GAPDH, respectively. Fig. 3*F* shows a time-dependent translocation of DNAS1L3 from the ER to the nucleus upon VP-16 treatment. Interestingly, VP-16-induced nuclear translocation of DNAS1L3 occurred concomitantly with PARP-1 (Fig. 3*F*) and ICAD (*G*, *upper panel*) cleavage and INDF (*C*). PARP-1 and ICAD cleavage also reflected timely caspase-3 activation as assessed by immunoblot analysis with antibodies to the active peptide of the cysteine protease (Fig. 3*G*, *lower panel*)

It is noteworthy that PARP-1 cleavage is required for DNAS1L3 endonuclease activity (12, 13). DNAS1L3, to the best of our knowledge, is the first Ca^{2+}-dependent endonuclease that translocates from the ER to the nucleus during apoptosis. Therefore, DNAS1L3 constitutes a new endonuclease that translocates to the nucleus during apoptosis, in addition to the already known endoG and apoptosis-inducing factor (18). Similarly to the latter endonucleases, DNAS1L3 harbors a nuclear localization signal located at its C terminus (26, 30), which is highly similar to that found in SV40 proteins (31). Such signal peptide appears to be effective only after induction of apoptosis. Altogether, these results suggest that a potential cooperative activity exists between CAD and DNAS1L3 for the processing of chromatin during apoptosis.

Efficient Translocation of DNAS1L3 from the ER to the Nucleus Requires the Activity of Cysteine and Serine Proteases—Proteolytic activity, including those of serine and cysteine proteases, has been shown to be pivotal for the manifestation of INDF during apoptosis (6, 32, 33). However, although cysteine proteases such as caspase-3 have been associated with both INDF and cleavage of DNA into large 50-kb fragments, the role of serine proteases has been linked chiefly to INDF (33, 34). Indeed, treatment of cells with serine chymotrypsin-like protease inhibitors such as TPCK or TLCK blocks only INDF during apoptosis (35). Accordingly, we examined whether the activation of caspases or serine proteases were critical for the translocation of DNAS1L3 from the ER to the nucleus during VP-16-induced apoptosis. Fig. 4*A* shows that induction of DNAS1L3 nuclear translocation in VP-16-treated HT29 cells was substantially reduced by treatment with the cell-permeable pan-caspase inhibitor z-VAD-fmk. The action of z-VAD-fmk on caspase activity was assessed by the blockade of PARP-1 (Fig. 4*A*, *center panel*). These results suggest that activation of caspases was required for the nuclear translocation of the endonuclease. The exclusion of membrane contamination was confirmed by the absence of the ER marker KDEL in the nuclear preparations (Fig. 4*A*, *bottom panel*). Interestingly, DNAS1L3 does not harbor a cleavage site for caspases. Hence, it is unlikely that caspases were directly responsible for the nuclear translocation of the endonuclease. Inhibition of serine proteases by TPCK was also effective in blocking DNAS1L3 nuclear translation in VP-16-treated cells. However, TPCK did not inhibit caspase activation in our experimental system, as indicated by the persistent cleavage of PARP-1 (Fig. 4*A*, *center panel*). These results suggest that caspase activation is necessary but not sufficient for VP-16-induced DNAS1L3 nuclear translocation. Recently, O'Connell *et al.* (32) reported that activation of chymotrypsin-like serine proteases during apoptosis may depend on caspase activity. Accordingly, it is conceivable that the blockade of VP-16-induced DNAS1L3 nuclear translocation by caspase inhibition was related to the requirement of these enzymes for the activation of serine proteases. Although it is unclear how serine proteases participate in nuclear translocation of DNAS1L3 during apoptosis, it is likely that a potential processing by these proteases may prompt an egression of the endonuclease out of the ER and its subsequent nuclear translocation. This becomes even more plausible when one considers the existence of at least two putative serine protease cleavage sites (Ser-14 and Ser-17) at the N terminus of human DNAS1L3. A closer examination of the blot displayed in Fig. 4*A* suggests a potential proteolytic processing, as revealed by the faster migrating band. More interestingly, the two cleavage sites are situated within the first 20 amino acid residues of the N terminus constituting the ER-targeting motif. Accordingly, it is

rather plausible to speculate that cleavage at this site(s) may be the determining factor for DNAS1L3 nuclear translocation. This speculation is supported by the finding reported by Napirei *el al*. (27) eloquently showing that deletion of the 25 amino acid-long N-terminal peptide of the mouse DNAS1L3 resulted in a nuclear accumulation of the mutant protein with some cytosolic distribution. Although the much slower-migrating band that appears in the nuclear fractions may constitute a nonspecific signal, the possibility that it is a precursor or a post-translationally modified version of DNAS1L3 cannot be ruled out. Obviously, more experimentation is needed to unequivocally reach such a conclusion.

It is rather important to note that the reduction in nuclear levels of DNAS1L3 upon VP-16 treatment achieved by inhibition of cysteine and serine proteases was not accompanied by a sequestration of the endonuclease in the ER (Fig. 4*B*). Rather, and only upon inhibition of serine proteases by TPCK, the endonuclease appeared to accumulate in the cytosolic fractions (Fig. 4*C*). These results suggest that protease activity is not responsible for the exit of DNAS1L3 from the ER but is important for its accumulation in the nucleus during apoptosis. At this point, however, the location of the endonuclease when caspases are inhibited is uncertain. It is conceivable that the endonuclease may be secreted out of the cell. If so, the mechanism and purpose for such a translocation are unclear and merit additional investigations.

Altogether, our results show for the first time that INDF is a cooperative activity between CAD and a Ca^{2+}-dependent endonuclease such as DNAS1L3. Such cooperation may also exist with other Ca^{2+}-dependent endonucleases that have been suggested to play a role in apoptotic INDF. Additionally, these results show for the first time the translocation of DNAS1L3 from the ER to the nucleus during apoptosis. Hence, DNAS1L3 represents a new endonuclease that translocates to the nucleus during apoptosis.

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