Caspase-3 Is the Primary Activator of Apoptotic DNA Fragmentation via DNA Fragmentation Factor-45/Inhibitor of Caspase-activated DNase Inactivation*

(Received for publication, July 14, 1999)

Beni B. Wolf‡§, Martin Schuler†, Fernando Echeverri, and Douglas R. Green¶

From the Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121 and the Department of Internal Medicine, University of California, San Diego, California 92103

Caspase-3 initiates apoptotic DNA fragmentation by proteolytically inactivating DFF45 (DNA fragmentation factor-45) (inhibitor of caspase-activated DNase), which releases active DFF40/CAD (caspase-activated DNase), the inhibitor's associated endonuclease. Here, we examined whether other apoptotic proteases initiated DNA fragmentation via DFF45/CAD inactivation. In a cell-free assay, caspases-3, -6, -7, -8, and -9, and granzyme B initiated benzoxyloxy carbonyl-Asp-Glu-Val-Asp (DEVD)-cleaving caspase activity, DFF45/CAD inactivation, and DNA fragmentation, but calpain and cathepsin D failed to initiate these events. Strikingly, only the DEVD cleaving caspases, caspase-3 and caspase-7, inactivated DFF45/CAD and promoted DNA fragmentation in an in vitro DFF40/CAD assay, suggesting that granzyme B, caspase-6, and caspase-8 promote DFF45/CAD inactivation and DNA fragmentation indirectly by activating caspase-3 and/or caspase-7. In vitro, however, caspase-3 inactivated DFF45/CAD and promoted DNA fragmentation more effectively than caspase-7 and endogenous levels of caspase-7 failed to inactivate DFF45/CAD in caspase-3 null MCF7 cells and extracts. Together, these data suggest that caspase-3 is the primary inactivator of DFF45/CAD and therefore the primary activator of apoptotic DNA fragmentation.

Caspase proteinases drive apoptotic signaling and execution by cleaving critical cellular proteins solely after aspartate residues (reviewed in Ref. 1). Caspases exist as latent zymogens, which are activated by cleavage at the N-terminal caspase site (Asp117) is both necessary and sufficient for caspase-8 autoactivation. Once activated, initiator caspases in turn activate the executioner caspases, caspases-3, -6, and -7. The active executioners promote apoptosis by cleaving cellular substrates that induce the morphological and biochemical features of apoptosis (1).

DFF45 (DNA fragmentation factor-45/ICAD) is a caspase-3 substrate that must be cleaved before apoptotic internucleosomal DNA fragmentation can proceed (2, 3). DFF45/CAD exists as a complex with a 40-kDa endonuclease termed DFF40/caspase-activated nuclease/CAD (caspase-activated DNase) that promotes apoptotic DNA fragmentation (3–5). DFF45/CAD serves as both a specific inhibitor of DFF40/CAD and as a molecular chaperone to ensure proper folding of the endonuclease (3, 4, 6, 7). DFF40/CAD remains inactive while bound to DFF45/CAD; however, caspase-3 cleaves DFF45/CAD at two sites, thereby releasing the endonuclease, which then cleaves DNA. DFF45/CAD cleavage at the N-terminal caspase site (Asp117) is both necessary and sufficient for DFF40/CAD activation; however, DFF45/CAD cleavage only at the C-terminal caspase site (Asp152), retains DFF40/CAD inhibitory activity (6). In contrast to DFF40/CAD, a mitochondrial protein termed apoptosis inducing factor, may induce high molecular weight DNA fragmentation in a caspase-independent manner (8).

Besides caspase-3, caspases-6, -7, -8, -9, the cytotoxic T cell proteinase granzyme B, the calcium-dependent proteinase calpain, and the lysosomal proteinase cathepsin D may function during apoptosis (9–12). These proteinases have all been suggested to participate in apoptotic DNA fragmentation; however, the mechanism(s) by which they promote DNA fragmentation remain unclear. Here, we examined whether these proteinases induced DNA fragmentation by inactivating DFF45/CAD. We find that caspase-3 and caspase-7 are the only direct inactivators of DFF45/CAD. However, endogenous levels of caspase-7 failed to inactivate the inhibitor or promote DNA fragmentation in an intact cell, suggesting that caspase-3 is the primary regulator of apoptotic DNA fragmentation via proteolysis of DFF45/CAD.

EXPERIMENTAL PROCEDURES

Materials—Benzyloxy carbonyl-Asp-Glu-Val-Asp-amino-4-trifluoromethyl-coumarin (DEVD-AFC) and N-acetyl-Leu-Leu-Val-Asp-AFC (LEHD-AFC) were from Enzyme Systems Products. Benzoyloxy carbonyl-Val-Asp-fluoromethyl ketone (ZVAD-fmk) was from Bachem. Antibodies against the following proteins were purchased commer-

* This is publication 325 from the La Jolla Institute for Allergy and Immunology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Mentored Clinical Scientist Development Award CA75268-01. To whom correspondence should be addressed: La Jolla Institute for Allergy and Immunology, 10355 Science Center Dr., San Diego, CA 92121. Tel.: 858-558-3500; Fax: 858-558-3525; E-mail: 102251.1444@compuserve.com.

‡ Recipient of a postdoctoral fellowship from the Dr. Mildred Scheel Stiftung für Krebsforschung.

¶ Supported by National Institutes of Health Grants CA69831 and AI46746.

The abbreviations used are: APAF-1, apoptotic proteinase activating factor-1; DFF40, DNA fragmentation factor-40; DFF45, DNA fragmentation factor-45; CAD, caspase-activated DNase; ICAD, inhibitor of caspase-activated DNase; DEVD-AFC, benzyloxy carbonyl-Asp-Glu-Val-Asp-amino-4-trifluoro methyl-coumarin; LEHD-AFC, N-acetyl-Leu-Leu-Val-Asp-AFC; ZVAD-fmk, benzyloxy carbonyl-Val-Asp-4-fluoromethyl ketone; GST, glutathione S-transferase; PIPES, 1,4-piperazine diethane sulfonic acid; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

This paper is available on line at http://www.jbc.org

Vol. 274, No. 43, Issue of October 22, pp. 30651–30656, 1999
Printed in U.S.A.
cally: caspases-3, -6, -7, -8 (Pharmingen) and actin (ICN Biomedicals). Dr. Xiaodong Wang (University of Texas Southwestern Medical Center) provided anti-DFF45/ICAD antibodies (2). Antibodies against APAF-1 and caspase-9 have been described previously (13).

RESULTS

Caspases, Granzyme B, and Cytochrome C plus dATP Promote DFF45/ICAD Cleavage and DNA Fragmentation in a Cell-free Assay—We first assessed whether various apoptotic proteinases could promote DNA fragmentation in a cell-free assay. Proteinases were incubated with cytosolic extracts from Jurkat T cells and subsequently examined for DNase activity using rat liver nuclei as a substrate. We also examined whether the proteinase-treated extracts cleaved DFF45/ICAD and the fluorogenic caspase substrate DEVD-afc.

As shown in Fig. 1, all proteinases examined promoted DFF45/ICAD cleavage as demonstrated by Western blotting. However, not all cleavage events inactivated the inhibitor. Caspases-3, -6, and -7 produced DFF45/ICAD cleavage products of ~23 and ~12 kDa. Note that the ~23-kDa band likely represents intermediate cleavage products resulting from cleavage at one of the two caspase cleavage sites; whereas, the broad band at ~12 kDa probably represents a composite of the three DFF45/ICAD fragments observed when the inhibitor is cleaved at both caspase sites (2).

The control lane shows a small amount of the intermediate cleavage products, likely due to background caspase activity. With granzyme B and cytochrome c plus dATP, only the ~12-kDa fragments were observed, suggesting cleavage had occurred at both caspase cleavage sites. By contrast, calpain prompted loss of DFF45/ICAD immunoreactivity and caspase D produced a ~19-kDa DFF45/ICAD fragment. Strikingly, only the caspases, granzyme B, and cytochrome c plus dATP elicited DEVD cleaving caspase activity (Fig. 1A), DFF45/ICAD inactivation, and DNA fragmentation (Fig. 1C). As in apoptotic cells (2), DNA fragmentation correlated with the production of ~12-kDa DFF45/ICAD fragments. Although calpain and caspase D induced DFF45/ICAD cleavage, they failed to inactivate the inhibitor, induce DNA fragmentation, or activate DEVD cleaving caspases. This suggests that DEVD cleaving caspase activity is not necessary for DFF45/ICAD cleavage, but is required for inactivation of the inhibitor and DNA fragmentation.

We also examined whether the initiator caspases functioned in the cell-free assay. Caspase-8 (50 nM) induced DEVDAse activity, DFF45/ICAD cleavage, and DNA fragmentation (not shown). Caspase-9 failed to promote these events; however, our caspase-9 preparation showed little activity against the fluorogenic caspase substrate LEHD-afc. This is probably due to lack of cofactors since caspase-9 demonstrates little activity in the absence of cytochrome c, dATP, and cytosolic factors (APAF-1) (18).

Caspase-3 and Caspase-7, but Not Other Apoptotic Proteinases, Promote DFF45/ICAD Inactivation and DNA Fragmentation in Vitro—We next asked whether the preceding apoptotic proteinases directly cleaved DFF45/ICAD or DFF46/ICAD that was bound to the inhibitor. To accomplish this, we treated ~35S-DFF45/ICAD and ~35S-pro-DFF45/ICAD with each proteinase and examined the products by SDS-PAGE and autoradiography. As shown in Fig. 2, DFF45/ICAD was susceptible to each protease; however, the extent and pattern of DFF45/ICAD cleavage varied with each proteinase.

Caspase-3, caspase-7, and cytochrome c plus dATP-treated cytosol produced a similar cleavage pattern with products of ~24, 22, and 12 kDa. Caspase-6 and granzyme B produced a small amount of ~24- and ~12-kDa products. Caspase D and calpain produced distinct products of ~19 and 25 kDa, respectively.

Cell Culture and Induction of Apoptosis—Dr. Margret Hufniga (La Jolla Institute for Allergy and Immunology) provided the MCF7 breast cancer cell line. Cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with fetal calf serum (10% v/v), l-glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml). For induction of apoptosis, cells were incubated for 24 h with 50 ng/ml tumor necrosis factor-α (Genentech) plus 10 μg/ml cycloheximide (Sigma) or with 50 μM paclitaxel (Sigma). DNA fragmentation was assessed by agarose gel electrophoresis with ethidium bromide staining (13, 17) and fluorescence-activated cell sorter analysis with propidium iodide staining (17).
FIG. 1. Caspases, granzyme B, and cytochrome c plus dATP initiate DEVDase activity, DFF45/ICAD cleavage, and DNA fragmentation in a cell-free assay. Jurkat cytosolic extracts (final concentration 12 mg of protein/ml) were prepared as described in the text and incubated with caspase-3 (final concentration 0.25 μM), caspase-6 (0.25 μM), caspase-7 (0.25 μM), and caspase-8 (0.25 μM) plus dATP (1 mM) at 37 °C. After 30 min at 37 °C, aliquots (40 μg of protein) of each reaction mixture were analyzed for DEVD cleaving caspase activity (A) and subjected to Western blotting (12% gels) with anti-DFF45/ICAD antibodies (B). The asterisk (*) indicates the long and short DFF45 isoforms, respectively. By contrast, only granzyme B cleaved DFF40/CAD, producing a small amount of a ~35-kDa product (Fig. 2B). Thus, several apoptotic proteinases cleave DFF45/ICAD, however, DFF40/CAD resists proteolysis.

To determine whether DFF45/ICAD cleavage inactivated the inhibitor and released active DFF40/CAD, we treated pro-DFF40/CAD (prepared by in vitro transcription and translation of pBS-mCAD in the presence of GST-DFF45/ICAD) with caspase-3 and/or -7. After 30 min at 37 °C, 35S-DFF45/ICAD was incubated with caspase-3 (0.25 μM), caspase-6 (0.25 μM), caspase-7 (0.25 μM), and caspase-8 (0.25 μM) plus dATP (1 mM), cathepsin D (50 μg/ml), or calpain (10 nM). After 1 h at 30 °C, 35S-DFF45/ICAD (A) and 35S-DFF40/CAD (B) proteolysis were assessed by SDS-PAGE (15% slabs) and autoradiography. In C, pro-DFF40/CAD was incubated with proteinases or cytochrome c plus dATP for 30 min at 30 °C. 106 rat liver nuclei and EGTA (final concentration 5 mM) were then added and after 2 additional hours DNA fragmentation was assessed by agarose gel electrophoresis and ethidium bromide staining. Unless indicated otherwise, reactions were conducted in CAD buffer. Note that DFF45/ICAD has a predicted molecular mass of 36.5 kDa based on amino acid sequence (2); however, the protein and its alternatively spliced short isoform run as bands of ~45 and ~38 kDa, respectively (2). Cleavage at the N-terminal caspase site gives predicted products of ~23.7 and 12.8 kDa; whereas, cleavage at the C-terminal caspase cleavage site yields products of ~24.8 and 11.7 kDa. Hydrolysis at both caspase sites therefore yields fragments of ~12.8, 12.0, and 11.7 kDa. In B, L and S indicate the long and short DFF45 isoforms, I represents the intermediate caspase cleavage products (~23.7, 24.8 kDa), and the asterisk (*) represents the ~12-kDa DFF45 fragments that correspond with DNA fragmentation. In A, error bars represent the S.E., n = 3. In B and C, the data are representative of three independent experiments.

**FIG. 2. Limited proteolysis of DFF45/ICAD by caspase-3 and caspase-7, but not other apoptotic proteinases, releases active DFF40/CAD in vitro.** 35S-DFF45/ICAD and 35S-pro-DFF40/CAD (prepared by transcription and translation of pBS-mCAD in the presence of GST-DFF45/ICAD) were incubated with caspase-3 (0.25 μM), caspase-6 (0.25 μM), caspase-7 (0.25 μM), and caspase-8 (0.25 μM) plus dATP (1 mM), cathepsin D (50 μg/ml), or calpain (10 nM). After 1 h at 30 °C, 35S-DFF45/ICAD (A) and 35S-DFF40/CAD (B) proteolysis were assessed by SDS-PAGE (15% slabs) and autoradiography. In C, pro-DFF40/CAD was incubated with proteinases or cytochrome c plus dATP for 30 min at 30 °C. 106 rat liver nuclei and EGTA (final concentration 5 mM) were then added and after 2 additional hours DNA fragmentation was assayed by agarose gel electrophoresis and ethidium bromide staining. Unless indicated otherwise, reactions were conducted in CAD buffer. L indicates the long isoform of DFF45/ICAD used for in vitro transcription and translation. Cleavage of DFF45/ICAD at both caspase sites gives predicted products of 12.8, 12.0, and 11.7 kDa (*), with intermediate cleavage products of 23.7 and 24.8 kDa (†). The presented data are representative of three independent experiments.
fig. 3. caspase-3 inactivates dff45/icad and activates dna fragmentation more efficiently than caspase-7. in a, 35s-dff45/icad (2 μl) was incubated with caspase-3 (40 nM) or caspase-7 (40 nM) for the indicated times at 37 °C. Reactions were terminated by denaturing samples in Laemmli sample buffer and the products subjected to SDS-PAGE (12% slabs) and autoradiography. in b, 35s-dff45/icad (2 μl) was incubated with the indicated concentrations of caspase-3 and caspase-7 for 30 min at 37 °C. Samples were then denatured and analyzed by SDS-PAGE (12% slabs) and autoradiography. in c, pro-DFF40/CAD was incubated with 106 rat liver nuclei for 2.5 h at 30 °C and the indicated concentrations of caspase-3 and caspase-7. DNA fragmentation was then assessed by agarose gel electrophoresis and ethidium bromide staining. The data are representative of three independent experiments.

Dff45/icad cleavage was evident within 10 min following caspase-3 treatment and complete by 2 h. By contrast, caspase-7 did not produce detectable DFF45/ICAD cleavage until 2 h and only ~50% of the DFF45/ICAD was cleaved at 4 h. In concentration dependence experiments (Fig. 3B), caspase-3 also cleaved DFF45/ICAD more efficiently than caspase-7. Following a 30-min incubation, DFF45/ICAD cleavage was detectable with as little as 8.8 nM caspase-3; however, DFF45/ICAD cleavage by caspase-7 was not detectable until the caspase concentration was 88 nM.

To determine if DFF45/ICAD cleavage corresponded with release of active DFF40/CAD, we incubated various concentrations of caspase-3 or caspase-7 with pro-DFF40/CAD and rat liver nuclei and then monitored DNA fragmentation. As shown in Fig. 3C, caspase-3 induced detectable DNA fragmentation at 50 nM, whereas with caspase-7, DNA fragmentation required 150 nM caspase-7. Together, the data indicate that caspase-3 inactivates DFF45/ICAD and induces DNA fragmentation more efficiently than caspase-7.

Caspase-7 Activation Does Not Induce DNA Fragmentation in Caspase-3 Null MCF7 Cells or Cytosolic Extracts—To determine whether endogenous levels of caspase-7 could cleave DFF45/ICAD and promote DNA fragmentation in the absence of caspase-3, we used caspase-3-null MCF7 cell extracts in a cell-free assay. The MCF7 cells lacked caspase-3 as demonstrated by Western blotting, although they contained caspases-6, -7, -8, and -9, and APAF-1 at comparable levels to Jurkat cells (not shown). We treated the extracts with cytochrome c plus dATP and then examined DEVDAse activity, procaspase-7 processing, DFF45/ICAD cleavage, and DNA fragmentation as a function of time. For comparison, we also analyzed these events in Jurkat cell extracts, which contain caspase-3.

As shown in Fig. 4, cytochrome c plus dATP initiated procaspase-7 processing and the onset of DEVDA cleaving caspase activity in Jurkat and MCF7 extracts. However, procaspase-7 processing occurred earlier and was more extensive in the Jurkat extracts. Procaspase-3 processing occurred with identical kinetics to procaspase-7 processing in the Jurkat extracts (not shown). The Jurkat extracts demonstrated ~10-fold greater DEVDAse activity than the MCF7 cells at 2 h, likely due to the combined activity of caspase-3 and caspase-7. Although DEVDAse activity declined with time in the Jurkat extracts, the Jurkat DEVDAse activity remained substantially greater than the MCF7 DEVDAse activity at all time points. Caspase-7 processing and DEVDAse activity correlated with the extent of DFF45/ICAD cleavage and DNA fragmentation in both extracts (Fig. 4, B and C). In the Jurkat extracts, DFF45/ICAD cleavage was complete by 2 h; whereas, even after 6 h, only ~50% of DFF45/ICAD had been cleaved in the MCF7 extracts. Strikingly, while DNA fragmentation had occurred by 2 h in the Jurkat extracts, little DNA fragmentation had occurred in the MCF7 extracts even after 6 h of incubation, despite caspase-7 activation and partial DFF45/ICAD cleavage. Thus, cytochrome c plus dATP initiate caspase-7 activation in the MCF7 extracts, but this does not inactivate DFF45/ICAD or promote significant DNA fragmentation. Similarly, we detected caspase-7-like DEVDAse activity in extracts prepared from apoptotic MCF7 cells, but we detected no DNA fragmentation by agarose gel analysis or propidium iodide staining and fluorescence-activated cell sorter analysis (not shown).

We next incubated caspase-3 with MCF7 extracts and nuclei to determine whether this protease could initiate DNA fragmentation. Fig. 4D shows that 125 nM caspase-3 induced DNA fragmentation in the extracts. Similarly, addition of exogenous caspase-7 to the extracts initiated DNA fragmentation, although this required a higher concentration than caspase-3 (not shown). Together, these data suggest that in the absence of caspase-3, endogenous levels of caspase-7 do not inactivate DFF45/ICAD.

Discussion

In this paper, we examined whether various apoptotic proteinases could protect internucleosomal DNA fragmentation by inactivating DFF45/ICAD, thereby releasing active DFF40/CAD. Of the eight proteinases examined, we find that only caspase-3 and caspase-7 are direct inactivators of DFF45/ICAD. However, caspase-3 was the more efficient inactivator and endogenous levels of caspase-7 failed to release active DFF40/CAD, suggesting that caspase-3 is the primary activator of apoptotic DNA fragmentation. These findings confirm and extend those of Liu et al. (19) who recently demonstrated that caspases-3 and -7, but not caspases-6 and -8, inactivate recombinant DFF45, releasing active DFF40 (19). Additionally,
our work emphasizes the central importance of caspase proteinases, particularly caspase-3, as the principal mediators of apoptosis.

Three lines of evidence support our finding that caspase-3 is the primary inactivator of DFF45/ICAD and thus the primary activator of apoptotic DNA fragmentation. First, only proteinases that initiated DEVDase activity in Jurkat extracts, which is due primarily to caspase-3 inactivated DFF45/ICAD and initiated DNA fragmentation (Fig. 1). Second, caspase-3 cleaved DFF45/ICAD and promoted DNA fragmentation more effectively than caspase-7, the only other direct DFF45/ICAD inactivator (Figs. 2 and 3). Third, caspase-3 null MCF7 cells failed to fragment DNA during apoptosis and cytochrome c plus dATP-activated MCF7 extracts did not inactivate DFF45/ICAD or promote DNA fragmentation, despite caspase-7 activation (Fig. 4). However, addition of caspase-3 to these extracts restored their caspase-dependent DNase activity. These findings are consistent with the delayed or absent apoptotic DNA fragmentation observed in caspase-3 null cells and mice (20–23).

Caspase-3 activation is therefore fundamentally important for DFF45/ICAD inactivation.

Although caspase-7 initiated DNA fragmentation in a cell-free assay (Fig. 1) and inactivated DFF45/ICAD in an in vitro nuclease assay (Fig. 2), our data suggest that caspase-7 plays a secondary role in inactivating the inhibitor. Like caspase-3, caspase-7 cleavage of DFF45/ICAD more effectively than caspase-7 (Fig. 3) in vitro and the low level of caspase-7 DEVDase activity generated in MCF7 cells and extracts (Fig. 4) did not inactivate DFF45/ICAD. This suggests that the two caspases cleave macromolecular substrates with different effi-

FIG. 5. A model of apoptotic DNA fragmentation. The model emphasizes release of active DFF40/CAD via caspase-3 dependent inactivation of DFF45/ICAD and highlights how other apoptotic proteinases may intersect this pathway. Note that the initiator caspases and granzyme B function primarily via activation of executioner caspases. Active caspase-3 is the primary inactivator of DFF45/ICAD, but caspase-7, which may be localized to mitochondria, plays a secondary role in DFF45/ICAD inactivation. Caspase-3 in turn activates caspase-6, which aids in nuclear apoptosis via cleavage of nuclear lamins. Caspase-6 can also activate caspase-3 activity via activation of procaspase-3. Cathepsin D and calpain apparently function independently of caspases and DFF40/CAD, although calpain could potentially initiate high molecular weight DNA fragmentation via release of mitochondrial apoptosis inducing factor.

FIG. 4. Endogenous levels of caspase-7 cleave DFF45/ICAD inefficiently and fail to promote DNA fragmentation. Cytosolic extracts were prepared from Jurkat cells and caspase-3 null MCF7 cells as described in the text. Extracts (final concentration 12 mg of protein/ml) were incubated at 37 °C with buffer or cytochrome c (10 µM) plus dATP (1 mM) and 10⁶ rat liver nuclei. At the indicated times, the nuclei were pelleted by centrifugation and analyzed for DNA fragmentation (C). Aliquots of the supernatant (40 µg protein) were analyzed for DEVD cleaving caspase activity (A) and subjected to Western blotting with antibodies against caspase-7, DFF45/ICAD, and actin (B). In D, MCF7 extracts were incubated with 10⁶ rat liver nuclei and the indicated concentrations of caspase-3. After 2.5 h at 37 °C, DNA fragmentation was assessed by agarose gel electrophoresis and ethidium bromide staining. In A, error bars represent the S.E., n = 3. In B, C, and D, the data are representative of three independent experiments.
chondria during apoptosis (28), could potentially induce high caspase cascades. However, calpain, which localizes to mitochondrial and microsomal membranes (24), the caspase may be physically sequestered from DFF45/ICAD and therefore unable to react with the inhibitor. Overall, the data suggest that caspase-7 is not a physiologic inactivator of DFF45/ICAD and that critical caspase-7 substrates remain unidentified.

Caspase-6 and granzyme B induced DFF45/ICAD inactivation and DNA fragmentation in Jurkat extracts (Fig. 1); however, although they cleaved DFF45/ICAD, they failed to inactivate the inhibitor in an in vitro nuclease assay (Fig. 2). Since these proteinases effectively activate caspase-3 (16, 25), these results suggest that they inactivate DFF45/ICAD via caspase-3 activation. Non-activating DFF45/ICAD cleavage was also observed with calpain and cathepsin D (Figs. 1 and 2); however, since these proteinases do not activate caspases (Fig. 1 and Ref. 13), they were unable to initiate DNA fragmentation in a cell-free assay (Fig. 1). Caspase-6, granzyme B, calpain, and cathepsin D probably cleave DFF45/ICAD at or near the C-terminal caspase cleavage site (Asp224) since DFF45/ICAD mutants that can only be cleaved at this site retain DFF40/CAD inhibitory activity (6). Non-activating cleavage of DFF45/ICAD by these proteinases could be synergistic with activating cleavage events mediated by caspase-3 or caspase-7 if the partially cleaved inhibitor is more susceptible to cleavage by caspases-3 and -7. Alternatively, non-activating cleavage might alter interaction of DFF40/CAD with cofactors such as histone H1 (19) or target the endonuclease for destruction. These possibilities are currently under investigation.

In summary, our data indicate that caspase-3 is the primary inactivator of DFF45/ICAD and suggest that proteolytic pathways that induce apoptotic internucleosomal DNA fragmentation must involve this proteinase. Fig. 5 summarizes our findings and presents a model of apoptotic internucleosomal DNA fragmentation. In this model, the initiator caspases, caspases-8 and -9, promote apoptotic signaling and activate the executioner caspases, which in turn degrade apoptotic substrates. Similarly, granzyme B functions primarily to activate executioner caspases and not to cleave apoptotic substrates. Studies demonstrating that granzyme B activates caspase-3 more efficiently than caspase-8 (16) and the lack of DNA fragmentation observed in the absence of caspase activation during granzyme B-mediated apoptosis (9) support this hypothesis. Once activated, caspase-3 plays a central role in apoptotic DNA fragmentation by inactivating DFF45/ICAD, thereby releasing active DFF40/CAD. Caspase-3 also activates caspase-6 (26), which in turn promotes nuclear apoptosis by degrading lamins (27). Caspase-6 can also activate procaspase-3 (25), providing the opportunity for amplification of caspase-3 activity. Caspase-7, which may be localized to mitochondria and microsomes plays a secondary role in DNA fragmentation as a back up DFF45/ICAD inactivator. By contrast, calpain and cathepsin D do not activate caspases and function downstream or independently of caspase cascades. However, calpain, which localizes to mitochondria during apoptosis (28), could potentially induce high molecular weight DNA fragmentation via release of mitochondrial apoptosis inducing factor. Although lysosomes release cathepsin D during some forms of apoptosis (29), this proteinase does not inactivate DFF45/ICAD and its cellular substrates remain undefined. Thus, many proteinases may function in nuclear apoptosis, but caspase-3 is the key determinant of DFF45/ICAD inactivation and apoptotic internucleosomal DNA fragmentation.

Acknowledgments—We thank Drs. Xiadong Wang, Shigezi Nagata, Guy Salvesen, Vishva Dixit, and Emad Alnemri for reagents.

REFERENCES

1. Wolf, B. B., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052
2. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) Cell 99, 175–184
3. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) Nature 391, 43–50
4. Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W. T., and Wang, X. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8461–8466
5. Halenbeck, R., MacDonald, H., Houlston, A., Chen, T. T., Conroy, L., and Williams, L. T. (1998) Curr. Biol. 8, 537–540
6. Sakahira, H., Enari, M., and Nagata, S. (1998) Nature 391, 96–99
7. Sakahira, H., Enari, M., and Nagata, S. (1999) J. Biol. Chem. 274, 15740–15744
8. Susin, S. A., Lorenzo, H. K., Zanazzi, M., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goulietti, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397, 441–446
9. Orth, K., O’Rourke, K., Salvesen, G. S., and Dixit, V. M. (1996) J. Biol. Chem. 271, 20977–20980
10. Darmon, A. J., Ley, T. J., Nicholson, D. W., and Bleakley, R. C. (1996) J. Biol. Chem. 271, 21709–21712
11. Pike, B. R., Zhao, X., Newcomb, J. K., Wang, K. K. W., Posmantur, R. M., and Hayes, R. L. (1998) J. Neurosci. Res. 52, 505–520
12. Deiss, L. P., Galinka, H., Berissi, H., Cohen, O., and Kimchi, A. (1996) EMBO J. 15, 3861–3870
13. Wolf, B. B., Goldstein, J. C., Stennicke, H. R., Beere, H., Amarante-Mendes, G., Salvesen, G. S., and Green, D. R. (1999) Blood, 94, 1683–1692
14. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) J. Biol. Chem. 272, 2952–2956
15. Srinivasula, S. S., Ahmad, M., Fernandes-Allemri, T., and Alnemri, E. S. (1998) Mol. Cell 1, 949–957
16. Stennicke, H. R., Jugensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, M. H., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) J. Biol. Chem. 273, 27984–27990
17. Martin, S. J., Newmeyer, D. D., Mathias, S., Faraschov, D. M., Wang, H.-G., Reed, J. C., Kolesnick, R. N., and Green, D. R. (1995) EMBO J. 14, 2491–5200
18. Stennicke, H. R., Deveraux, Q. L., Humke, E. W., Reed, J. C., Dixit, V. M., and Salvesen, G. S. (1999) J. Biol. Chem. 274, 8359–8362
19. Liu, X., Zou, H., Widlak, P., Garrard, W., and Wang, X. (1999) J. Biol. Chem. 274, 13836–13840
20. Zheng, T. S., Schlosser, S. F., Dao, T., Hingorani, R., Crispe, I. N., Boyer, J., and Flavell, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13618–13623
21. Wof, M., Haken, R., Soengas, M., Duncan, G. S., Shahnian, A., Kagi, D., Kakem, A., McCurrrach, M., Kho, W., Kaufman, S. A., Senaldi, G., Howard, T., Lowe, S. W., and Mak, T. W. (1998) Genes Dev. 12, 806–819
22. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) J. Biol. Chem. 273, 9357–9360
23. Tang, D., and Koid, V. J. (1998) J. Biol. Chem. 273, 28549–28552
24. Chandler, J. M., Cohen, G. M., and MacFarlane, P. (1998) J. Biol. Chem. 273, 10815–10818
25. Sree, E. A., Harte, T. M., Kluk, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H.-G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999) J Cell Biol. 144, 281–292
26. Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16443–16446
27. Liu, X., Kim, C. N., Pohl, I., and Wang, X. (1996) J. Biol. Chem. 271, 13737–13742
28. Wood, E. D., Thomas, A., Devi, L. A., Berman, Y., Beavis, R. C., Reed, J. C., and Newcomb, E. W. (1998) Oncogene 17, 1069–1078
29. Roberg, K., and Ollinger, K. (1998) Am. J. Pathol. 152, 1151–1156