Requirements for Proteolysis during Apoptosis

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The key effector proteins of apoptosis are a family of cysteine proteases termed caspases. Following activation of caspases, biochemical events occur that lead to DNA degradation and the characteristic morphological changes associated with apoptosis. Here we show that cytoplasmic extracts activated in vitro by proteinase K were able to cleave the caspase substrate DEVD-7-amino-4-methylcoumarin, while neither proteinase K nor nonactivated extracts were able to do so alone. Caspase-like activity was inhibited by the specific caspase inhibitor DEVD-aldehyde and by the protease inhibitor iodoacetamide, but not by *N*-ethylmaleimide. When added to isolated nuclei, the activated extracts caused internucleosomal DNA degradation and morphological changes typical of apoptosis. As DNA cleavage and morphological changes could be inhibited by *N*-ethylmaleimide but not by iodoacetamide, we conclude that during apoptosis, caspase activation causes activation of another cytoplasmic enzyme that can be inhibited by *N*-ethylmaleimide. Activity of this enzyme is necessary for activation of endonucleases, DNA cleavage, and changes in nuclear morphology.

Experiments with *Caenorhabditis elegans* and mammals have shown that the key effector molecules of apoptosis are a family of cysteine proteases, called caspases, that cleave their substrates at certain aspartate residues (2). Several other proteases have also been implicated in apoptosis and may have roles either upstream of caspases—for example, in the activation of these enzymes—or downstream, possibly in activating other enzyme systems, such as nucleases.

In order to determine the requirements for proteases during various stages of apoptosis, we have used protease inhibitors to study biochemical events associated with apoptosis in a cell-free system activated by the nonspecific protease proteinase K.

In these experiments, we have used the sulfhydryl (SH) reagents iodoacetamide (IAA) and *N*-ethylmaleimide (NEM), which inhibit cysteine proteases by reacting with them, but it should be kept in mind that they also react with low-molecular-weight SH compounds and all of the SH enzymes (14). To inhibit the apoptotic caspases specifically, we have used acetyl DEVD-aldehyde (Ac-DEVD-CHO), which inhibits caspase-3 (CPP32), caspase-6, and caspase-7 by acting as a pseudosubstrate (10, 11, 20).

The activation of cysteine proteases from the caspase family appears to be essential for the implementation of apoptosis (29). These enzymes cleave their substrate after certain recognition sequences ending with an aspartate residue. At least 10 of these enzymes are known, and they seem to cleave with different substrate specificities (2). Several caspases, in particular those thought to be essential for cell death, have been reported to be able to cleave the cellular enzyme poly(ADP-ribose) polymerase after the sequence DEVD, a specificity originally attributed to caspase-3 (CPP32) (19). The fluorogenic substrate DEVD-7-amino-4-methylcoumarin (DEVD-AMC) can therefore be used to detect the activity of these caspases in cellular extracts.

We examined whether proteinase K is able to induce DEVD-cleaving activity in normal extracts. As apoptosis is commonly accompanied by internucleosomal degradation of DNA (27), we also tested whether the activated extracts could cause DNA cleavage in isolated nuclei. In order to determine which proteases were necessary for endonuclease activity, we treated the cytosolic extracts with the protease inhibitors Ac-DEVD-CHO, IAA, and NEM.

MATERIALS AND METHODS

Preparation of extracts and nuclei. The murine T-cell lymphoma cell lines LB (18) and BW5147 (American Type Culture Collection, Rockville, Md.) and the human T-cell leukemia line Jurkat (American Type Culture Collection) were grown in RPMI medium supplemented with 5% fetal calf serum and 5 mM glutamine (Biochrom, Hamburg, Germany). For LB cells, the medium was further supplemented with insulin (10 μg/ml; Sigma, Munich, Germany). Extracts and nuclei were prepared as described for S/M extracts (15). Briefly, cells for extracts were grown in Falcon roller bottles (Becton Dickinson, Heidelberg, Germany) at 37°C in 5% CO_2 to a density of approximately 5×10^6 cells/ml. For some experiments, Jurkat cells were incubated with etoposide (50 μM) for 8 h before extracts were prepared. Cells were collected by centrifugation; washed once in phosphate-buffered saline and once in extraction buffer {50 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] [pH 7.0], 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20 μM cytochalasin B} containing the protease inhibitors phenylmethylsulfonyl fluoride, chymopain, leupeptin, antipain, and pepstatin (all from Sigma); and lysed in a centrifugal Dounce homogenizer. The pellet was subjected to two cycles of freezing and thawing in an ethanol-dry ice bath. The cell lysate was centrifuged for 90 min at $150,000 \times g$. Supernatants from this step are referred to as extracts here. Extracts were stored at −80°C for up to 2 months.

Nuclei from HeLa cells were prepared as described previously (15). Briefly, after being harvested, the cells were washed once with phosphate-buffered saline and once with nucleus buffer (NB; 10 mM PIPES [pH 7.4], 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B). Cells were allowed to swell on ice for 20 min in 10 volumes of NB and then lysed with a Dounce homogenizer. The resulting homogenate was layered over NB–30% sucrose. Nuclei were pelleted by centrifugation at $800 \times g$ for 10 min; washed once in NB; resuspended in a buffer containing 10 mM PIPES (pH 7.4), 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermine, 0.2 mM spermidine, protease inhibitors as described above, and 50% glycerol; and stored at −20°C for up to 2 weeks. Nuclei were washed twice in mitotic dilution buffer (MDB; 10 mM HEPES [pH 7.0], 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT) before addition to extracts.

Assay system for internucleosomal cleavage. Extracts were diluted to 50% with MDB. As a source of ATP, reagents were added to yield final concentrations of 2 mM ATP, 10 mM creatine phosphate, and 10 mM creatine kinase (Sigma). Individual reaction mixtures contained 1×10^6 to 2×10^6 nuclei in 50 μl of diluted extracts in 1.5-ml tubes and were incubated at 37°C for 30 min. Proteases were added at various concentrations immediately prior to the start of

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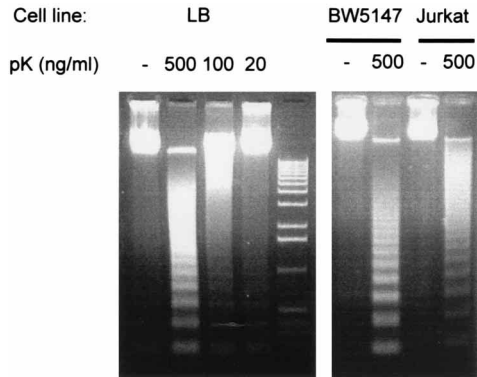
FIG. 1. Proteinase K induces DNA fragmentation in a cell-free system. Extracts were prepared from the lymphoid cell lines LB, BW5147, and Jurkat and incubated for 30 min with isolated nuclei at 37°C alone or with proteinase K (pK) as shown. The DNA was extracted and run on a 1% agarose gel. The experiment was done at least three times with independently prepared extracts from each cell line. The unlabelled lane contains DNA size standards.

incubation. The protease inhibitors NEM (Sigma), IAA (Sigma), and Ac-DEVD-CHO (Bachem Biochemica, Heidelberg, Germany) were added and allowed to equilibrate on ice for 5 min before the protease was added and the tube was shifted to 37°C. At the end of the incubation period, the reaction mixtures were centrifuged and the supernatants were removed and stored on ice. The pelleted nuclei were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.5% sodium dodecyl sulfate (SDS), 500 μg of proteinase K per ml) and digested for 30 min at 56°C. The supernatant and the digested pellet were then recombined, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol. DNA was dissolved in 50 μl of Tris-EDTA buffer containing RNase A (10 μg/ml; Sigma), incubated at 37°C for 1 h to digest RNA, and run on a 1% agarose gel containing ethidium bromide. DNA was visualized with UV light, and the gels were photographed with a video imaging system (Eagle Eye II; Stratagene, Heidelberg, Germany).

Microscopy. Two microliters was taken out of each reaction mixture, stained with acridine orange (Sigma; final concentration, 40 μg/ml), and examined under a Leica DM RBE fluorescence microscope. Pictures were taken with a Leica MPS52/48 camera.

Assay system for caspase activity. Extracts were diluted to final concentrations of 2 to 5% (each extract was titrated to optimize for a protease-induced signal and a low background) in MDB supplemented with 0.1% CHAPS {3-[(3-chol-amidopropyl)-dimethylammonio]-1-propanesulfonate} and 2 mM DTT (in some initial experiments bovine serum albumin [BSA] [100 μg/ml] was included, with identical results). Reactions were performed in triplicate in a 100-μl volume in 96-well flat-bottomed plates (Nunc plates; M & B Stricker, Oberschleissheim, Germany). For inhibitor experiments, the inhibitors were added to the diluted extract and preincubated at room temperature for 5 min. Then protease and a fluorogenic substrate (DEVD-AMC) (10 μM; Biomol, Hamburg, Germany) were added simultaneously, and the reactions were shifted to 37°C and allowed to proceed for 30 min. Fluorescence was then measured in a Millipore Cytofluor 96-well reader with filter settings of 390 (excitation) and 460 (emission). Values (means ± 3 standard errors of the means) are given as arbitrary units.

Assays for proteinase K and DNase I activity. To assess proteinase K activity, BSA (1 mg/ml) was incubated in MDB in a 50-μl reaction volume for 20 min at 37°C alone and in the presence of proteinase K, proteinase K plus NEM (5 mM), or proteinase K plus IAA (5 mM). The proteinase K concentration was 200 ng/ml. Twenty microliters of each reaction mixture was resolved by SDS-poly-acrylamide gel electrophoresis on a 12% polyacrylamide gel, and the protein was stained with Coomassie brilliant blue.

To assess DNase I activity, genomic DNA was prepared from day 14 embryonic fibroblasts by proteinase K digestion and phenol-chloroform-isoamyl alcohol extraction. Fifty micrograms of DNA was incubated in a 50-μl reaction volume in restriction enzyme buffer L (Boehringer Mannheim, Mannheim, Germany) for 20 min at 37°C alone, with DNase I (Sigma), or with DNase I and either NEM (5 mM) or IAA (5 mM). The DNase concentration was 10 μg/ml. Twenty microliters of the reaction mixture was resolved on a 1% agarose gel as described above.

RESULTS

Proteinase K can activate cytosolic extracts, leading to DNA degradation. In order to activate the apoptosis effector mechanism, proteinase K was added to cytosolic extracts from the three different cell lines, which were then incubated with nuclei

from HeLa cells for 30 min at 37°C. The amount of DNA cleavage that resulted was measured by extracting the DNA and subjecting it to gel electrophoresis (Fig. 1). When unaltered cytosolic extracts from the three cell lines were incubated with HeLa cell nuclei, no DNA fragmentation was seen. However, if proteinase K was added with the cytosolic extracts, the DNA in the nuclei became degraded in the pattern typical of internucleosomal DNA cleavage. Concentrations of proteinase K between 2 μg/ml and 100 ng/ml yielded ladders of degraded DNA. At higher concentrations, no fragmentation was observed, and the reaction mixture became highly viscous, most likely due to digestion of the nuclear matrix by the protease. Staining with intercalating dyes indicated that in this case the DNA was intact, and when subjected to electrophoresis little DNA migrated into the gel (not shown).

Proteinase K itself did not directly cause cleavage of the nuclear DNA, since in the absence of cytosolic extract, when any concentration of proteinase K was added to the nuclei, DNA fragmentation did not occur (data not shown). Similarly, the proteases trypsin and chymotrypsin were unable to trigger DNA cleavage in the presence or absence of cytosolic extract despite titration over a wide range of concentrations (100 mg/ml to 10 ng/ml) that ran well into concentrations where the nuclear matrix was digested and the reaction acquired a viscous appearance (data not shown).

The cytosol from etoposide-treated Jurkat cells was apoptotically activated, as it could induce internucleosomal DNA cleavage (Fig. 2, leftmost lane) and typical apoptotic morphological changes (Fig. 3, top middle) in HeLa cell nuclei. Activation of caspase-3 (CPP32)-like proteases occurred in the extracts, as they were able to cleave the fluorogenic substrate DEVD-AMC (not shown).

The DNA-cleaving activity of the extracts from etoposide-treated Jurkat cells was labile at 37°C, as incubation of the extracts for 20 to 40 min prior to the addition of nuclei greatly reduced the extent of DNA cleavage (Fig. 2). This decline was not due to a consumption of the ATP regeneration system, as replenishment of these components did not restore apoptotic activity (not shown). Also, it is unlikely that the effect was the consequence of diminishing caspase activity, as DEVD-AMC-
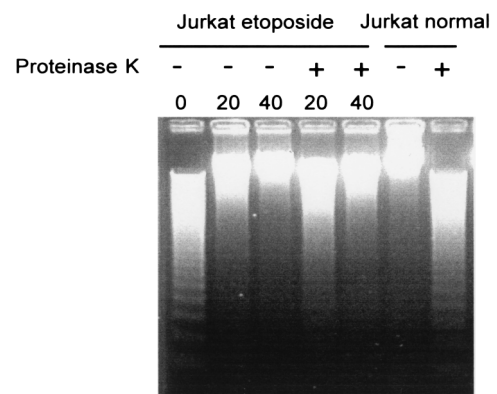

FIG. 2. Proteinase K requires labile components from the cytosol to induce DNA fragmentation. Extracts were prepared from Jurkat cells cultured for 8 h with etoposide (50 μM) or from normal Jurkat cells. Aliquots of extract from etoposide-treated cells were diluted in MDB as described in Materials and Methods and incubated at 37°C. Nuclei were added either at the beginning of the reaction or after 20 or 40 min of incubation at 37°C; proteinase K (500 ng/ml) was added to two samples at the same time as the nuclei (as indicated). The reaction was then allowed to continue for 60 min at 37°C. For comparison, nuclei were incubated with extract from normal cells with or without proteinase K for 60 min. DNA was then extracted and analyzed by electrophoresis.
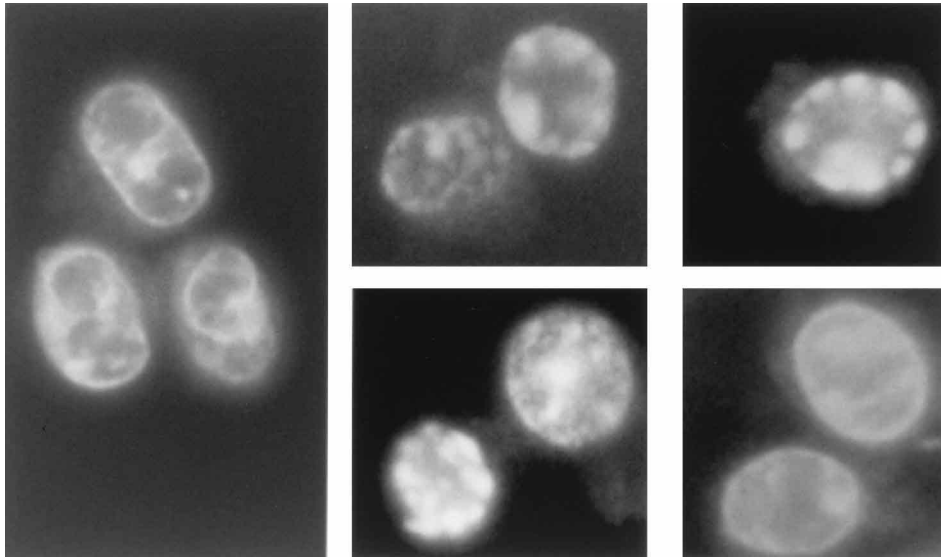
FIG. 3. Morphology of nuclei after incubation with extract and proteinase K. Nuclei from HeLa cells were incubated for 30 min at 37°C in either normal extract (i.e., extract prepared from normal Jurkat cells [left]), the same extract supplemented with 500 ng of proteinase K per ml (top right), or extract prepared from Jurkat cells treated with etoposide (50 μM) for 8 h (top middle). Additional samples contained normal extract supplemented with proteinase K and either IAA (bottom middle) or NEM (bottom right).

cleaving activity in such extracts was measured over 4 h and found to be constant during this period (data not shown).

Significantly, the addition of proteinase K to these extracts after preincubation for 20 min had little effect and the addition of proteinase K after 40 min of preincubation did not induce DNA fragmentation (Fig. 2), indicating that for proteinase K to cause internucleosomal DNA cleavage, cytosolic components which are used up or destroyed during the 40-min incubation period are required. Addition of the same concentration of proteinase K to extracts from Jurkat cells before etoposide treatment did cause fragmentation (Fig. 2). Very similar results were obtained when extracts from irradiated LB cells were used (not shown).

Proteinase K-activated extracts can cause altered morphology of nuclei. Characteristic of cells undergoing apoptosis are changes in nuclear morphology, such as chromatin condensation and disintegration of the nucleus. We examined the morphology of HeLa cell nuclei during and after incubation with normal extract alone or with cytosolic extract in the presence of proteinase K.

Nuclei incubated with extract alone at 37°C for 30 min (or up to 120 min [data not shown]) did not change their morphology (Fig. 3, left) and were indistinguishable from untreated nuclei (not shown). When proteinase K plus cytosolic extract was added, the nuclei started to disintegrate rapidly. Figure 3 (top right) depicts a typical nucleus at the end stage, which is reached in about 15 min at 37°C after the addition of proteinase K. The appearance of the nuclei was very similar to the classical apoptotic morphology seen in intact cells undergoing apoptosis or in nuclei exposed to extracts from Jurkat cells treated with etoposide (Fig. 3, top middle). In both cases, the classical morphology of chromatin condensation along the margins of the nuclei was observed.

The proteinase K-induced morphological changes appear to be caused by an extract component, as in the absence of extract no chromatin condensation could be observed although some deformation of nuclei was induced (not shown). Also, when NEM was present in the extract, proteinase K was unable to provoke the apoptotic changes (Fig. 3, bottom right), while

IAA was unable to prevent the proteinase K effect (Fig. 3, bottom middle). High concentrations of proteinase K (above 2 μg/ml) led to swelling of the nuclei and leakage of long streaks of DNA into the extracts (not shown).

Activation of cytosolic caspases. To determine whether proteinase K could activate caspase-3-like proteases in extracts from nonapoptotic cells, serial dilutions of proteinase K were incubated for 30 min at 37°C with DEVD-AMC. AMC fluorescence was then measured and found to be strongly induced over a wide range of concentrations of proteinase K (Fig. 4a). Proteinase K could not cleave the DEVD-AMC substrate directly, as in the absence of extract it did not cause AMC fluorescence at any concentration (Fig. 4a). Neither of the serine proteases trypsin and chymotrysin was able to activate caspases within the extracts (Fig. 4b). Proteinase K did not lead to activation of caspase-1 (ICE)-like proteases in BW5147 cell extracts, as the caspase-1 substrate YVAD-AMC was not cleaved (not shown).

IAA strongly inhibited the induction of DEVD-AMC-cleaving activity by proteinase K, whereas the inhibition by NEM was only marginal (Fig. 4c).

In order to determine the abilities of NEM and IAA to inhibit DNA fragmentation caused by proteinase K-activated extracts, both agents were added to extracts and nuclei prior to proteinase K. NEM at 5 mM completely inhibited DNA fragmentation, while IAA had no inhibitory effect (Fig. 5). The tetrapeptide inhibitor of PARP-cleaving caspases Ac-DEVD-CHO inhibited proteinase K-induced DNA fragmentation only at the extremely high (>500 μM) concentrations at which it could directly inhibit proteinase K when BSA was used as a substrate (not shown). When added to extracts from etoposide-treated Jurkat cells, NEM completely inhibited DNA fragmentation, while IAA had a distinct but not complete inhibitory effect (not shown). This finding suggests that the NEM-inhibitable enzyme is also required for the physiological apoptosis pathway.

It was clear that NEM and IAA do not inhibit proteinase K directly, as neither NEM nor IAA inhibited the digestion of BSA by proteinase K (Fig. 6a). Therefore, NEM must act on a
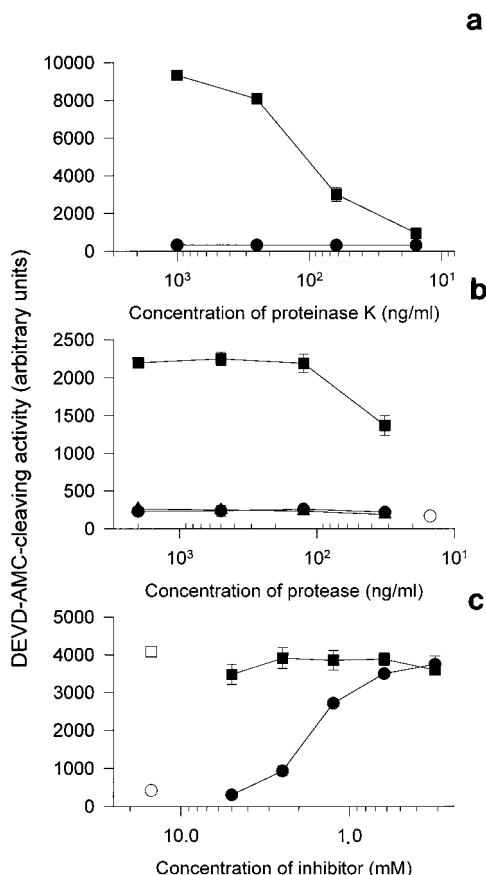
FIG. 4. Activation of latent cytosolic caspases. (a) Proteinase K leads to the activation of caspases. A constant concentration of DEVD-AMC (10 μM) in MDB containing 2 mM DTT and 0.1% CHAPS was incubated for 30 min at 37°C with proteinase K at the concentrations shown either without (circles) or with (squares) normal extract from BW5147 cells (diluted to 1:40). (b) Trypsin and chymotrypsin do not activate DEVD-AMC-cleaving activity. The proteases trypsin (solid circles), chymotrypsin (triangles), and proteinase K (squares) were incubated for 30 min at 37°C with DEVD-AMC in the presence of normal extract from LB cells (diluted to 1:20). As a negative control, reactions without protease were included (open circle). (c) IAA but not NEM inhibits proteinase K-induced caspase activation. Extract from LB cells was incubated with proteinase K at a constant concentration (200 ng/ml) and with various concentrations of IAA (closed circles) or NEM (closed squares) at 37°C in the presence of DEVD-AMC, and after 30 min fluorescence was determined. The negative control (open circle) was the same reaction mixture without proteinase K; the positive control (open square) contained proteinase K without the inhibitor. Reactions were performed in triplicate, and the results are given as means ± 3 standard errors of the means. The data shown here are representative of at least three independent experiments using individually prepared extracts.

cytosolic or nuclear enzyme activated by proteinase K that is required for DNA fragmentation.

While it remains uncertain which nuclease is responsible for DNA fragmentation during apoptosis, DNase I has been suggested as one candidate. Neither NEM nor IAA was able to inhibit the cleavage of murine genomic DNA by DNase I when added at a concentration of 5 mM (Fig. 6b).

DISCUSSION

This study demonstrates that the proteolytic action of proteinase K, a protease with low substrate specificity, can provoke activation of enzyme systems in vitro which are normally activated during apoptosis in vivo. Activation of both cell death-associated proteases (caspases) and nucleases was ob-
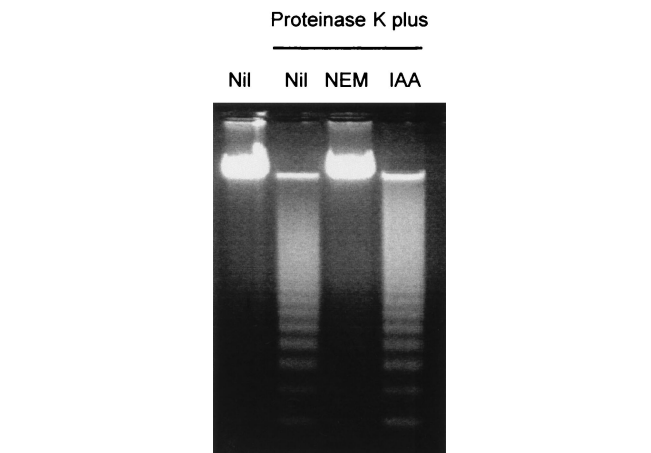

FIG. 5. NEM but not IAA inhibits proteinase K-induced DNA fragmentation. Extract from LB cells was incubated with HeLa cell nuclei at 37°C for 30 min alone (lane 1) or in the presence of proteinase K (500 ng/ml). One reaction mixture also contained 5 mM NEM, and one reaction mixture contained 5 mM IAA. DNA was extracted and analyzed by gel electrophoresis.

served. Since the cysteine protease inhibitors NEM and IAA affected the outcome of the assays differently, it is clear that the enzymes they inhibit play roles at several points in the apoptotic process.

Earlier studies have suggested that proteases other than caspases also play a role in cell death. For example, calpain, cathepsin D, the proteasome, and proteases sensitive to the inhibitors diisopropylfluorophosphate, N-tosyl-L-phenylalanine chloromethyl ketone, and Nα-p-tosyl-L-lysine chloromethyl ketone, and others have been implicated (6, 7, 12, 13, 22–24, 28). Although it is widely accepted that caspases are obligatory for physiological cell death, our data indicate that other enzymes can act either upstream to activate caspases or downstream to be activated as a consequence of proteolysis by caspases.
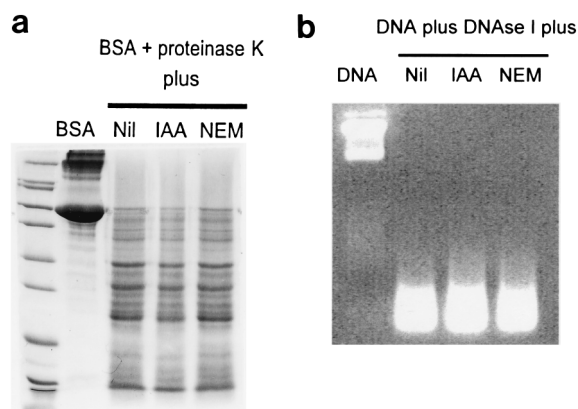

FIG. 6. NEM and IAA do not inhibit proteinase K or DNase I directly. (a) BSA (1 mg/ml) was incubated in MDB in a 50-μl reaction volume for 20 min at 37°C either alone or in the presence of proteinase K, proteinase K plus 5 mM IAA, or proteinase K plus 5 mM NEM. The proteinase K concentration was 200 ng/ml. Twenty microliters of each reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel, and the protein was stained with Coomassie mixture brilliant blue. (b) Fifty micrograms of genomic DNA was incubated in a 50-μl reaction volume in restriction enzyme buffer for 20 min at 37°C either alone, with DNase I (Sigma), or with DNase I and either 5 mM IAA or 5 mM NEM. The DNase concentration was 10 μg/ml. Twenty microliters of the reaction mixture was resolved on a 1% agarose gel.
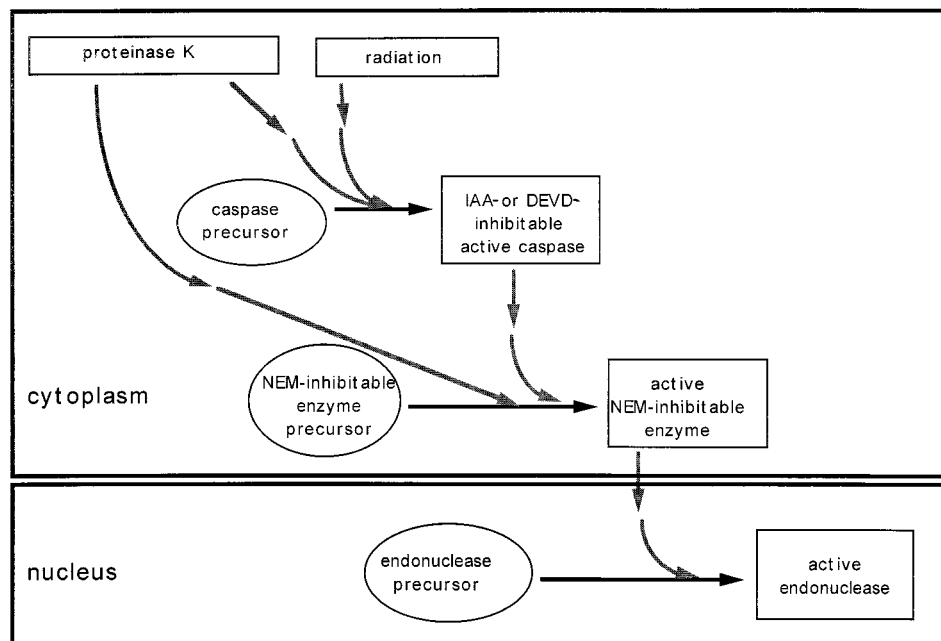
FIG. 7. Model of the action of proteinase K and inhibition by IAA and NEM. IAA blocks the appearance of caspase activity but not nuclear fragmentation; therefore, proteinase K must be able to bypass this stage. NEM did not block caspase activity but prevented DNA fragmentation, so its point of action must be further downstream. Proteinase K did not cause DNA fragmentation in the absence of cytoplasmic extract, so the NEM-inhibitable enzyme precursor is most likely cytoplasmic.

Several groups have proposed that the proteasome may be involved in apoptosis. However, the role of the proteosome is unclear; some have concluded that inhibition of the proteasome causes apoptosis, whereas others have concluded that the proteasome plays an essential role in apoptosis (8, 13, 23).

It is not clear at which step in the apoptotic pathway noncaspase proteases act. One possibility is that they play a role in the upstream stages of apoptosis induction; for example, calpain has been found to be important in some but not other forms of apoptosis (24). This finding suggests that unlike caspases, calpain is involved in a part of the pathway which is not common to all forms of apoptosis. Cathepsin D has been found to be required for cell death induced by gamma interferon, tumor necrosis factor, and CD95 signal (7). An interesting feature of this protease is that it is strongly induced during treatment of cells with gamma interferon or tumor necrosis factor (7). Since in other cases of apoptosis new synthesis of proteins is not required, this suggests that cathepsin also is necessary for some but not other forms of cell death, and this could be the case for most noncaspase proteases.

Caspases are present in all nucleated cells as inactive precursors and become activated upon initiation of cell death. It is possible that caspase molecules activate each other, but the primary cause for activation of the first molecule is unknown. Adapter molecules such as MORT1/FADD (3, 4), RAIDD/CRADD (1, 9), or homologs of ced-4 (5) could be involved in this activation. One model is that physiological or experimental activation of proteases in intact cells can lead to the proteolytic destruction of adapter/inhibitor molecules, which in turn activates caspases. This process could then be mimicked by the addition of proteinase K to extracts. It is remarkable how resistant to proteolysis caspases themselves seem to be; at concentrations of proteinase K as high as 2 μg/ml, caspase activity can still be measured.

It has been reported that introduction of the proteases proteinase K, trypsin, and chymotrysin via the osmotic lysis of pinosomes induced cells to undergo apoptosis (26). Since trypsin and chymotrypsin were unable to trigger either caspase or nuclease activity in cell extracts (data not shown), it is likely that these serine proteases act in an indirect fashion in that system, where cellular components mimicking one or more signals for apoptosis induction are digested (26).

It has been speculated that the caspase-mediated cleavage of lamins during apoptosis accounts for some of the changes in nuclear morphology (16). Interestingly, for both the morphological changes and the DNA fragmentation induced by proteinase K, extract components were required, and both effects could be blocked by NEM. This indicates that both effects are mediated by one effector molecule or at least by one pathway which can be blocked by NEM.

The properties of the NEM-inhibitable enzyme are remarkably similar to those described for DFF, a heterodimeric protein that is activated by caspase-3 and functions in the pathway leading to DNA fragmentation (17). Both proteins are thought to reside in the cytoplasm as inactive precursors. Both appear to be activated by caspases following the induction of apoptosis. Both are required for endonuclease activation and DNA degradation, but neither appears to be an endonuclease. We think that it is likely that the NEM-inhibitable enzyme is one of the components of DFF, and we predict that DFF activity will be susceptible to inhibition by NEM. Since NEM targets proteases with catalytic cysteines, if this is true then DFF is likely to be a noncaspase cysteine protease. The subunit of DFF cloned to date does not have any residues that resemble the catalytic cysteines in any of the ~20 known families of cysteine proteases (21), so if the NEM-inhibitable enzyme is DFF, the yet-to-be-cloned subunit is likely to contain the catalytic residue.

The agents IAA and NEM have both been described as inhibitors of caspase-3-mediated PARP cleavage in extracts from cells undergoing apoptosis (20). Both enzyme inhibitors target cysteine residues and can inhibit a broad range of en-

zymes. While we found that IAA could inhibit DEVD-cleaving activity at concentrations of 2 to 5 mM and that NEM was not able to inhibit DEVD-cleaving activity at any concentration up to 5 mM, Nicholson et al. found that both inhibitors could suppress PARP cleavage by apoptotic extracts at 5 mM. This observation raises the possibility that IAA acts on an upstream protease that is required for some apoptosis activation pathways but is not needed when proteinase K is used to artificially activate them.

In our system, IAA inhibited all the DEVD-AMC-cleaving activity that was induced by proteinase K, whereas the effect of NEM was only marginal. Conversely, NEM completely inhibited the DNA fragmentation induced by proteinase K, while if anything IAA enhanced DNA fragmentation.

Digestion of nuclear DNA can be observed in most cases of apoptosis; however, exceptions have been reported. For example, one cell line has been observed to undergo apoptosis without detectable nuclease activation (25). This phenotype could be the result of a lack of either the responsible nuclease itself or the NEM-inhibitable enzyme.

With the caveats that DEVD-AMC may not be efficiently cleaved by all caspases and that some caspases (e.g., caspase-1 [ICE]) may not be activated in this system, we interpret these results as shown in Fig. 7: both caspases and nucleases can be activated either directly or indirectly by generalized proteolysis caused by proteinase K. IAA can inhibit caspases, most probably directly. This inhibition cannot prevent activation of nucleases by proteinase K, which means that endonuclease activation does not require IAA-sensitive caspases. However, as cytoplasmic extract was needed for proteinase K to cause DNA degradation, proteinase K does not activate the endonucleases directly. NEM inhibits nuclease activation independently of caspase activity and therefore must act at a point downstream of both the caspases and proteinase K. The simplest explanation is that NEM acts on an enzyme whose precursor exists in the cytoplasm. This enzyme can be activated by proteinase K or the caspases and is able to activate endonucleases in the nucleus, ultimately resulting in DNA fragmentation.

As preincubation of activated extracts at 37°C for 40 min resulted in the loss of the ability of proteinase K to induce laddering when it and the extracts were added together to the nuclei, some cytosolic component required for DNA degradation must be labile. This labile component is not likely to be the caspase precursors, as preincubation did not destroy DEVD-cleaving activity (not shown). Furthermore, this demonstrates that proteinase K uses components of the physiological cell death pathway.

Although caspases are necessary for the normal implementation of cell death, the results reported here show that there are other essential enzyme systems involved in the intracellular apoptosis pathway. The development of alternative methods of activating this pathway in cell extracts may lead to the molecular definition of such essential components.

REFERENCES

1. **Ahmad, M., S. M. Srinivasula, L. J. Wang, R. V. Talanian, G. Litwack, T. Fernandes-Alnemri, and E. S. Alnemri.** 1997. CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL tumor necrosis factor receptor-interacting protein RIP. Cancer Res. **57:**615–619.
2. **Alnemri, E. S.** 1997. Mammalian cell death proteases—a family of highly conserved aspartate specific cysteine proteases. J. Cell. Biochem. **64:**33–42.
3. **Boldin, M. P., E. E. Varfolomeev, Z. Pancer, I. L. Mett, J. H. Camonis, and D. Wallach.** 1995. A novel protein that interacts with the death domain of fas/apo1 contains a sequence motif related to the death domain. J. Cell. Biochem. **270:**7795–7798.
4. **Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit.** 1995. FADD, a novel death domain-containing protein, interacts with the death domain of fas and initiates apoptosis. Cell **81:**505–512.
5. **Chinnaiyan, A. M., K. O'Rourke, B. R. Lane, and V. M. Dixit.** 1997. Interaction of ced-4 with ced-3 and ced-9—a molecular framework for cell death. Science **275:**1122–1126.
6. **Chow, S. C., M. Weis, G. Kass, T. H. Holmstrom, J. E. Eriksson, and S. Orrenius.** 1995. Involvement of multiple proteases during fas-mediated apoptosis in T lymphocytes. FEBS Lett. **364:**134–138.
7. **Deiss, L. P., H. Galinka, H. Berissi, O. Cohen, and A. Kimchi.** 1996. Cathepsin d protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. EMBO J. **15:**3861–3870.
8. **Drexler, H.** 1997. Activation of the cell death program by inhibition of proteasome function. Proc. Natl. Acad. Sci. USA **94:**855–860.
9. **Duan, H., and V. M. Dixit.** 1997. RAIDD is a new death adaptor molecule. Nature **385:**86–89.
10. **Fernandes-Alnemri, T., A. Takahashi, R. Armstrong, J. Krebs, L. Fritz, K. J. Tomaselli, L. Wang, Z. Yu, C. M. Croce, G. Salveson, W. C. Earnshaw, G. Litwack, and E. S. Alnemri.** 1995. Mch3, a novel human apoptotic cysteine protease highly related to CPP32. Cancer Res. **55:**6045–6052.
11. **Fernandes-Alnemri, T., G. Litwack, and E. S. Alnemri.** 1995. Mch2, a new member of the apoptotic ced-3/ICE cysteine protease gene family. Cancer Res. **55:**2737–2742.
12. **Fujita, E., T. Mukasa, T. Tsukahara, K. Arahata, S. Omura, and T. Momoi.** 1996. Enhancement of CPP32-like activity in the TNF-treated U937 cells by the proteasome inhibitors. Biochem. Biophys. Res. Commun. **224:**74–79.
13. **Grimm, L. M., A. L. Goldberg, G. G. Poirier, L. M. Schwartz, and B. A. Osborne.** 1996. Proteasomes play an essential role in thymocyte apoptosis. EMBO J. **15:**3835–3844.
14. **Katunuma, N., and E. Kominami.** 1995. Structure, properties, mechanisms, and assays of cysteine protease inhibitors: cystatins and E-64 derivatives. Methods Enzymol. **251:**382–397.
15. **Lazebnik, Y. A., S. Cole, C. A. Cooke, W. G. Nelson, and W. C. Earnshaw.** 1993. Nuclear events of apoptosis in vitro in cell free mitotic extracts—a model system for analysis of the active phase of apoptosis. J. Cell Biol. **123:**7–22.
16. **Lazebnik, Y. A., A. Takahashi, R. D. Moir, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw.** 1995. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. Proc. Natl. Acad. Sci. USA **92:**9042–9046.
17. **Liu, X. S., H. Zou, C. Slaughter, and X. D. Wang.** 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell **89:**175–184.
18. **Lugasi, H., S. Hajos, J. R. Murphy, T. B. Strom, J. Nichols, C. Penarroja, and D. Naor.** 1990. Murine spontaneous T-cell leukemia constitutively expressing IL-2 receptor—a model for human T-cell malignancies expressing IL-2 receptor. Int. J. Cancer **45:**163–167.
19. **Nagata, S.** 1997. Apoptosis by death factor. Cell **88:**355–365.
20. **Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, N. A. Munday, S. M. Raju, M. E. Smulson, T. T. Yamin, V. L. Yu, and D. K. Miller.** 1995. Identification and inhibition of the ICE/ced-3 protease necessary for mammalian apoptosis. Nature **376:**37–43.
21. **Rawlings, N. D., and A. J. Barrett.** 1994. Families of cysteine peptidases. Methods Enzymol. **244:**461–486.
22. **Sarin, A., D. H. Adams, and P. A. Henkart.** 1993. Protease inhibitors selectively block T cell receptor-triggered programmed cell death in a murine T cell hybridoma and activated peripheral T cells. J. Exp. Med. **178:**1693–1700.
23. **Shinohara, K., M. Tomioka, H. Nakano, S. Tone, H. Ito, and S. Kawashima.** 1996. Apoptosis induction resulting from proteasome inhibition. Biochem. J. **317:**385–388.
24. **Squier, M., and J. J. Cohen.** 1997. Calpain, an upstream regulator of thymocyte apoptosis. J. Immunol. **158:**3690–3697.
25. **Ucker, D. S., P. S. Obermiller, W. Eckhart, J. R. Apgar, N. A. Berger, and J. Meyers.** 1992. Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes. Mol. Cell. Biol. **12:**3060–3069.
26. **Williams, M. S., and P. A. Henkart.** 1994. Apoptotic cell death induced by intracellular proteolysis. J. Immunol. **153:**4247–4255.
27. **Wyllie, A. H.** 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature **284:**555–556.
28. **Yoshida, A., R. Takauji, M. Inuzuka, T. Ueda, and T. Nakamura.** 1996. Role of serine and ICE-like proteases in induction of apoptosis by etoposide in human leukemia HL-60 cells. Leukemia **10:**821–824.
29. **Yuan, J. Y., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz.** 1993. The *C. elegans* cell death gene ced 3 encodes a protein similar to mammalian interleukin 1 beta converting enzyme. Cell **75:**641–652.