Purification of a 24-kD Protease from Apoptotic Tumor Cells That Activates DNA Fragmentation

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Summary

We report the purification of a protease from tumor cells undergoing apoptosis that is involved in activating DNA fragmentation. Initial studies revealed that two inhibitors of serine proteases, N-1-tosylamide-2-phenylethylchloromethyl ketone and carbobenzoxy-Ala-Ala-borophenylglycinamide (DK120), suppressed tumor necrosis factor or ultraviolet (UV) light-induced DNA fragmentation in the U937 histiocytic lymphoma as well as UV light-induced DNA fragmentation in the BT-20 breast carcinoma, HL-60 myelocytic leukemia, and 3T3 fibroblasts. The protease was purified by affinity chromatography with DK120 as ligand and showed high activity on a synthetic substrate preferred by elastase-like enzymes (Ala-Ala-Pro-Val-p-nitroanilide), but was inactive on the trypsin substrate, N-α-benzylxocarbonyl-l-lysine thiobenzyl ester, or the chymotrypsin substrate, Ala-Ala-Pro-Phe-p-nitroanilide. The activity of the DK120-binding protease purified from U937 cells undergoing apoptosis was increased ~10-fold over that recovered from normal cells. Further purification to homogeneity by heparin-Sepharose affinity chromatography followed by reverse phase high-performance liquid chromatography revealed a single band of 24 kD on a silver-stained sodium dodecyl sulfate gel. In addition to protease activity, the purified enzyme induced DNA fragmentation into multiples of 180 basepairs in isolated U937 nuclei. These findings suggest the 24-kD protease is a novel enzyme that activates DNA fragmentation in U937 cells undergoing apoptosis.

Apoptosis is the normal physiological process of cell death that functions to control cell populations during embryogenesis, immune responses, hormone withdrawal from dependent tissues, and normal tissue homeostasis (for review see references 1–3). Unlike necrosis, during apoptosis, endonucleases present in the cell cut the DNA in the linker regions to release DNA fragments in multiples of 180 bp (4). However the biochemical basis of signal transduction as well as the identification of the relevant nuclease(s) have not yet been elucidated. Our relatively limited understanding of the mechanism of apoptosis suggests it may differ depending on the cell type and inducing agent. Some (4–6) but not all (7, 8) examples of apoptosis require new gene expression and protein synthesis. Differences have also been reported in the requirement for extracellular calcium (4, 9–11). These findings may reflect diverse signaling events functioning at early stages of a pathway that subsequently converge to a final biochemical response common to many or all forms of apoptosis.

Our laboratory has been studying the mechanism of apoptosis using primarily the human histiocytic lymphoma, U937, as a model system. This cell line undergoes apoptosis in response to a variety of stimuli, including TNF, UV light, heat shock, oxidative stress, and chemotherapeutic drugs (12, 13). Most of our studies have used TNF or UV light, which rapidly (within 2–3 h) induce apoptosis in U937 cells. In this system, one can prepare populations in which >90% of the cells are undergoing apoptosis, and thus U937 is a valuable resource for identifying, isolating, and characterizing enzymes activated during this process. Thus far, our studies indicate that both TNF and UV light activate signals converging to a final common pathway leading to DNA fragmentation. This process does not require protein synthesis (12) or extracellular calcium, in agreement with a previous report (14). PMA inhibits apoptosis induced by TNF (15) or UV light, suggesting a regulatory role for protein kinase C in agreement with other examples of apoptosis (16–20). Augmentation of intracellular protein phosphorylation by inhibitors of serine/threonine-dependent phosphatases promoted TNF-induced apoptosis and overcame the resistance of a U937 variant (21), suggesting a critical role for protein kinases in signal transduction. This is further supported by the finding that an inhibitor of certain serine/threonine-dependent protein kinases (KT5926) blocked TNF- (21) and UV light-induced apoptosis (our unpublished observations). However, the specific kinase involved has not yet been identified. Apoptosis in U937 cells is also blocked by 3-aminobenzamide (12), an inhibitor...
Materials and Methods

Cell Lines. The human histiocytic lymphoma, U937, the human mammary carcinoma BT-20, the human myelocytic leukemia HL-60, and the murine fibroblast cell line 3T3 were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in antibiotic-free RPMI 1640 supplemented with 10% FCS and L-glutamine (2 mM). All cell lines were routinely tested for mycoplasma and always found to be negative according to the Mycotect kit (GIBCO BRL, Gaithersburg, MD).

Reagents. Purified human TNF (sp act = 10^10 U/mg) was purchased from R&D Systems, Inc. (Minneapolis, MN). DNase I was purchased from Worthington Biochemical Corp. (Freehold, NJ), and micrococcal nuclease (116 U/mg) was from Calbiochem Corp. (San Diego, CA). All substrates for the protease assays and protease inhibitors (except DK120) were purchased from Sigma Chemical Co., and human leukocyte elastase was from Calbiochem Corp.

DNA Fragmentation Assay. This assay was described in detail previously (12). Briefly, target cells were labeled with [3H]thymidine (5 Ci/mmol) and plated in triplicate in flat-bottomed microtiter plates. Cells were incubated for the indicated length of time for each experiment in the presence of TNF or various inhibitors. In some experiments, cells were treated with UV light at 245 nm by use of a UV cross-linker (Fisher Scientific Co., Pittsburgh, PA). Assays were terminated and the radioactivity counted on a beta counter (model Matrix 96; Packard Instrument Co., Inc., Meriden, CT). Percent DNA fragmentation was calculated as follows: [(total cpm-test cpm)/total cpm] x 100. The spontaneous release of [3H]thymidine relative to the total counts at the initiation of the assay did not exceed 2%/h for up to 7 h.

Visualization of DNA Fragmentation on Gel Electrophoresis. This assay was described in detail previously (12). The assay was adapted to assess the effects of compounds on DNA fragmentation in isolated nuclei. Nuclei were prepared from normal unlabeled U937 cells as described above. After the desired treatments of aliquots of 5 x 10^6 nuclei, 1.0 ml of harvesting buffer was added, and debris was removed by centrifugation at 13,000 g for 10 s. DNA in the supernatant was ethanol precipitated after phenol extraction. Equivalent amounts of material from a fixed number of cells were loaded and electrophoresed on a 1.0% agarose slab gel. DNA was visualized by ethidium bromide staining.

Nuclease Assay with U937 DNA as Substrate. [3H]thymidine-labeled DNA was prepared by incubating U937 cells with isotope at 0.5 μCi/ml for 24 h. The cells were washed, and DNA was isolated according to a previously published procedure (25). [3H]labeled U937 DNA was diluted to 75 μg/ml in 50 mM Tris, 10 mM MgSO4, 1 mM CaCl2, pH 7.5. 0.05 ml of substrate and 0.05 ml of sample were mixed in Eppendorf tubes and incubated at 37°C for 20 h. The assay was terminated by the addition of 0.05 ml of 5.0% BSA plus 0.05 ml of 7% perchloric acid and placed on ice for 15 min. The intact DNA was pelleted by centrifugation at 13,000 g for 15 min, and the radioactivity in 0.1 ml of the supernatant was assessed by scintillation counting. The percentage of DNA fragmentation was calculated as the counts per minute in the supernatant divided by the total counts per minute of the original DNA added x 100.

Protease Assays. Proteolytic activity was assayed with synthetic substrates selective for different known proteases. All assays were set up by adding 0.02 ml of sample at the desired concentration (or known enzyme as a positive control) plus 0.18 ml of appropriate substrate dissolved in PBS, pH 7.5, in triplicate in flat-bottomed microtiter plates. After incubation for the indicated length of time at room temperature, the OD at 405 nm was measured with a plate reader.

To measure chymotrypsin-like activity, we used N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAP) at 0.1 mM as a substrate. Trypsin-like activity was measured by use of N-α-benzoxylcarbonyl-4-lysine thiobenzyll ester (BLT) at 0.2 mM plus 0.11 mM nitrobenzic acid.

Elastase-like activity was measured with N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MAAPV) at 0.25 mM used as a substrate. For convenience and maximum sensitivity, these assays were routinely incubated for 20 h to monitor protease purification. Under these conditions, the dye response of commercially obtained leukocyte elastase was linear up to a concentration of 6 μg/ml, producing an OD_405 of 1 in the protease assay. The activity of the protease sample during purification did not exceed an OD_405 of 1 under these assay conditions.
To quantitate the activity of the purified protease, identical assays were set up, but kinetic readings of OD40s were taken every hour from 1 to 6 h. U of enzyme activity was calculated as the amount of enzyme that hydrolyzes 1 nM substrate/h.

Asp-ase activity was measured by use of the tert-butylxocarbonyl-Ala-Ala-Asp-thiobenzyl ester substrate (Enzyme Systems Products, Dublin, CA). Protease samples (0.02 ml) were added to 0.18 ml from 1 to 6 h. 1 U of enzyme activity was calculated as the amount of optical density at 405 nm. A control for nonspecific indicator substrate and incubated at room temperature for 20 rain before reading was performed by omitting the substrate from the mixture. Negative controls contained all assay components except the protease.

Preparation of DK120 Affinity Resin. The synthesis and characterization of the boronic acid amino acid analogue protease inhibitor (carbobenzoxy-Ala-Ala-borophenylpropionate; DK120) as well as the non-inhibitory boronic acid compound isopropylboronic acid (IBA), was described previously (26, 27). DK120 is a tripeptide reversible inhibitor of chymotrypsin in which a boronic acid takes the place of COOH at the site of enzymatic serine OH attack. Affinity resin was prepared in a manner similar to that described previously (28, 29), except that epoxy-activated Sepharose 6B was used instead of cyanogen bromide-activated resin (30). DK120 was N deprotected by hydrogenation in the presence of 5% Pd/carbon (10% weight) in 95% ethanol. Catalyst was removed by filtration through celite, and the ethanol was evaporated. The resulting oily residue was dried under vacuum for at least 1 h to remove excess alcohol. The deprotected product was dissolved in dimethylformamide (DMF) for coupling to the resin. Sepharose 6B (Sigma Chemical Co.) was swollen with water and then reacted with DK120 at pH 10 in 0.1 M NaOH/DMF (1:1) for 16–24 h at 37°C. The resin was subsequently washed with DMF followed by water. The derivatized resin was then treated with 1 M ethanolamine at room temperature for 4 h. The resin was then rinsed sequentially with water, acetate buffer, pH 4.0, and finally with borate buffer, pH 8.0. Affinity resin was stored in 20% ethanol until use. A control resin was prepared that lacked the DK120 ligand but was treated with ethanolamine and washed as described above.

Preparation of Cell Lysates for Protease Purification. Normal or UV light-treated (0.2 J/cm² in a UV cross-linker) cells were pelleted and resuspended at 2 X 10⁶/ml in ice-cold lysing buffer (50 mM Tris, 0.3% NP-40, pH 7.0). The debris was pelleted by centrifugation in a microfuge at 14,000 g for 15 min. The supernatant was used immediately or stored at −70°C until further purification.

DK120 Affinity Chromatography. 1 ml of affinity resin was equilibrated with starting buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1.0 mM CaCl₂, 0.1% NaN₃, 0.05% Tween 20). Cell lysate was mixed with the resin and allowed to bind for 60 min. The column was then washed with starting buffer at 0.5 ml/min until the OD₃₈₀ returned to baseline. Bound material was eluted with starting buffer plus 0.1 M HCl, pH 4.5. During elution, 1-ml fractions were collected and immediately neutralized to pH 7.5. The column was regenerated by washing with 2 M NaCl (no additional protease was eluted during this step).

Heparin-Sepharose Affinity Chromatography. Heparin-Sepharose (Pharmacia Inc., Piscataway, NJ) was equilibrated in starting buffer (50 mM Tris, pH 7.5, 1.0 mM CaCl₂, 0.05% Tween 20, 0.1% NaN₃). Active fractions eluted from the DK120 column were applied and then washed with starting buffer at 0.5 ml/min until the OD₃₈₀ returned to baseline. Bound material was eluted with a linear gradient from 0 to 25 M NaCl in starting buffer. 1-ml fractions were collected and tested for protease activity. All flow-through fractions were negative for protease activity, whereas proteases showing activity against the MAAPV substrate eluted at 1.0–1.5 M NaCl.

Reverse Phase HPLC. Separations were performed on a liquid chromatography system (model 325; Waters Associates, Milford, MA) with a 3-cm × 2.1-mm cartridge (reverse phase C4; Brownlee, Applied Biosystems, Inc., Foster City, CA). Buffer A was water plus 0.1% TFA, and buffer B was 80% 2-propanol plus 0.1% TFA. Bound material was eluted by use of a linear gradient from 75% A/25%B to 35%A/65%B over 60 min at a flow rate of 0.2 ml/min. 0.2-ml fractions were collected and immediately neutralized to pH 7.5. All fractions were tested for protease activity as well as nuclear DNA fragmenting activity.

SDS-PAGE. Protease purity was assessed with 15% Laemmli SDS-PAGE followed by silver staining (Daiichi II kit; ISS, Hyde Park, MA).

Amino Acid Analysis. The amino acid composition was determined at the Stanford University Medical Center Protein and Nuclear Acid Facility (Stanford, CA) by use of an analyzer (model 6300; Beckman Instruments, Inc., Fullerton, CA). Liquid phase hydrolysis was performed at 110°C for 24 h under vacuum.

Results

Certain Serine Protease Inhibitors Suppress DNA Fragmentation in Cells Undergoing Apoptosis in Response to TNF or UV Light. To examine the role of proteases in the mechanism of apoptosis, we tested the effects of a variety of protease inhibitors on DNA fragmentation induced by TNF or UV light in the U937 cell line. Two serine protease inhibitors, N-1-tosylamide-2-phenyllycloromethyl ketone (TPCK) and DK120, were found to be potent inhibitors of DNA fragmentation. U937 cells were pretreated with the inhibitors for 1 h, then exposed to TNF (Fig. 1 A) or UV light (Fig. 1 B) at doses that preliminary studies established to be in the linear portion of the dose–response curve. Both DK120 and TPCK could dose dependently inhibit DNA fragmentation, with maximum suppression occurring at 2 μM. TPCK is a known potent inhibitor of chymotrypsin-like enzymes, whereas DK120 is a boronic acid–containing tripeptide substrate analog that also potently inhibits chymotrypsin-like enzymes (26, 27). Isopropylboronic acid, a boronic acid analog devoid of protease inhibitory activity, does not inhibit DNA fragmentation (not shown), indicating that the inhibition by DK120 is not a property of any boron compound.

Additional studies revealed that not all inhibitors of serine proteases can suppress DNA fragmentation since N-α-p-tosyl-l-lysine-chloromethyl (TLCK), N-α-p-tosyl-l-arginine methyl ester (TAME), PMSF, chymostatin, diisopropyl fluorophosphate, benzamide, and leupeptin were without effect (data not shown). In addition, the inhibitor of sulfhydryl-dependent enzymes, N-εthylmaleimide, and the aminopeptidase inhibitor bestatin, were inactive. It should be noted that in this type of experiment, negative results are inconclusive since they may be due to inefficient penetration of the inhibitor to the presumably intracellular site of protease action.

Further experiments were performed to determine if these findings were unique to U937 or if other cell lines also require protease activity to undergo apoptosis. We tested the
light-induced DNA fragmentation as HL-60 and U937 cells, they received a higher UV dose and longer incubation time. The results demonstrate that both TPCK and DK120 effectively blocked DNA fragmentation. Taken altogether, these results support the hypothesis that the activity of a serine protease(s) is essential for at least one apoptotic pathway operating in different cell types responding to different stimuli.

All inhibitors used in these studies were at nontoxic concentrations for the duration of the assays, as determined by trypan blue exclusion.

**Affinity Purification of the U937 Protease.** Previous studies showed that adsorption to an immobilized protease inhibitor can be used successfully to purify proteases (29). Therefore, to purify the putative apoptotic protease, a DK120 affinity column was prepared as described in Materials and Methods. Preliminary experiments verified that this matrix efficiently binds commercially available chymotrypsin, which could be eluted with 0.1 M HCl. After neutralization, the eluted material still exhibited high levels of protease activity measured on the SAAPP substrate (data not shown).

To prepare starting material, U937 cells were exposed to UV light (0.2 J/cm²) and incubated at 37°C until ~50–70% of the cells exhibited the apoptotic morphology (this usually required 1.5–2 h incubation). Numerous cytoplasmic membrane blebs characteristic of apoptosis were easily discernable by light microscopy as documented previously (12). Cells were harvested at this time point while they were >95% viable by trypan blue exclusion, however, 90–100% of the cells were destined to die if the incubation were continued another 2–3 h. Cytoplasmic extracts were prepared from apoptotic as well as normal untreated U937 cells. Ma-
terial from equal numbers of control and UV light-treated cells containing equal amounts of protein were chromatographed in an identical fashion on DK120 affinity columns. The results in Fig. 2 show that most of the protein as detected by absorption at 280 nm passed through the column in unbound fractions 2-17, whereas a relatively small amount of material eluted with 0.1 M HCl. Proteolytic activity present in the eluted fractions was tested by use of several different synthetic substrates. The eluate from UV light-irradiated cells showed high proteolytic activity against the elastase substrate, MAAPV, but relatively low activity against the chymotrypsin substrate, SAAPP, or the trypsin substrate, BLT. The affinity-purified protease from irradiated cells was also inactive on the Asp-ase substrate, Ala-Ala-Asp-thiobenzyl ester (not shown). The eluate from control cells was also inactive on the Asp-ase substrate, Ala-Ala-Asp-thiobenzyl ester (not shown). Therefore, proteolytic activity was monitored during protein purification with the MAAPV substrate. To maximize sensitivity, this assay was carried out at room temperature for 20 h. However, the dose response of commercially available leukocyte elastase was still linear up to an OD of 1, even at this prolonged incubation time (not shown).

The profiles of protease activity showed high levels in the unbound fractions, suggesting that the columns may have been overloaded. However, when the flow-through fractions were pooled and reapplied to the DK120 column, all protease activity was still found in the unbound fractions and none in the eluate (data not shown). Therefore, it is concluded that lysates from both control and UV light-treated U937 cells contain substantial amounts of proteases that do not bind to the DK120 column. It is possible that proteases found in the unbound fractions were also activated during apoptosis. However, since our primary objective was to characterize the DK120-binding protease, the enzymes in the unbound fractions were not further analyzed in this study.

The protease activity profiles suggested that enzyme activity in the eluate from UV light-irradiated cells was significantly increased over that recovered from control cells. To quantitate activity, the active eluted fractions from each column were pooled separately and tested in a kinetic assay. The results presented in Fig. 3 show a linear increase in optical density monitored from 1 to 6 h. Units of enzyme activity calculated as described in Materials and Methods revealed a total recovery of 27.8 U from control cells versus 248 U from UV light-irradiated cells. These results indicate that UV light treatment caused almost a 10-fold increase in the activity of DK120-binding enzymes. We were unable to isolate any UV light-activated protease using control columns of Sepharose 6B, indicating that this enzyme does not bind to the unconjugated resin (data not shown).

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![Figure 3](image-url)

Figure 3. Kinetic assay to quantitate DK120-binding protease. Active fractions of protease purified in the figure from normal U937 cells (A) or UV light-irradiated cells (B) were pooled and tested in a kinetic protease assay against the MAAPV substrate.

![Figure 4](image-url)

Figure 4. Semipurified protease activates DNA fragmentation in isolated nuclei. (A) Protease preparations pooled as described in Fig. 3 were tested at the indicated dilutions along with several commercially available proteases for induction of DNA fragmentation in isolated U937 nuclei as described in Materials and Methods. (B) The protease pool isolated from UV light-irradiated cells was tested in the presence of the protease inhibitors DK120 and TPCK at 50 μM and α1 antiprotease at 5 μg/ml.
**Semipurified Protease Activates DNA Fragmentation in Isolated U937 Nuclei.** The semipurified protease in material pooled as described in Fig. 3 did not induce apoptosis in whole U937 cells (not shown). However, when incubated with isolated U937 nuclei, the results presented in Fig. 4 A show that both samples caused DNA fragmentation, although the pool derived from UV light–treated cells had much higher activity than that from control cells. The highest total protein concentration of the pools tested was 80 μg/ml, only a fraction of which was attributed to the semipurified protease. In contrast, elastase, chymotrypsin, and trypsin, which tested at concentrations as high as 0.1 mg/ml, were completely inactive. This argues against the possibility that DNA-fragmenting activity is a nonspecific effect of any protease incubated with isolated nuclei. Furthermore, the nuclear DNA-fragmenting activity of protease isolated from UV light–treated cells was inhibited by DK120, TPCK, and an α1 antiprotease (Fig. 4 B). This suggests that the semipurified protease may directly or indirectly activate a nuclease endogenous to U937 nuclei. Alternatively, it may be argued that the protease modifies chromatin structure to make the DNA more susceptible to digestion by a nuclease that may contaminate the protease preparation. This possibility was tested by incubating both pools with naked 3H-labeled DNA isolated from U937 cells. The results shown in Table 2 indicate that neither pool could digest DNA, in contrast to the commercially available nucleases tested in parallel. The sensitivity of the assay is demonstrated by the fact that DNase I at only 0.1 ng/ml and micrococcal nuclease at 2 U/ml gave clearly detectable signals. Therefore, it is unlikely that a contaminating nuclease accounts for the ability of the protease preparations to activate DNA fragmentation in isolated nuclei.

During apoptosis, endonucleases cleave DNA in the linker regions to release fragments in multiples of 180 bp. Therefore, gel electrophoresis was used to analyze DNA extracted from isolated nuclei after exposure to DK120 affinity-purified protease obtained from U937 cells exposed to UV light. The results show that isolated nuclei incubated with the semipurified protease released internucleosomal-sized DNA fragments to produce the electrophoretic “ladder” pattern typical of apoptosis (Fig. 5, lane 2). However, if the protease preparation was pretreated with α1 antiprotease, DK120, or TPCK for 1 h before addition to the nuclei, DNA fragmentation was abolished (Fig. 5, lanes 4–6). The DNA from untreated nuclei (lane 3) or from nuclei treated with protease inhibitors alone (lanes 7–9) remained in high molecular weight form. These results confirm that a DK120-binding protease can activate internucleosomal DNA cleavage in isolated U937 nuclei.

**Table 2. Semipurified Protease Preparations Do Not Have DNase Activity**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Percent DNA Fragmentation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937 control protease</td>
<td>0</td>
</tr>
<tr>
<td>UV light–activated</td>
<td>0</td>
</tr>
<tr>
<td>U937 protease</td>
<td>0</td>
</tr>
<tr>
<td>DNase 1 1.0 ng/ml</td>
<td>61</td>
</tr>
<tr>
<td>DNase 1 0.1 ng/ml</td>
<td>29</td>
</tr>
<tr>
<td>DNase 1 0.01 ng/ml</td>
<td>11</td>
</tr>
<tr>
<td>Micrococcal nuclease 50 U/ml</td>
<td>72</td>
</tr>
<tr>
<td>Micrococcal nuclease 10 U/ml</td>
<td>65</td>
</tr>
<tr>
<td>Micrococcal nuclease 2 U/ml</td>
<td>50</td>
</tr>
<tr>
<td>Micrococcal nuclease 0.4 U/ml</td>
<td>0</td>
</tr>
</tbody>
</table>

* Affinity-purified protease was tested at a final dilution of 1:2 in the assay.
† DNase activity was measured using purified U937 DNA as a substrate as described in Materials and Methods.
Figure 5. Western blot analysis of purified 24-kD U937 protease. Protease was purified from UV light-treated U937 cells, and the active fraction 41 from the reverse phase separation shown in Fig. 6 was analyzed by SDS-PAGE under non-reducing conditions (identical results were observed under reducing conditions, not shown).

Discussion

This investigation examined the role of protease activity in the mechanism of apoptosis. The findings indicate that a 24-kD protease is involved in activating DNA fragmentation in U937 cells undergoing apoptosis. This conclusion is supported by the following lines of evidence. (a) DNA fragmentation in U937 and several other cell lines exposed to TNF or UV light is suppressed by the protease inhibitors TPCK and DK120. (b) Activation of apoptosis by UV light caused an ~10-fold increase in the activity of a protease isolated by binding to DK120 affinity columns. (c) Purification to homogeneity revealed a single peptide of 24 kD that had protease activity and also activated DNA fragmentation in isolated U937 nuclei.

These findings provide the basis for a model of the mechanism of apoptosis in U937 cells. We propose that normal U937 cells contain the 24-kD protease in an inactive proenzyme form or else in an active form that is normally sequestered from its substrate. This is supported by the observation that DNA fragmentation in U937 cells triggered by TNF (12)
Table 3. Purification of a 24-kD U937 Protease

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein mg</th>
<th>Protease activity U/ml</th>
<th>Total protease U</th>
<th>Specific activity U/mg</th>
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<tbody>
<tr>
<td>Cell lysate</td>
<td>245</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DK120 affinity</td>
<td>4.10</td>
<td>27.6</td>
<td>4,960</td>
<td>1,210</td>
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<tr>
<td>Heparin-Sepharose</td>
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<td>38.9</td>
<td>777</td>
<td>5,557</td>
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<tr>
<td>Reverse phase HPLC C4</td>
<td>0.0018</td>
<td>95</td>
<td>507</td>
<td>28,167</td>
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</table>

ND, not determined.

or UV light (our unpublished observations) is not blocked by inhibitors of protein synthesis. Low levels of a DK120-binding protease were recovered from normal U937 cells. This may be due to the artifactual activation of a proenzyme during cell lysis and purification. Alternatively, it may reflect the low incidence of apoptosis occurring spontaneously in cell culture.

Agents inducing apoptosis, such as UV light, may directly or indirectly activate the 24-kD protease. It is known that UV light can cause structural modifications to proteins and could conceivably activate a latent form of the 24-kD protease. Alternatively, UV light may activate a protease inhibitor that would normally function to protect a cell from apoptosis. If the UV light effect is indirect, the signal could be transduced by second messengers that activate the protease (or inactivate the postulated protease inhibitor). Such signals could involve protein phosphorylation, since UV light has been shown to activate certain protein kinases participating in the UV response in other cells (31), and ionizing irradiation activates protein kinases leading to apoptosis in B lymphocytes (32). Alternatively, the UV signal may be transduced through the generation of free radicals, which are known to modify the function of many proteins. This possibility is supported by our unpublished observations that free radical scavengers (e.g., ascorbate, iron chelators, etc.) can protect U937 and other tumor cells from UV light–induced apoptosis.

Once activated, the 24-kD protease presumably acts on a substrate located in the nucleus, since we have shown that this enzyme activates DNA fragmentation in isolated U937 nuclei. One possible substrate is an endonuclease that would normally be latent in the nucleus. Alternatively, the protease may cleave other molecules that in turn activate endogenous nucleases. One possible candidate is the nuclear enzyme pADPRp. This enzyme is activated in cells undergoing apoptosis, and its inhibitors block DNA fragmentation in U937 cells (12). Furthermore, a recent report indicates that pADPRp is proteolytically cleaved, yielding enzymatically active fragments in cells undergoing apoptosis induced by chemotherapeutic drugs (33). However, a role for pADPRp in mediating nuclear events leading to DNA fragmentation has yet to be established.

Previous studies have reported both generalized proteolysis (34) as well as specific proteolytic processing of precursor IL-1 (35) in cells undergoing apoptosis. However, in these studies, experiments were not performed to address the question of whether the protease activity is an essential step in the apoptotic pathway or just an epiphenomenon. More recently, it has been shown that overexpression of recombinant IL-1β–converting enzyme (ICE) induced apoptosis in rat fibroblasts (36), implicating this protease in signaling DNA fragmentation. ICE is a cysteine protease consisting of two active 20- and 10-kD subunits (37) that requires Asp at the P1 site (38). Since proteolytically active ICE was isolated from the cytosol of monocytes and the monocyte line THP-1 (39), it appears that synthesis of this protease is not sufficient to induce apoptosis in any cell line. The possible interaction of ICE with other proteases (e.g., with 24-kD U937 protease) required for signaling apoptosis deserves further investigation.

Table 4. Amino Acid Composition

<table>
<thead>
<tr>
<th>Residue</th>
<th>Residues/Molecule*</th>
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</thead>
<tbody>
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<tr>
<td>Thr</td>
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</tr>
<tr>
<td>Ser</td>
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</tr>
<tr>
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<tr>
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<td>Phe</td>
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</tr>
<tr>
<td>His</td>
<td>7</td>
</tr>
<tr>
<td>Lys</td>
<td>8</td>
</tr>
<tr>
<td>Trp</td>
<td>ND</td>
</tr>
<tr>
<td>Arg</td>
<td>13</td>
</tr>
</tbody>
</table>

* The number of residues/molecule was calculated based on a molecular mass of 24 kD.
† ND, not determined. Acid hydrolysis does not allow quantitative recovery of tryptophan.

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Other studies that used protease inhibitors have implicated a role for proteolysis in apoptosis. In agreement with our findings, it was reported that TPCK inhibits chemotherapeutic drug–induced apoptosis in HL-60 cells (40). However, since that study did not purify and characterize the putative protease, it is not clear whether the observed effects could be attributed to a protease comparable to the one purified in this investigation.

The suppressive effects of certain protease inhibitors have implicated the involvement of a serine protease in TNF-mediated tumor cell lysis (41, 42). However, neither report indicated whether the target cells (L929 fibroblasts and SK-MEL-109) died by necrosis or apoptosis, which is a critical issue since TNF can induce either mode of cell death in different target cell types (43). A more recent report indicated that expression of increased levels of plasminogen activator inhibitor-2 in HT-1080 fibrosarcoma cells correlated with resistance to lysis by TNF (44). Again, it was not specified whether the sensitive cells died by apoptosis, nor was the putative protease isolated.

Evidence from a recent study of the effect of a variety of protease inhibitors on T lymphocyte apoptosis induced by antibodies against the T cell receptor suggested two proteases may be involved (45). One was postulated to be calpain or some other cysteine protease, whereas the other appeared to be a serine protease inhibited by diisopropyl fluorophosphate or PMSF. Since the serine protease was not isolated and characterized, it is not known if it may be related to the U937 protease. Taken together, these findings raise the possibility that apoptosis signal transduction may involve a proteolytic cascade. Indeed, we have unpublished evidence for activation of multiple proteases that cleave substrates different from the elastase-like substrate preferred by the 24-kD protease from apoptotic U937 cells. It is now clear that mechanisms of apoptosis may differ depending on the inducing agent, and therefore the protease described in this communication may be unrelated to enzymes involved in anti-T cell receptor–induced apoptosis (45).

Analysis of the mechanism of cell-mediated cytotoxicity has led to the discovery of several proteases that may be involved in apoptosis in this system. According to the granule exocytosis model of cell-mediated cytotoxicity (for review see references 46, 47), after recognition of the target cell, the effector cell (CTL or NK cell) releases cytoplasmic granules that contain lytic mediators, including cytolysin and a family of serine proteases (granzymes). Cytolysin is a pore-forming molecule that may act to promote entry of the proteases into the target cell. Recent studies have shown that in the presence of cytolysin, or using detergent-permeabilized target cells, purified granzyme A/fragmentin 1 (48, 49) and fragmentins 2 (50) and 3 (49) can activate DNA fragmentation. Further studies suggest that fragmentin 2 may act by inducing the premature activation of the p34γt2 protein kinase that normally functions to control cell entry into mitosis (51). However, the characteristics of these enzymes, summarized in Table 5, indicate that they are clearly distinct from the protease we isolated from U937 with regard to molecular weight and substrate preference. Granzyme B and fragmentin 2 cleave tripeptide thiobenzyl ester substrates after aspartic acid, in contrast to the 24-kD U937 protease, which lacks Asp-ase activity. Further evidence suggests that our enzyme is not closely related to human granzyme B since the latter was reported to be inactive on methoxysuccinyl-Ala-Ala-Pro-Val-thiobenzyl ester (52), a substrate preferred by elastase-like enzymes. Granzyme A/fragmentin 1 and fragmentin 3 are tryp-tases that cleave the BLT substrate. The completely purified U937 protease was inactive on the BLT substrate while mediating high activity on a synthetic substrate preferred by elastase-like enzymes. U937 cells are known to contain high levels of leukocyte elastase that can be isolated in two forms of 30 and 60 kD (53). In addition to the difference in molecular weight, the fact that commercially available leukocyte elastase

Table 5. The U937 24-kD Protease Differs from Leukocyte Elastase and Other Proteases Implicated as Inducers of DNA Fragmentation

<table>
<thead>
<tr>
<th>Protease</th>
<th>Source</th>
<th>Molecular Mass</th>
<th>Substrate</th>
<th>Homolog</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 kd protease</td>
<td>Humn U937 cells</td>
<td>24 kD reduced or nonreduced</td>
<td>Ala-Ala-Pro-Val</td>
<td>Fragmentin 1</td>
<td>47</td>
</tr>
<tr>
<td>Granzyme A/Hanakah</td>
<td>Human CTL</td>
<td>30 kD reduced</td>
<td>BLT</td>
<td>Fragmentin 2</td>
<td>50</td>
</tr>
<tr>
<td>factor</td>
<td></td>
<td>60 kD nonreduced</td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>CCPI/Granzyme B</td>
<td>Murine CTL</td>
<td>35 kD reduced</td>
<td>Asp-ase</td>
<td>Fragmentin 2</td>
<td>50</td>
</tr>
<tr>
<td>Fragmentin 1</td>
<td>Rat NK cell</td>
<td>30 kD reduced</td>
<td>BLT</td>
<td>Granzyme A</td>
<td>48</td>
</tr>
<tr>
<td>Fragmentin 2</td>
<td></td>
<td>31 kD nonreduced</td>
<td>Asp-ase</td>
<td>CCPI/Granzyme B</td>
<td>49</td>
</tr>
<tr>
<td>Fragmentin 3</td>
<td>Rat NK cell</td>
<td>27 kD nonreduced</td>
<td>BLT</td>
<td>Granzyme 3</td>
<td>48</td>
</tr>
<tr>
<td>Leukocyte elastase</td>
<td>Human U937 cells</td>
<td>30 kD reduced</td>
<td>Ala-Ala-Pro-Val</td>
<td>None</td>
<td>53</td>
</tr>
<tr>
<td>ICE</td>
<td>Monocytes</td>
<td>10- and 20-kD subunits</td>
<td>IL-1β at Asp16-Ala47'</td>
<td>None</td>
<td>36, 37</td>
</tr>
</tbody>
</table>

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cannot induce DNA fragmentation in U937 nuclei (Fig. 4A) indicates that the protease we purified is not identical to leukocyte elastase. It is likely that the high levels of protease activity that did not bind to the DK120 affinity column may be attributed in part to leukocyte elastase.

It is somewhat surprising that the DK120-binding protease did not cleave the chymotrypsin substrate, SAAPP, which, like DK120, contains Phe at P1. This suggests that the peptide-binding activity of this enzyme does not strictly correlate with proteolytic substrate specificity. Work underway to clone and express the gene for this enzyme should provide quantities of protease sufficient for further characterization of substrate preferences and other activities. To the best of our knowledge, the 24-kD U937 protease appears to be distinct from other known proteases. Efforts are underway to repurify this enzyme and obtain amino acid sequences from tryptic fragments. The results of this investigation provide the basis for further analysis of the mechanism of action of this protease in the U937 model system as well as exploration of its involvement in other examples of apoptosis.

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