Activation of CPP32-like Proteases Is Not Sufficient to Trigger Apoptosis: Inhibition of Apoptosis by Agents that Suppress Activation of AP24, but Not CPP32-like Activity

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Summary

The 24-kD apoptotic protease (AP24) is a serine protease that is activated during apoptosis and has the capacity to activate internucleosomal DNA fragmentation in isolated nuclei. This study examined the following: (a) the functional relationship between AP24 and the CPP32-like proteases of the caspase family; and (b) whether activation of CPP32-like proteases is sufficient to commit irreversibly a cell to apoptotic death. In three different leukemia cell lines, we showed that agents that directly (carbobenzoxy-Ala-Ala-borophenylalaninechloromethylketone, and caspase inhibitors) protected cells from apoptosis induced by TNF or UV light. Only the caspase inhibitors, however, prevented activation of CPP32-like activity as revealed by cleavage of the synthetic substrate, DEVD-pNa, by cell cytosols, and also by in vivo cleavage of poly (ADP-ribosyl) polymerase, a known substrate of CPP32. Activation of DEVD-pNa cleaving activity without apoptosis was also demonstrated in two variants derived from the U937 monocytic leukemia in the absence of exogenous inhibitors. Cell-permeable peptide inhibitors selective for CPP32-like proteases suppressed AP24 activation and apoptotic death. These findings indicate that CPP32-like activity is one of several upstream signals required for AP24 activation. Furthermore, activation of CPP32-like proteases alone is not sufficient to commit irreversibly a cell to apoptotic death under conditions where activation of AP24 is inhibited.

The process of apoptosis, which normally functions to maintain homeostasis in cell populations, can be initiated by a variety of stimuli, including withdrawal of growth factors, various cytokines, ionizing radiation, and cytotoxic drugs (for review see reference 1). Although these diverse stimuli initially engage different signal transduction pathways, it is postulated that they may converge to activate a final common death pathway found in all forms of apoptosis. In contrast, it is possible that different parallel pathways are activated by different stimuli that do not overlap from the initial stimulus to the ultimate death of the cell. The actual cause of death or the point in the pathway where the cell is irreversibly committed to die has not yet been established.

Intense investigation into the biochemical mechanism of apoptosis has produced evidence implicating a variety of enzymes and signal transduction molecules, including protein kinases and phosphatases, sphingomyelinase (SMase), transglutaminase, ADP ribosyl transferase (ADPR T), and endonucleases (for review see reference 2). Many recent studies have focused on the role of proteases that may function in either signal transduction or as the mediators of apoptotic cell death (for review see references 3 and 4). The best characterized of these proteases is the family of interleukin-1β converting enzymes (ICE), now called caspases. The growing family of ICE-related proteases consists of at least 10 members that can be divided into 3 subfamilies: ICE (caspases 1,4,5), CPP32 (caspases 3,6,7,8,10), and Ich-1 (caspases 2,9) (for review see reference 5). All these en-

1Abbreviations used in this paper: ADPR T, ADP ribosyl transferase; CaM-K II, calcium/calmodulin-dependent protein kinase II; cpDEVD-CHO, cell-permeable Asp-Glu-Val-Ala-dehydroalaninechloromethylketone; ICE, interleukin-1β converting enzyme; PARP, poly (ADP-ribosyl) polymerase; SMase, sphingomyelinase; TPCK, tosylphenylalananinechloromethylketone; VAD-cmk, acetyl-Val-Ala-Asp-chloromethylketone.

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zymes are synthesized as inactive proenzymes that require cleavage at specific Asp residues to form the active protease. They require Asp at the P1 site, and based on their substrate specificity, synthetic substrates and inhibitors (with YVAD specific for the ICE subfamily and DEVD specific for the CPP32 subfamily) have been designed. Some of these proteases may autoprocess themselves or other members of the family, resulting in a cascade of proteolytic events that may amplify the initial apoptotic signal. Although the details have yet to be worked out, there is abundant evidence that some combination of the ICE family of proteases is part of a common core death pathway in apoptosis (reviewed in reference 6).

How the death pathway is engaged after activation of ICE-related enzymes is not clear (7). Some intracellular substrates such as poly (ADP-ribose) polymerase (PARP) have been identified that may be involved in cellular repair mechanisms (8). Whether the cleavage of these substrates is an irreversible cause of death is not known. Certainly, internucleosomal DNA cleavage could be considered an irreversible lethal event. Certain recombinant proteases such as CPP32 can cause DNA fragmentation in normal nuclei in the presence of cell cytosol, but not in the absence of cytosol (9). Thus, there appears to be a missing link between activation of caspases in the cytosol and the transmission of apoptotic signals to initiate DNA fragmentation in the nucleus. Evidence generated in our laboratory suggests that one candidate for this missing link may be AP24, which is able to activate internucleosomal DNA fragmentation in normal nuclei in the absence of cytosol (10). AP24 is activated in and was isolated from the cytosol or nuclei of U937 cells undergoing apoptosis in response to TNF or UV light (10). AP24 is a serine protease that binds to and is inhibited by carboxbenzoxo-Ala-Ala-borophene (DK120), a tripeptide boronic acid–containing inhibitor of chymotrypsin. In fact, a DK120 affinity resin was used to purify AP24 (10). While efforts are ongoing to clone the cDNA encoding this protease, recent studies have focused on the mechanism of activation of AP24 during apoptosis.

Since protein synthesis is not needed for apoptosis in the U937 model, AP24 must exist in normal cells as an inactive proenzyme, or else is active but sequestered from its substrate(s). Analysis of signal transduction events leading to activation of AP24 revealed the participation of several enzymes, some of which have been reported in other models of apoptosis. For example, activation of SMase and production of ceramide, which was previously shown to signal apoptosis in other systems (11, 12), was found to be an essential event for AP24 activation by TNF or UV light (13). ADP ribosylation reaction inhibition has been shown to block some forms of apoptosis (14–17), and NAD-dependent enzymes such as ADPRT are required to signal AP24 activation (18). Recently, we demonstrated that calcium/calmodulin-dependent protein kinase II (CaM-KII) is rapidly activated by TNF or UV light in U937 cells, and that inhibition of this enzyme prevents AP24 activation and cell death (19, 19a). A recent report described that a novel form of CaM-K functions in apoptotic signal transduction (20). These findings at least partially integrate AP24 activation with other signaling events shown previously to participate in apoptosis.

CPP32 is activated in U937 cells undergoing apoptosis in response to TNF or ionizing radiation (21). Although ICE is present in U937 cells and may be constitutively active, its activity is not increased by TNF (unlike CPP32 which is proteolytically processed to its active form in apoptotic U937 cells) (22). These studies examined two questions: (a) the possible functional relationship between the CPP32 subfamily and AP24; and (b) whether activation of CPP32-like proteases by apoptotic stimuli is sufficient to commit irreversibly a cell to apoptotic death. Our findings show that activation of CPP32-like proteases is one signal that contributes to AP24 activation. Activation of the former protease alone, however, is not sufficient to trigger apoptotic cell death under circumstances where AP24 activation is suppressed.

Materials and Methods

Cell Lines. All cell lines were obtained from the American Type Culture Collection (Rockville, MD), and were cultured in RPMI-1640 plus 10% FCS in the absence of antibiotics. All lines were routinely tested for mycoplasma contamination, and were always found to be negative. The TNF- and UV light-resistant U9-TR variant was derived by culturing parental U937 cells in the presence of TNF, and has been described previously (13, 19). The NAD-depleted U9-NAD variant was generated by culture of U937 cells for 2 wk in the absence of nicotinamide, as described in detail previously (18).

Reagents. KN62 was from Calbiochem Corp. (San Diego, CA). KT5926 was from Kamiya Biomedical Co. (Thousand Oaks, CA). Tosylphenylalaninechloromethylketone (TPCK), and the methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (AAPV-pNA) substrate were from Sigma Chemical Co. (St. Louis, MO). Tosylphenylalaninechloromethylketone (TPCK), and the methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (AAPV-pNA) substrate were from Sigma Chemical Co. (St. Louis, MO). DK120 was synthesized by Dr. D.H. Kinder (Ada, OH) as described previously (23, 24). Cell-permeable Asp-Glu-Val-Asp-adehyde (cpDEVD-CHO) was obtained from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Side chain fully methylated carboxbenzoxo-Asp-Glu-Val-Asp-chloromethylketone (DEVD-meth-cmk), acetyl-Val-Ala-Asp-chloromethylketone (VAD-cmk), and DEVD-cmk were obtained from Panorama Research (Mountain View, CA). Human recombinant bFGF was obtained from R&D Systems (Minneapolis, MN).

Measurement of DNA Fragmentation and Percent of Apoptotic Cells. To induce apoptosis, U937 cells were exposed to UV light in a Stratalinker™ or to TNF plus cycloheximide 0.5 μg/ml. Percent DNA fragmentation was determined by measuring release of 3H-labeled DNA fragments as described in detail previously (17). In some experiments the percent of morphologically apoptotic cells was enumerated microscopically as described previously (17). Morphologically apoptotic cells were defined as those exhibiting at least two or more prominent membrane protuberances. This change occurs before cell death (defined as the inability to exclude trypan blue). The kinetics of appearance of morphologically apoptotic cells correlates with DNA fragmentation as previously documented (17).

In every experiment that cells were exposed to the various inhibitors, controls were examined microscopically at the end of
the assay to verify that all inhibitors were used at nontoxic concentrations.

A assay of DEVD-pNa cleavage. CPP32-like activity was measured in the cytosol of cells treated with various inhibitors and inducers of apoptosis. Cells were lysed at a density of 2 × 10^6/ml in 50 mM Tris, pH 7.5, 0.03% N-P-40, 1.0 mM DTT. As assays were set up in flat-bottomed 96-well plates containing 0.2 mM DEVD-pNa in PBS, and 0.01 ml of cytosol in a total volume of 0.1 ml. Assays were incubated at 37°C with periodic readings of OD at 405 nm taken from 0-3 h. Specific activity was calculated as the amount of enzyme that hydrolyzes 1 nmol substrate/h per mg total protein.

Partial Purification and Quantitation of AP24 Activity. Proteolytic activity of AP24 was quantitated as described in detail previously (18). In brief, cells were incubated with or without inducing agents for 1.5 h, and lysates were prepared, from which AP24 was purified by affinity chromatography using DK120 as ligand. Total units of AP24 proteolytic activity eluted from the column were quantitated using the AAPV-pNa substrate (18). In some experiments, AP24 activity was quantitated using the nuclear DNA fragmentation assay described below. In this case, AP24 units were defined as the reciprocal of the dilution of affinity-purified enzyme required to mediate 30% DNA fragmentation in isolated nuclei.

A assay of DNA Fragmentation in Isolated Nuclei. DNA fragmentation in isolated nuclei was assessed as described in detail previously (10). [3H]thymidine-labeled U937 cells were pelleted, and the cytoplasmic membrane was lysed by resuspending the cells in assay buffer (50 mM Tris, pH 7.5, 250 mM sucrose, 10 mM M(gso) plus 0.02% N-P-40). Nuclei were then pelleted and resuspended in assay buffer at 1 × 10^9/ml. The assay was set up in triplicate in flat-bottomed microtiter plates under sterile conditions. Nuclei (0.05 ml) were mixed with 0.05 ml of sample diluted in 50 mM Tris, pH 7.5, or buffer alone, to determine total counts. Plates were incubated for 5 h at 37°C, and were then harvested by adding 0.1 ml of harvesting buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.3% Triton X-100). High molecular weight DNA was collected by filtration onto fiberglass paper, and the radioactivity was counted on a Packard liquid scintillation counter (Packard Instrs., Meriden, CT). Percent DNA fragmentation was calculated as follows: [(total cpm - test cpm)/total cpm] × 100. The spontaneous release of [3H]thymidine relative to the total counts at the initiation of the assay did not exceed 5%.

In some experiments, nuclei were further purified by centrifugation through a cushion of 30% sucrose, and were compared to nuclei isolated as above. We found that both preparations of nuclei were equally sensitive to DNA fragmentation induced by affinity-purified AP24 (unpublished observations). This finding argues against the possibility that contamination of the nuclei by cytosolic components is required for the AP24 capacity to induce nuclear DNA fragmentation.

Immunoblot Analysis. For PARP detection, 8 × 10^4 cells were lysed in sample buffer (62.5 mM Tris/HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, and 0.00125% bromophenol blue), electrophoresed in 10% denaturing polyacrylamide gels, and then transferred to Immobilon-P blotting membrane (Millipore Corp., Bedford, MA). The membranes were incubated with rabbit polyclonal antibody against PARP (Biotium, Inc., Pleasanton, CA) at 1/2,500 dilution. This incubation was followed by incubation with biotinylated goat anti-rabbit IgG (1/3,000; Gibco BRL, Gaithersburg, MD). After reaction with streptavidin-alkaline phosphatase conjugate (1/3,000; Gibco BRL), the blots were developed with Sigma-Fast BCIP/NBT solution as recommended by the vendor (Sigma Chemical Co.).

Results

Agents That Inhibit Apoptosis Do Not Necessarily Prevent Activation of CPP32-like Activity. Our studies on the mechanism of apoptosis have identified a variety of compounds that inhibit TNF or UV light–induced apoptosis in U937 cells. Since CPP32 has been shown to be activated as an essential step in TNF (22) or ionizing radiation (21)–induced apoptosis in U937 cells, experiments were performed to determine if these apoptosis inhibitors block activation of CPP32-like enzymes. In preliminary experiments, we verified that CPP32-like activity was indeed increased by treatment with TNF or UV light in U937 cells, as measured on the synthetic substrate, DEVD-pNa. Kinetic studies revealed that the peak of DEVD-pNa cleaving activity in U937 cytosol extracts is found 60–90 min after exposure to either TNF or UV light (data not shown). Therefore, in the following experiments, U937 cells were pretreated for 1 h with various inhibitors, exposed to TNF or UV light, and incubated at 37°C for 60 min. Cytosol extracts were assayed for proteolytic activity on the DEVD-pNa substrate. Aliquots of cells treated identically were incubated for 1.5 h, and then the percentage of morphologically apoptotic cells were enumerated microscopically. Our previous studies showed that the development of apoptotic morphology coincides with DNA fragmentation, which precedes cell death (measured by trypan blue exclusion) by about 1 h (17). Two serine protease inhibitors, TPCK and DK120, which we previously reported to block apoptotic DNA fragmentation (10), did not prevent TNF or UV light activation of DEVD-pNa cleaving activity, even at concentrations that completely prevented apoptosis (Table 1, experiment 1). In contrast, the broad spectrum inhibitor of the ICE family, VAD-cmk, completely prevented activation of DEVD-pNa cleaving activity, and also inhibited apoptosis, as expected (experiments 2 and 4). Other agents tested included two mechanistically different inhibitors of CaM-KII, KN62 (25) and KT5926 (26), both of which suppressed apoptosis (Table 1, 19, 19a) but had no significant effect on UV light activation of DEVD-cleaving activity (experiments 2 and 3). We also found that bFGF protected cells from apoptosis without blocking activation of CPP32-like proteases (experiment 4). All the inhibitors also suppressed DNA fragmentation, measured by release of [3H]labeled DNA fragments at 1.5 h (data not shown). These results indicate that activation of CPP32-like proteolytic activity is not sufficient to induce apoptosis within the time frame examined. This is not unique to U937 cells, since we found that DK120 and TPCK could also inhibit apoptosis in the CEM T cell leukemia and the HL-60 myeloid leukemia without preventing UV light activation of DEVD-pNa-cleaving activity (Table 2).

To confirm the results obtained with the synthetic substrate, we also examined the effects of the apoptosis inhibi-
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In vivo cleavage of PARP, a known substrate of CPP32, in apoptotic U937 cells. U937 cells were pre-treated for 1 h with the inhibitors, except for bFGF, which was pre-treated 3 h. After exposure to TNF or UV light, cells were incubated 1.5 h to determine percent apoptotic cells, and 1 h to measure cleavage of DEVD-pNa by the cytosol.

| Experiment | Cell treatment* | Apoptotic cells | DEVD-pNa cleaving activity (nmol/h/mg) |
|------------|-----------------|----------------|----------------------------------------|
| 1          |                 | %              |                                        |
| 1          | TNF 2 ng/ml     | 46             | 12.8                                   |
| 1          | TNF + DK120 2 μM| 0              | 13.1                                   |
| 1          | TNF + DK120 1 μM| 29             | 13.9                                   |
| 1          | TNF + TPCK 2 μM | 0              | 9.9                                    |
| 1          | TNF + TPCK 1 μM | 30             | 9.5                                    |
| 1          | UV light 0.06 J/cm² | 64             | 16.8                                   |
| 2          | UV light 0.06 J/cm² | 58             | 63.3                                   |
| 2          | UV light + VAD-cmk 2 μM | 12             | 2.0                                    |
| 2          | UV light + DK120 2 μM | 15             | 59.1                                   |
| 2          | UV light + TPCK 2 μM | 15             | 79.9                                   |
| 2          | UV light + KN 62 20 μM | 11             | 70.8                                   |
| 3          | UV light 0.06 J/cm² | 59             | 80.4                                   |
| 3          | UV light + KT5926 5 μM | 0              | 76.2                                   |
| 4          | TNF 2 ng/ml     | 46             | 8.8                                    |
| 4          | TNF + VAD-cmk 5 μM | 0              | 1.4                                    |
| 4          | TNF + bFGF 1.0 ng/ml | 0              | 6.3                                    |
| 4          | UV light 0.06 J/cm² | 68             | 13.1                                   |
| 4          | UV light + VAD-cmk 5 μM | 0              | 0                                      |
| 4          | UV light + DK120 5 μM | 0              | 10.5                                   |
| 4          | UV light + bFGF 1.0 μM | 0              | 17.6                                   |

*U937 cells were pretreated for 1 h with the inhibitors, except for bFGF, which was pretreated 3 h. After exposure to TNF or UV light, cells were incubated 1.5 h to determine percent apoptotic cells, and 1 h to measure cleavage of DEVD-pNa by the cytosol.

Table 1. Inhibition of Apoptosis by Agents That Do Not Prevent Activation of CPP32-like Activity in U937 Cells

Do the protective inhibitors that suppress AP24 activation actually prevent apoptosis, or do they just delay it? This cannot be tested directly since most of the inhibitors alone except for bFGF are toxic to cells upon long-term exposure. In a 24-h experiment, we found that all of the cells exposed to TNF or UV light had died, with high levels of DNA fragmentation (Table 3). Approximately 50% of cells pretreated with bFGF followed by TNF or UV light survived at 24 h, and appeared morphologically normal. Almost 50% of the bFGF-treated cells exposed to TNF or UV light, however, died a necrotic death as evidenced by trypan blue uptake in the absence of DNA frag-
mentation. Therefore, bFGF prevented apoptosis in all of the cells, and conferred 24-h survival in ~50% of the cells (as opposed to 0% survival in cells without bFGF). The fact that half the bFGF-treated cells still died indicates that apoptotic stimuli may also activate a delayed mechanistically different necrotic death that is at least partially resistant to protection by bFGF.

U937 Variants are Resistant to UV Light-induced Apoptosis, Although They Still Show Normal Activation of DEVD-cleaving Activity.

Since the above studies all used exogenous inhibitors to prevent apoptosis while leaving DEVD-cleaving activity intact, it was of interest to determine if this phenomenon can ever be observed in the absence of exogenous inhibitors. We previously described two variants derived from U937 that are both relatively resistant to apoptosis induced by either TNF or UV light. The U9-TR variant was generated by the prolonged culture of parental U937 cells in the presence of TNF (19), and the U9-NAD variant (18) was produced by nutritional depletion of nicotinamide, the biosynthetic precursor of NAD. Both of these variants responded normally to UV light-induced activation of CPP32-like protease activity with kinetics similar to that of normal U937 cells (Fig. 2 A). In contrast, both of the variants were almost completely resistant to apoptosis (Fig. 2 B), in agreement with previous findings (18, 19). Similar results were obtained when apoptosis was activated by TNF (data not shown). Although we usually incubate U937 cells for 1.5–2 h to measure morphological apoptotic changes as well as DNA fragmentation, cell death does not occur until later time points. In this experiment, 6 h after exposure to UV light, 51% of U937 cells were dead, according to trypan blue exclusion, whereas both variants were still ~95% viable. This experiment provides further evidence that activation of CPP32-like proteases is not sufficient to lead to apoptosis, even in the absence of exogenous inhibitors.

Cell-permeable Peptide Inhibitors of CPP32 Prevent TNF or UV Light Activation of DEVD-pNa Cleaving Activity and Apoptosis.

The above findings led us to question whether activation of CPP32-like proteases is, in fact, essential for apoptosis in this system. In preliminary experiments, we found that apoptosis of U937 is quite refractory to inhibition by conventional tetrapeptide inhibitors of CPP32 (e.g., DEVD-CHO), even up to 500 μM. This finding is in agreement with another report (27), and is probably due

**Table 2.** Inhibition of apoptosis by agents that do not prevent activation of CPP32-like activity in CEM and HL-60 cells

| Cell line | Cell treatment | Apoptotic cells | DEVD-pNa cleaving activity |
|-----------|----------------|-----------------|----------------------------|
| CEM       | —              | 0               | 22                         |
| CEM       | UV light 0.06 J/cm² | 48             | 83                         |
| CEM       | UV light + VAD-cmk 2 μM | 31             | 9.2                        |
| CEM       | UV light + DK120 2 μM | 18             | 53                         |
| CEM       | UV light + TPCK 2 μM | 8              | 56                         |
| HL-60     | —              | 0               | 15                         |
| HL-60     | UV light 0.06 J/cm² | 51             | 27                         |
| HL-60     | UV light + VAD-cmk 2 μM | 29             | 5.4                        |
| HL-60     | UV light + DK120 2 μM | 16             | 41                         |
| HL-60     | UV light + TPCK 2 μM | 9              | 26                         |

*Cells were pretreated for 1 h with the inhibitors. After exposure to UV light, cells were incubated 1.5 h to determine percent apoptotic cells and 1 h to measure cleavage of DEVD-pNa by the cytosol.

**Table 3.** bFGF Prevents Apoptotic Death and Partially Protects from Necrosis 24 h after Exposure to Apoptosis Inducers

| Cell treatment | Viability | DNA fragmentation |
|----------------|-----------|-------------------|
| TNF 2 ng/ml    | 0         | 86 ± 6.5          |
| TNF + bFGF 2 ng/ml | 58       | 0                |
| UV light 0.06 J/cm² | 0         | 78 ± 7.0          |
| UV light + bFGF 2 ng/ml | 50       | 0                |

*U937 cells were pretreated 3 h with bFGF before exposure to TNF or UV light. 24 h later, viability was determined by trypan blue exclusion and DNA fragmentation was measured by release of 3H-labeled DNA fragments.

**Figure 1.** Some inhibitors of apoptosis do not prevent cleavage of PARP. U937 cells were pretreated for 1 h with the indicated inhibitors, exposed to TNF or UV light, and incubated for 1.5 h. Cell lysates were analyzed for PARP by immunoblotting.
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Table 4. Inhibition of CPP32-like Proteases by the Broad Spectrum Inhibitor VAD-cmk and the Chymotrypsin Inhibitor DK120

| Inhibitors | IC50 (mM) |
|------------|-----------|
| VAD-cmk    | 0.01      |
| DK120      | 1.0       |

Discussion

Of all the caspases studied thus far, most of the evidence favors a role for CPP32 and/or other members of this sub-family as regulators of apoptosis. However, the specific role of CPP32 in the activation of apoptosis is not yet fully understood. It is possible that CPP32 may function as a mediator in the activation of other caspases, such as CPP32-like proteases, which play a crucial role in the execution of apoptosis.

Future studies will be needed to clarify the exact role of CPP32 in the apoptosis pathway, as well as to investigate the potential for targeting CPP32 as a therapeutic strategy for diseases associated with apoptosis.

Figure 2. Activation of CPP32-like proteases in normal and resistant U937 variants. (A) Normal U937 cells, TNF-selected variants (U9-TR), and NAD-depleted variants (U9-NAD) were exposed to UV light 0.06 J/cm², incubated for the indicated time, and cytosol extracts were assayed for proteolytic activity on the DEVD-pNa substrate. (B) U937 and the variants were incubated for 90 min after exposure to UV light, and the morphologically apoptotic cells were enumerated microscopically.

Specificity of Protease Inhibitors Used in This Study. Both the broad spectrum inhibitor of the ICE family, VAD-cmk, and the selective inhibitor of the CPP32 subfamily, DEVD-CHO, potently inhibited DEVD-pNa cleaving activity in the cytosol from apoptotic cells (Table 6). It should be noted that the cell-permeable inhibitor cpDEVD-CHO was just as active in vitro as was the unmodified tetrapeptide inhibitor. In contrast, the two chymotrypsin inhibitors, DK120 and TPCK, were inactive at 20 μM. This result shows that the AP24 inhibitor, DK120, does not crossreact with CPP32-like proteases found in apoptotic U937 cytosol at concentrations used in this study. Furthermore, only DK120, and not DEVD-CHO or VAD-cmk, inhibited AP24 induction of nuclear DNA fragmentation, confirming that this protease is probably not related to the caspase family.
family in the mechanism of apoptosis. One issue that has not been previously addressed is whether activation of CPP32-like activity is sufficient to commit a cell to apoptosis in response to various stimuli. Although overexpression of each of the members of the ICE family has been shown to induce apoptosis (6), this is not convincing evidence that activation of these enzymes will irreversibly commit a cell to apoptosis. These effects could be nonspecific, since introduction of high concentrations of proteases such as trypsin or chymotrypsin into the cytosol will also induce apoptosis (29). Clearly, activation of ICE can occur in monocytes engaged in the production of IL-1β in the absence of apoptotic death (30). However, a similar example of TNF or UV light activation of CPP32-like activity in the absence of apoptosis has not been reported. Thus, it is possible that activation of CPP32-like proteases is an irreversible step in the apoptotic pathway.

These studies took a different approach to evaluate the role of CPP32-like enzymes by asking whether apoptotic death could be prevented in cells that still showed full activation of DEVD-pNa cleaving activity in response to apoptotic stimuli. Treatment with various agents was shown to protect several leukemia cell lines from apoptosis induced by TNF or UV light. None of these treatments (with the exception of the caspase inhibitors), however, prevented activation of CPP32-like proteases as revealed by cleavage of the DEVD-pNa a substrate by the cell extracts. The lack of inhibition of CPP32 was confirmed in intact cells by demonstration of the cleavage of PARP to its characteristic 85-kD fragment.

This phenomenon was also observed in the absence of exogenous inhibitors in the case of the U9-TR and NAD-depleted U937 variants. These cells showed UV light activation of DEVD-pNa a cleaving activity with kinetics and magnitude identical to parental U937. Both of these variants, however, were highly resistant to TNF or UV light-induced apoptosis, and have a defect in the signaling pathway leading to activation of AP24, as shown in our previous studies (13, 18). U9-TR has a defect in signals leading to SMase activation (13), whereas NAD-depleted U937 variants appear to be resistant due to a lack of ADP-ribosylation reactions (18). According to our model, both ADPRT and SMase function upstream of activation of AP24 in the apoptotic pathway. The results with the variants show that even in the absence of exogenous inhibitors, cells may avoid apoptotic death, despite the fact that CPP32-like proteases have been activated.

This investigation also examined the morphological changes that accompany apoptosis as observed by light microscopy, which include formation of protrusions of the cell membrane, and budding of apoptotic bodies. Inhibitors that prevented apoptosis also blocked these morphological changes, even though CPP32-like proteases were still activated. Likewise, the two resistant variants did not undergo the morphological alterations of apoptosis. Therefore, activation of CPP32-like proteases alone is not sufficient to cause these morphological changes associated with apoptosis.

This raised the question of whether activation of CPP32-like proteases is necessary, or is just an epiphenomenon in this model of apoptosis. Inhibition of DNA fragmentation

| Cell treatment* | Apoptotic cells | DEVD-pNa cleaving activity | DNA fragmentation |
|-----------------|-----------------|---------------------------|-------------------|
|                 | %               | nmol/h/mg                 | %                |
| —               | 0               | 9.5                       | 0                |
| TNF 2.0 ng/ml   | 41              | 78.8                      | 48 ± 4.8         |
| TNF + cpDEVD-CHO 1.0 μM | 8 | 1.1 | 0 |
| TNF + cpDEVD-CHO 0.5 μM | 11 | 38.6 | 21 ± 0.8 |
| TNF + cpDEVD-CHO 0.25 μM | 20 | 46.5 | ND |
| TNF + cpDEVD-CHO 0.125 μM | 31 | 80.5 | ND |
| —               | 0               | 0                         | 0                |
| UV light 0.06 J/cm² | 58           | 20.4                      | 53 ± 4.2         |
| UV light + cpDEVD-CHO 1.0 μM | 11 | 3.4 | 12 ± 0.6 |
| UV light + cpDEVD-CHO 0.25 μM | 38 | 8.8 | ND |
| —               | 0               | 3.5                       | 0                |
| UV light 0.06 J/cm² | 59           | 80.4                      | 46 ± 4.6         |
| UV light + DEVD(meth)-cmk 10 μM | 8 | 12.2 | 0 |
| UV light + DEVD(meth)-cmk 5 μM | 18 | 27.8 | 0 |
| UV light + DEVD(meth)-cmk 2.5 μM | 39 | 46.6 | 15 ± 4.5 |

*U937 cells were pretreated with inhibitors for 2 h before exposure to TNF or UV light. After 1 h of incubation, cells were pelleted, washed twice in PBS, and lysed to measure protease activity on the DEVD-pNa a substrate. The percent morphologically apoptotic cells and percent DNA fragmentation was determined after 1.5 h incubation. ND, not determined.
by low micromolar concentrations of cell-permeable peptide inhibitors of CPP32 (cpDEVD-CHO and DEVD (meth)-cmk), however, indicates that this subfamily of proteases is needed for optimal apoptotic signal transduction. Therefore, we hypothesize that the protective agents that do not inhibit CPP32 must be blocking events that occur downstream of this protease, or else they interfere with a separate parallel signal transduction pathway that is independent of CPP32.

How do the protective inhibitors prevent apoptosis if they do not block activation of CPP32? Although the mechanism of action of these inhibitors is not fully understood, one property they have in common is the direct inhibition of AP24 proteolytic activity, or the suppression of activation of intracellular AP24 by apoptotic stimuli (KT5926, TPCK, and bFGF). KT5926 (26) and KN62 (25) are two selective and mechanistically different inhibitors of CaM-KII. Our previous work demonstrated that both TNF and UV light rapidly activated CaM-KII in U937 cells (19a). Furthermore, inhibitors of this kinase blocked activation of AP24 and apoptotic death (19). TPCK is a chymotrypsin inhibitor that may have multiple sites of action, however, our evidence suggests that it blocks signaling events leading to activation of AP24. It is not clear how bFGF inhibits activation of AP24, however, one possibility is through activation of protein kinase C (PKC), a mechanism previously proposed for bFGF inhibition of apoptosis (31). Indeed, we have shown that brief pretreatment with phorbol myristate acetate, which activates PKC, will inhibit TNF or UV light–induced apoptosis in U937 cells (unpublished observations). Further work is in progress to analyze the mechanism by which bFGF inhibits activation of AP24.

The capacity of AP24 to induce internucleosomal DNA fragmentation in isolated nuclei distinguishes it from several other proteases that cleave at aspartic acid residues, such as Granzyme B (32), ICE (33), and CPP32 (9). Unlike AP24, all these enzymes require the presence of cytosolic components to activate nuclear DNA fragmentation in cell-free systems. We hypothesize that AP24 may play a key role in transmitting apoptotic signals from the cytosol to the nucleus, where it directly or indirectly activates endogenous endonucleases resulting in DNA digestion. We cannot, however, rule out the possible existence of cytosolic substrates of AP24, since high levels of this protease were originally detected in apoptotic cytosol (10), and purified AP24 can cleave several cytoskeleton-associated proteins in vitro (our unpublished observations).

Until recently, AP24 was the only protease reported to cause DNA fragmentation in isolated nuclei. Now, another

| Experiment | Cell treatment* | AP24† | AP24 nuclear activity | Apoptotic cells |
|------------|----------------|-------|-----------------------|----------------|
| 1          | —              | 362   | <2                    | ND             |
| TNF 2 ng/ml|                | 1395  | 8                     | ND             |
| TNF + cpDEVD-CHO 2 μM | 777   | 2     | ND                    |
| TNF + DK120 5 μM | 805   | 3     | ND                    |
| 2          | —              | 139   | ND                    | 0              |
| TNF 5 ng/ml|                | 722   | ND                    | 48             |
| TNF + VAD-cmk 2.5 μM | 389  | ND               | 29             |
| 3          | —              | 494   | <2                    | 0              |
| UV light 0.06 J/cm² | 947  | 8     | ND                    |
| UV light + TPCK 2 μM | 606  | <2   | 0                     |
| 4          | —              | 632   | ND                    | 0              |
| TNF 2 ng/ml|                | 1554  | ND                    | 48             |
| TNF + bFGF 2 ng/ml | 996  | ND   | 11                    |
| 5          | —              | 108   | ND                    | 1              |
| TNF 5 ng/ml|                | 348   | ND                    | 56             |
| TNF + KT5926 5 μM | 126  | ND  | 6                     |
| UV light 0.08 J/cm² | 352  | ND | 67                    |
| UV light + KT5926 5 μM | 128  | ND | 8                     |

*Cells were pretreated with inhibitors for 1 h except for cpDEVD-CHO and bFGF, which were pretreated for 3 h before exposure to TNF or UV light. †AP24 activity was quantitated using the AAPV-pNa substrate or the nuclear assay after purification of cell lysates on DK120 affinity columns as described in Materials and Methods. ND, not determined.
factor with similar activity has been partially characterized as a 50-kD protein released from mitochondria, whose activity on isolated nuclei is inhibited by VAD-fmk (34). Whether this protein is a new member of the ICE family has not been clarified, however, it is clearly different from AP24 with respect to molecular weight and susceptibility to protease inhibitors.

Another protein recently reported to activate DNA fragmentation in nuclei was isolated from HeLa cell cytosol, although it was not reported to be a protease (35). Interestingly, this protein, called DFF, exists as a precursor that is proteolytically activated by CPP32. The possible relationship between AP24 and DFF, or its activated fragments, is currently under investigation.

Recent findings in a different model of apoptosis indicate that overexpression of Bcl-xL will prevent anti-Fas-induced death of Jurkat cells without preventing activation of ICE-like proteases (36). One possible interpretation is that high levels of Bcl-xL act downstream of ICE-like proteases to prevent mitochondrial permeability transition, which has been proposed as an irreversible step in apoptosis (37). Alternatively, in a process that is blocked by overexpression of Bcl-2, mitochondria can release cytochrome c, which then promotes activation of CPP32 before the permeability transition (38, 39). If this model is correct, then AP24 activation may occur downstream of cytochrome c release and CPP32 activation. Studies are currently underway to examine the possible relationship between mitochondrial function and AP24 activation.

A recent report indicates that CPP32 is activated during PHA stimulation of T lymphocytes in the absence of apoptosis (40). This finding, as well as our results, suggest that CPP32 may have additional physiological functions that are unrelated to apoptosis.

The evidence that activation of DEVD-pN a cleavage does not irreversibly commit a cell to apoptosis, as well as the finding that activation of AP24 occurs downstream of CPP32-like activity, has important therapeutic implications. Inappropriate apoptosis contributes to the pathology of a variety of conditions (reviewed in 41). Inhibitors that block apoptosis may attenuate tissue damage, provided that subsequent necrosis does not negate the beneficial effects. Recently there has been considerable interest in the development of therapeutic inhibitors of ICE-related proteases to treat conditions involving inappropriate apoptosis (42). Apoptosis has been reported to be at least one component of cell death resulting from ischemia reperfusion injury in conditions such as neuronal stroke (43) and in heart failure (44, 45). Activation of CPP32 has been reported in cardiac myocytes of heart patients (46), and ICE was activated in an animal model of neuronal ischemia reperfusion injury (47). Furthermore, neuronal damage was ameliorated by administration of peptide inhibitors of ICE or CPP32, although there was a very short therapeutic window (<1 h) after reperfusion (47). In victims of acute ischemia reperfusion injury, cells may have received apoptotic signals hours before medical treatment is available. In these situations, drugs that block early apoptotic signaling events may not be useful. Since AP24 appears to function downstream of CPP32-like proteases, inhibition of AP24 may represent a superior therapeutic approach for treatment of acute apoptotic cell injury.

![Table 6. Specificity of Protease Inhibitors. Effects on CPP32-like Activity and on Nuclear DNA Fragmenting Activity by AP24 Assay	Inhibitor	IC$_{50}$
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Cleavage of DEVD-pN a by UV light-activated cytosol	cpDEVD-CHO 0.009
Cleavage of DEVD-pN a by UV light-activated cytosol	DEVD-CHO 0.007
Cleavage of DEVD-pN a by UV light-activated cytosol	VAD-cmk 0.010
Cleavage of DEVD-pN a by UV light-activated cytosol	DK120 >20.0
Cleavage of DEVD-pN a by UV light-activated cytosol	TPCK >20.0
DNA fragmentation in U937 nuclei by AP24	DEVD-CHO >20.0
DNA fragmentation in U937 nuclei by AP24	VAD-cmk >20.0
DNA fragmentation in U937 nuclei by AP24	DK120 0.016
DNA fragmentation in U937 nuclei by AP24	TPCK >20.0

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