Recent genetic and biochemical studies have implicated cysteine-dependent aspartate-directed proteases (caspases) in the active phase of apoptosis. In the present study, three complementary techniques were utilized to follow caspase activation during the course of etoposide-induced apoptosis in HL-60 human leukemia cells. Immunoblotting revealed that levels of procaspase-2 did not change during etoposide-induced apoptosis, whereas levels of procaspase-3 diminished markedly 2–3 h after etoposide addition. At the same time, cytosolic peptidase activities that cleaved DEVD-amidomethylcoumarin and VEDD-amidomethylcoumarin increased 100- and 20-fold, respectively; but there was only a 1.5-fold increase in YVAD-amidofluoromethylcoumarin cleavage activity. Affinity labeling with N-[(2,6-dimethylbenzoyl)oxy]methyl ketone indicated that multiple active caspase species sequentially appeared in the cytosol during the first 6 h after the addition of etoposide. Analysis on one- and two-dimensional gels revealed that two species comigrated with caspase-6 and three comigrated with active caspase-3 species, suggesting that several splice or modification variants of these enzymes are active during apoptosis. Polypeptides that comigrate with the cytosolic caspase species were also labeled in nuclei of apoptotic HL-60 cells. These results not only indicate that etoposide-induced apoptosis in HL-60 cells is accompanied by the selective activation of multiple caspases in cytosol and nuclei, but also suggest that other caspase precursors such as procaspase-2 are present but not activated during apoptosis.

Recent studies (reviewed in Refs. 1–5) indicate that caspases (now termed caspases)2 might play a critical role in initiating and sustaining the biochemical events that result in apoptotic cell death (reviewed in Refs. 5 and 13–15). Caspases are unusual in several respects. First, although they are cysteine-dependent proteases, the members that have been examined are insensitive to antipain and E64, two broad spectrum inhibitors of sulfhydryl proteases (16, 17). Second, caspases cleave at aspartate residues (16, 17). Third, activation of caspases might undergo autoactivation and/or activate each other in a cell death cascade (17, 22, 23).

Caspases are homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis.*

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Recent genetic and biochemical studies have implicated cysteine-dependent aspartate-directed proteases (caspases) in the active phase of apoptosis. In the present study, three complementary techniques were utilized to follow caspase activation during the course of etoposide-induced apoptosis in HL-60 human leukemia cells. Immunoblotting revealed that levels of procaspase-2 did not change during etoposide-induced apoptosis, whereas levels of procaspase-3 diminished markedly 2–3 h after etoposide addition. At the same time, cytosolic peptidase activities that cleaved DEVD-amidomethylcoumarin and VEDD-amidomethylcoumarin increased 100- and 20-fold, respectively; but there was only a 1.5-fold increase in YVAD-amidofluoromethylcoumarin cleavage activity. Affinity labeling with N-[(2,6-dimethylbenzoyl)oxy]methyl ketone indicated that multiple active caspase species sequentially appeared in the cytosol during the first 6 h after the addition of etoposide. Analysis on one- and two-dimensional gels revealed that two species comigrated with caspase-6 and three comigrated with active caspase-3 species, suggesting that several splice or modification variants of these enzymes are active during apoptosis. Polypeptides that comigrate with the cytosolic caspase species were also labeled in nuclei of apoptotic HL-60 cells. These results not only indicate that etoposide-induced apoptosis in HL-60 cells is accompanied by the selective activation of multiple caspases in cytosol and nuclei, but also suggest that other caspase precursors such as procaspase-2 are present but not activated during apoptosis.

A variety of experimental results suggest that ICE1 family proteases (now termed caspases) might play a critical role in initiating and sustaining the biochemical events that result in apoptotic cell death (reviewed in Refs. 5 and 13–15). Caspases are unusual in several respects. First, although they are cysteine-dependent proteases, the members that have been examined are insensitive to antipain and E64, two broad spectrum inhibitors of sulfhydryl proteases (16, 17). Second, caspases cleave at the carboxyl side of aspartate residues (17–20), a specificity that is unusual for mammalian proteases. Third, activation of caspases appears to require cleavage at Asp-X sequences in the proenzymes to yield the large and small subunits that are present in the active caspase tetramer (reviewed in Refs. 15, 17, and 21). The last two observations raise the possibility that caspases might undergo autoactivation and/or activate each other in a cell death cascade (17, 22, 23).

In Caenorhabditis elegans, deletion of the ced-3 gene, which encodes the single caspase known for this organism, abolishes

1 The abbreviations used are: ICE, interleukin-1-converting enzyme; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methyl-coumarin; cmk, chloromethyl ketone; caspase, cysteine-dependent aspartate-directed protease; DTT, dithiothreitol; E64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; Fmk, fluoromethylketone; Fmoc, N-(9fluorenyl)methoxycarbonyl; IRP, ICE-related protease; MOI, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; PMSF, p-methylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction; Z-EDCH(bio)-D-aomk, N-'-benzyloxycarbonylglutamyl-N'-'-biotinyllysyl-aspartic acid (2,6-dimethylbenzoyloxy)methyl ketone; Z-, benzoxycarbonyl; PIPES, 1,4-piperazinediethanesulfonic acid; HPLC, high pressure liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethyloammonium]-1-propanesulfonic acid; TLCK, 1-chloro-3-10-sulfoamido-7-amin-0-2-haptanone; aomk, Z-Glu-Leu-Glu-Asp(ω-c(arylmethyl)oxyl)methyl ketone.

2 In accordance with recent recommendations (72) members of the ICE family are now called caspases. Previous names of the proteases described in this manuscript are as follows: ICE (caspase-1), Ich-1 (caspase-2), CPP32/apopain (caspase-3), ICE-re1/Tx (caspase-4), ICE-re1/RY (caspase-5), Mch2 (caspase-6), Mch3 (caspase-7), FLICE/Mach/Mch5 (caspase-8), and Mch4 (caspase-10).

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Protease Activation in Chemotherapy-induced Apoptosis

cells were removed by sedimentation at 200 × g for 20 min on Ficoll-Hypaque step gradients (density = 1.119 g/cm³). Cells harvested from the interface were diluted with RPMI 1640, sedimented at 200 × g for 10 min, and resuspended in fresh growth medium. Etoposide (prepared as a 1000-fold concentrated stock in Me₂SO) was added to HL-60 cells at a concentration of 68 μM and incubated for 16 h. Cytosol was collected (7) and stored at −80°C until use. All protease substrates were added to cytosol at a final concentration of 10 μM.

Preparation of Cytosol and Nuclei (Modified from Ref. 38)—After drug treatment, all steps were performed at 4°C. Cells were sedimented at 200 × g for 10 min, washed twice in serum-free RPMI 1640 or RPMI 1640 containing 68 μM etoposide and resuspended in buffer C (25 mM HEPES (pH 7.5 at 4°C), 5 mM MgCl₂, 1 mM EGTA immediately before use with 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). After a 20-min incubation on ice, cells were lysed with 20–30 strokes in a tight fitting Dounce homogenizer. Following removal of nuclei by sedimentation at 800 × g for 10 min (see below) or 16,000 × g for 3 min, the supernatant was supplemented with 0.5 mM EDTA and sedimented at 280,000 × g for 60 min in a Beckman TL-100 ultracentrifuge. After the addition of DTT to a final concentration of 2 mM, the supernatant (cytosol) was frozen in 50-μl aliquots at −70°C. Experiments revealed that aliquots of cytosol retained DEVD-AFC cleavage activity without noticeable decrement for at least 3 months. All experiments described in the present study were performed within 1 month of extract preparation.

To isolate nuclei, the homogenates prepared above were sedimented at 800 × g. The pellet was washed once with buffer C, resuspended in 1 ml of buffer C, and layered over 4 ml of 50 mM Tris-HCl (pH 7.4) containing 2.1 mM sucrose and 5 mM MgSO₄. The nuclei were sedimented through the 2.1 mM sucrose at 80,000 × gₘₙₐₓ for 1 h and resuspended in storage buffer (39) consisting of 10 mM PIPES (pH 7.4), 80 mM KCN, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 50% (v/v) glycerol, 1 mM PMFS, 10 μM leupeptin, and 10 μM pepstatin. To assess purity of the nuclei, the activity of the cytosolic marker enzyme lactate dehydrogenase was assayed as described (40).

Organic Syntheses—To synthesize VED-TFA, the tetrapeptide portion (Z-VEID) was first synthesized from Fmoc-L-glutamic acid γ- butyryl ester, Fmoc-L-isoleucine, Fmoc-L-aspartic acid γ-butyryl ester, and Z-L-valine on an acid-labile 2-chlorotriyl chloride polystyrene resin (Advanced ChemTech, Louisville, KY). After assembly of the peptide on an ABI 431A peptide synthesizer using deprotection and coupling protocols provided by the instrument’s manufacturer (Perkin-Elmer), the t-butylylated tetrapeptide was released by treatment of the peptide–resin with 1% trifluoroacetic acid (TFA) and 5% H₂O for 30 min at 20°C. Following dilution with 50 volumes of cold H₂O, the protected peptide was lyophilized for 2 days to remove acetic acid and organic solvents, purified by gel filtration on LH-20 (Pharmacia Biotech Inc.) in 1:1 dichloromethane:propanol, and coupled to AMC through its free carboxyl group by high pressure liquid chromatography on a Vydac Analytical C₁₈ column (4.6 × 250 mm; Hesperia, CA) with a gradient of 0.1% trifluoroacetic acid containing 70% acetonitrile. Electrospores ionization mass spectrometry verified that the final product (1.2 mg) had the predicted molecular mass of 784.3.

The synthesis of Z-EKD(bio)D-aomk was initiated using the methodology described by Kranitz et al. (41–43). N’-(Z)-aspartic acid β-butyryl ester was activated as a mixed anhydride (1.4 equivalents of N-methylmorpholine, 1.3 equivalents of ethyl chloroformate in tetrahydrofuran at −15°C) and then treated with 2 equivalents of CH₂N₂ in Et₂O to yield the corresponding diamide, which was treated with a 1.1 mixture of excess glacial acetic acid and 45% HBr at 0°C to generate the bromo- methyl ketone. Treatment of the bromomethyl ketone with 1.2 equivalents of 2,6-dimethylbenzoic acid and 2.5 equivalents of KF in dimethylformamide yielded Z-Asp-(a-([a-carylsulfonyloxy]methyl) ketone). After removal of the Z- protecting group by catalytic hydrogenation (1 atmosphere, 10% Pd/C, 1 at solution of compound in ethanol) in the presence of 1.05 equivalents of HCl, the lysine residue was added according to conventional procedures.
Protease Activation in Chemotherapy-induced Apoptosis

The protease activation in chemotherapy-induced apoptosis was studied using various strategies. One approach involved the use of recombinant baculoviruses expressing caspases-1, 2, 4, 5, 6, 7, 8, 9, 10, and 12, which were used to induce apoptosis in HL-60 cells. The protease activity was measured using AMC substrate and by western blotting with antibodies against PARP and procaspase-3.

**Results:**

1. **Protease Activity Measurement:** The protease activity was measured using AMC substrate and by western blotting with antibodies against PARP and procaspase-3.
2. **Expression of Caspases:** The expression of caspases was confirmed using RT-PCR and western blotting.
3. **Inhibitor Competition Experiments:** Inhibitor competition experiments were performed using specific inhibitors for caspases-1, 2, 4, 5, 6, 7, 8, 9, 10, and 12.

**Conclusions:**

The results indicate that the activation of caspases is a critical factor in chemotherapy-induced apoptosis. The activation of caspases can be inhibited by specific inhibitors, suggesting that targeting caspases may be a potential therapeutic strategy for chemotherapy-induced apoptosis.
phase of apoptosis (5, 13, 14, 17, 27, 28, 32) prompted us to compare the expression and activation of caspase precursors in this cell line. RT-PCR revealed that the transcripts for the nine caspases examined are expressed constitutively in these cells (Fig. 1). Northern blot analysis (not shown) indicated that full-length messages for these transcripts are present. Sequencing of the PCR products confirmed that the observed gel bands corresponded to the indicated caspase precursors. In addition, this sequence analysis revealed that the multiple PCR products observed using procaspase-8 and procaspase-10 primers are derived from alternate splicing of the target mRNAs as described by Boldin et al. (64). These results indicated that HL-60 cells constitutively express at least nine procaspases.

Detection of Multiple Caspase Activities in Cytosol from Etoposide-treated HL-60 Cells—In order to follow caspase activation during apoptosis, cytosol from cells treated with 68 μm etoposide for increasing lengths of time was incubated with the fluorogenic substrate DEVD-AFC, VEID-AMC, or YVAD-AFC. The first two of these substrates correspond to the apoptotic cleavage sites of PARP (29) and lamin A (20), respectively, whereas the third substrate is derived from the cleavage site preferred by caspase-1 (21). Results of these assays are shown in Fig. 2.

Etoposide treatment of HL-60 cells was accompanied by a marked increase in activity that cleaved DEVD-AFC (Fig. 2A). The specific activity in cytosolic extracts from control HL-60 cells was 2 pmol of product released/min/mg of protein. There was a 20-fold increase in activity within 2 h of the addition of etoposide and a 100-fold increase within 3 h (Fig. 2A). Control experiments (Fig. 2B) revealed that the DEVD-AFC cleavage activity in cytosolic extracts from etoposide-treated HL-60 cells was extremely sensitive to DEVD-fmk (IC_{50} ~ 10 nm) compared with YVAD-cmk (IC_{50} ~ 10 μM) and completely insensitive to E64 at concentrations of 100 μM.

VEID-AMC cleavage activity also increased during the course of etoposide-induced apoptosis, albeit somewhat more modestly (Fig. 2C). The specific activity in extracts from control HL-60 cells was 15 pmol of product released/min/mg of protein. There was a 4-fold increase in activity 2 h after the addition of etoposide and a 20-fold increase over the 6-h time course. Examination of the effect of inhibitors (Fig. 2D) revealed that the VEID-AMC cleavage activity was at least 10-fold more sensitive to DEVD-fmk (IC_{50} < 1 nm) and 3-fold more sensitive to YVAD-cmk (IC_{50} ~ 3 μM) than the DEVD-AFC cleavage activity in the same extract, suggesting that the DEVD-AFC and VEID-AMC cleavages are mediated, at least in part, by different caspases (20). The VEID-AMC-cleaving enzyme was also somewhat more sensitive to 1-chloro-3-tosylamido-7-aminoo-2-heptanone (Fig. 2, compare TLCK in panels B and D).

The YEVD-AFC cleavage activity in cytosol from control HL-60 cells was <10 pmol of product released/min/mg of protein. In contrast to the marked increase in DEVD-AFC and VEID-AMC cleavage activities, there was little change in YEVD-AFC cleavage activity after the addition of etoposide (Fig. 2E). Additional experiments with cytosol from THP-1 cells, which are known to contain caspase-1 activity (38), not only confirmed that the assay could detect YEVD-AFC cleavage activity in cytosolic extracts, but also indicated that the inhibitor profile of the apoptotic proteases (Figs. 2, B and D) differed substantially from that of caspase-1 (Fig. 2F).

The Protease Activities Represent Activation of Preexisting Polypeptides—To determine whether the appearance of active apoptotic proteases depends on de novo synthesis of the proenzymes, HL-60 cells were treated with 68 μm etoposide in the absence or presence of cycloheximide or puromycin at concentrations that inhibited 90% methionine incorporation into protein by >90%. Results of this experiment (Fig. 3) revealed that neither cycloheximide nor puromycin inhibited the etoposide-induced increase in DEVD-AFC cleavage activity. Instead, treatment with cycloheximide alone or puromycin alone was associated with increased DEVD-AFC cleavage activity, a result that is consistent with previous observations that cycloheximide and puromycin induce apoptosis in HL-60 cells (30, 31, 61). These results suggest that de novo protein synthesis is not required for the appearance of caspase activity when HL-60 cells undergo apoptosis.

Sequential Labeling of Multiple Caspases in HL-60 Cells—To more completely delineate the spectrum of caspases activated in HL-60 cells, aliquots of cytosol were labeled with Z-EK(bio)D-aomk, an affinity labeling reagent (43, 65) designed to mimic the EVD motif preferred by the apoptotic proteases detected in Fig. 2. Control experiments revealed that Z-EK(bio)D-aomk covalently modified the larger subunits of all active caspases tested, including caspase-1, -2, -3, -4, and -6 (e.g., Fig. 7A). Titration experiments revealed that as little as 1 ng of purified caspase-1 could be detected with this reagent. When this reagent was utilized to label cytosol from control and etoposide-treated HL-60 cells, evidence for activation of multiple caspases was obtained. No active enzymes were detectable when cytosol from untreated HL-60 cells was incubated with Z-EK(bio)D-aomk (Fig. 4A, lane 1). In contrast, 6 h after the addition of etoposide to HL-60 cells, four discrete bands were detected by this affinity label (Fig. 4A, lane 6). These bands were termed IRP1 (relative mobility of 21.5 kDa), IRP2 (relative mobility of 19.1 kDa), IRP3 (relative mobility of 18.4 kDa), and IRP4 (relative mobility of 16.6 kDa). When cytosol from apoptotic HL-60 cells was pretreated with YVAD-cmk (a nonspecific caspase inhibitor at the concentration used), Z-EK(bio)D-aomk labeling of the proteases was abolished (Fig. 4B), providing further support for the view that these four bands are active caspases.

Interestingly, each band appeared at a characteristic time after etoposide addition (Fig. 4A, lanes 3–6). The bands denoted IRP2 and IRP4 were faintly visible 2 h after the addition of etoposide, increased dramatically by 3 h, and remained relatively constant thereafter. In contrast, IRP1 and IRP3 were first evident at 3 h and increased progressively during the remainder of the incubation. Similar results were obtained when cytosol was labeled with N-(acetyltyrosinylvalinyl-N)-
biotinyllysyl aspartic acid [(2,6-dimethylbenzoyl)oxy]methyl ketone (YV(bio)KD-aomk) (65) (data not shown), although Z-EK(bio)D-aomk could be used to label the IRPs at 10-fold lower concentrations.

Detection of Caspases in Nuclei of HL-60 Cells—Because several nuclear polypeptides are cleaved during the course of apoptosis (5, 13, 15, 30), we next examined the possibility that active caspases might be detected in nuclei as well as cytosol. For these experiments, HL-60 cells were treated with etoposide and then fractionated into cytosol and nuclei. The nuclei were purified by sedimentation through sucrose cushions as described previously (40). Electron microscopy of these nuclei failed to reveal any identifiable cytoplasmic organelles (data not shown). Furthermore, assays indicated that the activity of the marker enzyme lactate dehydrogenase was 4.6 ± 1.4 pmol of substrate consumed/min/mg of protein in nuclei compared...
with 85 ± 14 pmol/min/mg of protein in cytosol after 3 h of etoposide treatment, suggesting that contamination by cytosolic polypeptides was minimal. Nonetheless, nuclei prepared from etoposide-treated cells contained DEVD-AFC cleavage activity. Results of time course experiments (Fig. 5A) revealed that this activity was low or undetectable in nuclei from control cells (0 h) and increased to ~100 pmol of product released/min/mg of protein after 2 h. In additional experiments, VEID-AMC cleavage activity was also detected in nuclei from apoptotic HL-60 cells. Nuclei that were not treated with Z-EK(bio)D-aomk contained a number of polypeptides that reacted with streptavidin (Fig. 5B, lane 7), including a polypeptide of Mr;~17,000 that migrated just below the expected position of IRP3. These streptavidin-binding polypeptides did not change during the course of etoposide treatment (Fig. 5B, lanes 4–6, and data not shown). Etoposide treatment was, however, accompanied by the appearance of new bands that labeled with Z-EK(bio)D-aomk. These bands, which appeared beginning 3 h after the addition of etoposide to the cells, comigrated with IRP1 and IRP2 (Fig. 5B, lanes 4–6). Interestingly, IRP3 and IRP4 were detectable in cytosol from these cells but not in nuclei (cf. lanes 2 and 5).

Identification of Caspases That Are Activated during Etoposide-induced Apoptosis—Taken together, the results in Figs. 2–5 suggest that etoposide treatment is associated with activation of multiple caspases from preexisting proenzymes. In order to begin to identify the proenzymes that are activated, whole cell lysates from etoposide-treated HL-60 cells were examined by immunoblotting using antibodies that recognize...
cloned human caspases. To provide a frame of reference, the same blots were also probed with antibodies that recognize the caspase substrates PARP and lamin B1 as well as the nuclear protein B23, a polypeptide that does not cleaved during apoptosis in HL-60 cells (31). Results of these experiments are shown in Fig. 6.

Cleavage of PARP to its signature 89-kDa fragment (29–31) was evident within 3 h of the addition of etoposide to these cells (Fig. 6A, lane 7). A marked decrease in lamin B1 content, indicative of lamin B1 degradation (39, 66, 67), was also evident 3 h after etoposide addition (Fig. 6B, lane 7). At this time point, a decrease in procaspase-3 was evident (Fig. 6C, lane 7), suggesting that caspase-3 might be activated in these cells. In contrast, there was no significant change in procaspase-2 content in etoposide-treated HL-60 cells (Fig. 6D).

Despite the marked decrease in procaspase-3 signal (Fig. 6C), the appearance of a signal corresponding to active caspase-3 was not observed even after overexposure of the blots. To confirm that the disappearance of procaspase-3 from the immunoblots was associated with appearance of active caspase-3 and to attempt to identify the other IRPs activated in apoptotic HL-60 cells, the mobility of the large subunits of active caspases detected in HL-60 cytosol following Z-EK-(bio)D-aomk labeling was compared with the mobility of the corresponding subunits of cloned human enzymes expressed in SF9 cells (Fig. 7). Unidimensional SDS-PAGE (Fig. 7A) revealed that IRP1 and IRP3 comigrated with two bands observed in SF9 cells expressing caspase-6 (Fig. 7A, lane 4); IRP2 and IRP4 comigrated with two bands observed in SF9 cells expressing caspase-3 (Fig. 7A, lane 5); and IRP3 also comigrated with the band observed in SF9 cells expressing caspase-2 (Fig. 7A, lane 3). In contrast, the large subunits of caspase-1 and -4 did not comigrate with any of the IRPs. These results suggested the tentative interpretation of the HL-60 labeling pattern shown in Fig. 7A.

To further examine the spectrum of IRPs activated after etoposide treatment, labeled HL-60 cytosol was subjected to two-dimensional isoelectric focusing/SDS-PAGE. This analysis revealed as many as eight distinct spots in the M r 16,000–18,000 range (Fig. 7, B and C). For convenience, these spots have been termed A1 (pI 6.5), A2 (pI 6.3), A3 (pI 6.2), B (pI 6.6), C1 (pI 6.4), C2 (pI 6.2), C3 (pI 6.0), and C4 (pI 5.9), respectively. In mixing experiments, the mobilities of the large subunits of caspase-2, -3, and -6 expressed in SF9 cells were also determined (Fig. 7C, bottom). When apoptotic HL-60 cytosol and the recombinant caspases were compared, a number of the labeled spots comigrated (Fig. 7C, middle). This analysis leads to several conclusions. First, spots A1, C1, and C3 comigrate with three different active species of recombinant caspase-3 expressed in SF9 cells. Second, spot B comigrates with an active species of caspase-6. Finally, none of the spots detected in HL-60 cytosol comigrated with the major form of caspase-6 detected in insect cells or with recombinant caspase-2.

**DISCUSSION**

Previous studies have suggested that caspase activation plays a crucial role in the initiation or propagation of apoptotic events. Recent reports also indicate that genes encoding as many as nine caspases are simultaneously expressed in certain human leukemia cell lines. However, it has been unclear whether all of these proteases participate in apoptotic events. Two factors contribute to this uncertainty. First, activation of caspases, which involves proteolytic cleavage of zymogen precursors at Asp–X bonds to yield the large and small subunits of the active heterotetrameric proteases, is poorly understood (reviewed in Refs. 15 and 28). Second, high titer antibodies that are specific for the subunits of mature proteases are not available for all of the caspases. As a result, it has remained unclear whether all of the proenzymes are activated simultaneously, whether the appearance of active enzymes involves de novo synthesis of caspases or activation of preexisting proenzymes, and whether active caspases are localized exclusively to the cytoplasm or are found in other cellular compartments. The present study sheds light on all of these issues. Analysis of affinity labeling experiments by unidimensional SDS-PAGE revealed that HL-60 cells undergoing etoposide-induced apoptosis contain four distinct bands that covalently label with Z-EK(bio)D-aomk (Fig. 4). Although these polypeptides appear sequentially rather than simultaneously, the apparent stability of the larger bands (IRP3,4) at later time points suggests that the lower bands are not derived from the upper band(s) by proteolytic cleavage. Instead, these polypeptides appear to correspond to the larger subunits of the active forms of multiple discrete caspases. A similar repertoire of five endogenous caspases with distinct cleavage preferences was recently demonstrated by us in apoptosis-inducing S/M extracts prepared from chicken hepatoma cells (20). Thus, apoptosis in higher eukaryotic cells differs in detail from developmental cell death in *C. elegans*, where a single caspase appears to be
required (24, 25).

The results obtained with Z-EK(bio)D-aomk provide the first direct evidence that a number of caspases are activated during apoptosis in HL-60 cells. This caspase activation is accompanied by the appearance of at least two distinct enzyme activities. These activities cleave DEVD-AFC (Fig. 2A) and VEID-AMC (Fig. 4) but display different inhibitor sensitivities (Fig. 2, B and D). In contrast, activation of a caspase-1-like activity was not observed during etoposide-induced apoptosis in HL-60 cells (Fig. 2E), a result that differs from the recent observation that caspase-1-like protease activity is elevated early in the course of Fas-mediated apoptosis in T cells (68). Whether these disparate findings reflect differences between various cell types or variations in the pathways activated by different apoptotic stimuli requires further investigation.

The observation that several recombinant caspase polypeptides expressed in Sf9 cells comigrate with the IRPs present in HL-60 extracts following labeling with Z-EK(bio)D-aomk enables us to propose a tentative identification of the proteases acting in the course of etoposide-induced HL-60 apoptosis. IRP2 and IRP4 appear to correspond to caspase-3 (Fig. 7B). The appearance of these active species occurs with a time course (Fig. 4) that parallels the appearance of DEVD-AFC cleavage activity (Fig. 2A) and disappearance of procaspase-3 from immunoblots (Fig. 6). Our demonstration that caspase-3 is enzymatically active in HL-60 apoptosis (i.e. covalently labels Z-EK(bio)D-aomk) confirms and extends recent claims of caspase-3 activation based solely on appearance of immunoreactive P17 subunit (27, 69).

The two-dimensional analysis presented in Fig. 7 provides the first evidence for the presence of multiple active forms of caspase-3 during apoptosis. Spots A1, C1, and C2 all comigrate with species observed in Sf9 cells expressing human caspase-3. The various forms of the enzyme could arise from alternative splicing of the transcripts (70), alternative processing of the proenzymes, and/or posttranslational modification of the processed subunits. Whether these species correspond to functionally distinct subpopulations of caspase-3 that differ in their substrate recognition properties, time of activation during apoptosis, or intracellular location requires further study. Nonetheless, the observation that cells contain multiple active species of this caspase suggests that the spectrum of active caspases in apoptosis could be even more complicated than previously thought.

Measurements of enzyme activity also indicate that a discrete VEID-AMC cleavage activity appears in etoposide-treated HL-60 cells beginning ~2 h after the addition of etoposide (Fig. 2, C and D). Analysis by one- and two-dimensional isoelectric focusing/SDS-PAGE supports this observation by indicating that IRP/spot B has the same mobility as one species of active caspase-6 (Fig. 7). Caspase-6 was recently shown to be unique among caspases characterized to date in being able to cleave the VEID-X sequence (20). Although analysis by unidimensional SDS-PAGE strongly suggested that IRP1 might correspond to a second active species of caspase-6 (Fig. 7A), this assignment could not be confirmed by two-dimensional isoelectric focusing/SDS-PAGE because IRP1 did not focus in the two-dimensional gels (Fig. 7, B and C).
Two-dimensional analysis of HL-60 cytosol also revealed the presence of multiple additional Z-EK(bio)D-Aomk-reactive species (Fig. 7C) that did not comigrate with any of the recombinant caspases tested to date. Among these, spots C3 and C4 have mobilities on SDS-PAGE similar to species tentatively identified as caspase-3 but have different isoelectric points, suggesting that they might be posttranslationally modified versions of the caspase-3 large subunit. Alternatively, these minor species could correspond to other human caspases, either known or yet to be characterized.

In addition to the caspases detected in cytosol, Z-EK(bio)D-Aomk also selectively labeled two polypeptides in nuclei of etoposide-treated HL-60 cells (Fig. 5B). Despite the fact that several of the known caspase substrates are nuclear proteins (reviewed in Refs. 15 and 28), this is the first demonstration of multiple active caspases in nuclei. It is unlikely that the nuclear enzymes represent contaminating cytosolic proteins, since the isolated nuclei were shown to lack significant cytoplasmic contamination as detected either by electron microscopy or assays for the cytosolic marker enzyme lactate dehydrogenase. The comigration of the active caspase subunits in cytosol and nuclei (Fig. 5B) suggests the possibility that some (but not all) of the species activated in the cytosol might be transported into nuclei. Alternatively, we cannot rule out the possibility that certain caspase precursors present in nuclei are activated during the course of apoptosis. Further experiments are required to distinguish between these possibilities.

The present observations also shed light on the origin of the active caspases. We were unable to detect either DEVD-X cleavage activity (Fig. 2A) or Z-EK(bio)D-Aomk-labeled polypeptides corresponding to the large subunit of active caspases in cytosol from control HL-60 cells (Fig. 4A). These observations argue against the possibility that caspase-3 (or any related protease) is prefractionated in the cytosol of HL-60 cells and efficiently rule out a model in which the activity of prefractionated proteases is controlled in these cells by regulation of the levels of endogenous inhibitors.

An alternative possibility is that caspases are synthesized de novo in response to apoptotic stimuli (71). We did not observe a reproducible ectodomain-induced increase in content of any of the four caspases for which antibodies are currently available (Fig. 6 and data not shown). Moreover, the observation that high concentrations of the protein synthesis inhibitors cycloheximide and puromycin failed to prevent the appearance of DEVD-AFC cleavage activities in etoposide-treated HL-60 cells (Fig. 3) argues against the possibility that this activity results from de novo synthesis of the proenzyme. Instead, it appears that multiple caspase proenzymes are present in HL-60 cells and undergo the processing of the proenzyme. Alternatively, we cannot rule out the possibility that certain caspase precursors present in nuclei are activated during the course of apoptosis. Further experiments are required to distinguish between these possibilities.

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Protease Activation in Chemotherapy-induced Apoptosis

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