THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 11, Issue of March 14, pp. 7421–7430, 1997
Printed in U.S.A.

Activation of Multiple Interleukin-1β Converting Enzyme Homologues in Cytosol and Nuclei of HL-60 Cells during Etoposide-induced Apoptosis*

(Received for publication, May 30, 1996, and in revised form, December 27, 1996)

Luis M. Martins‡§, Timothy Kottke‡¶, Peter W. Mesner¶, Guriqbal S. Basi‖, Sukanto Sinha‖, Normand Frigon, Jr.‖, Eric Tatar‖, Jay S. Tung‖, Karin Bryant‖, Atsushi Takahashi, Phyllis A. Svingen¶, Benjamin J. Madden, Daniel J. McCormick**, William C. Earnshaw‡‡, and Scott H. Kaufmann¶§§¶¶**

*From the Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, EH9 3JR Scotland, United Kingdom, the ¶Division of Oncology Research and **Protein Core Facility, Mayo Clinic, Rochester, Minnesota 55905, and ‖Athena Neurosciences, Inc., S. San Francisco, California 94080*

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-aminotrifluoromethylcoumarin and VEID-aminomethylcoumarin increased 100- and 20-fold, respectively; but there was only a 1.5-fold increase in YVAD-aminotrifluoromethylcoumarin cleavage activity. Affinity labeling with *N*-(N^{α}-benzyloxycarbonylglutamyl-N^{ϵ}-biotinyllysyl)-aspartic acid [(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 caspases 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 the cytotoxicity of virtually all chemotherapeutic agents is accompanied by apoptosis in susceptible cell lines. Likewise, experiments in animals (6–9) and studies of circulating blasts from leukemia patients (10) have provided evidence that chemotherapy is accompanied by apoptosis *in vivo*. Moreover, it has been suggested that resistance to the cytotoxic effects of chemotherapeutic agents can result from resistance to chemotherapy-induced apoptosis (8, 11, 12). These observations highlight the potential importance of understanding the factors that control apoptosis.

A variety of experimental results suggest that ICE[1] family proteases (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 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 $\alpha_2\beta_2$ 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

* This work was supported in part by U.S. Public Health Service Grant CA69008 (to S. H. K. and W. C. E.) and a grant from the Wellcome Trust (to W. C. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The first two authors contributed equally to this work.
§ Supported by a "Programa Gulbenkian de Doutoramento em Biologia e Medicina" studentship.
‡‡ Principal Fellow of the Wellcome Trust.
§§ Leukemia Society of America Scholar.
¶¶ To whom correspondence should be addressed: Div. of Oncology Research, Guggenheim 1342C, Mayo Clinic, 200 First St., S.W., Rochester, MN 55901. Tel.: 507-284-8950; Fax: 507-284-3906; E-mail: Kaufmann.Scott@Mayo.edu.

[1] The abbreviations used are: ICE, interleukin-1β 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*-(9-fluorenyl)methoxycarbonyl; IRP, ICE-related protease; MOI, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; PMSF, α-phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction; *Z*-EK(bio)D-aomk, *N*-(N^{α}-benzyloxycarbonylglutamyl-N^{ϵ}-biotinyllysyl)aspartic acid [(2,6-dimethylbenzoyl)oxy]methyl ketone; *Z*-, benzyloxycarbonyl; PIPES, 1,4-piperazinediethanesulfonic acid; HPLC, high pressure liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TLCK, 1-chloro-3-tosylamido-7-amino-2-haptanone; aomk, *Z*-Glu-Lys-Asp-(α-((arylacyl)oxy)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-rel_{II}/Tx (caspase-4), ICE-rel_{III}/TY (caspase-5), Mch2 (caspase-6), Mch3 (caspase-7), FLICE/Mach/Mch5 (caspase-8), and Mch4 (caspase-10).

all developmentally regulated cell death (24, 25). In higher eukaryotes, the situation appears to be more complex. First, proenzyme forms of multiple caspases are expressed in many cell types (17, 22, 26, 27). Second, deletion of the genes for several of these proteases does not appear to alter the features of apoptosis induced by many stimuli, perhaps because of functional redundancy in the substrate recognition properties of these proteases (14, 28).

Previous studies have also identified a number of cellular polypeptides that are cleaved during apoptosis (reviewed in Refs. 5, 14, 15, and 28). These include the DNA damage recognition protein PARP, which is cleaved at the sequence DEVD ↓ G (29), and the lamins, which are cleaved by a protease that recognizes the sequence VEID ↓ N (20). After treatment of HL-60 human acute myelomonocytic leukemia cells with a variety of chemotherapeutic agents, PARP and lamin B_1 are cleaved just prior to or concomitant with the appearance of other apoptotic changes (30, 31). Other polypeptides that appear to be cleaved by caspases during apoptosis in various cell types include the DNA-activated protein kinase DNA-PK, the retinoblastoma gene product, the 70-kDa polypeptide subunit of U1 small nuclear ribonucleoprotein complexes, steroid response element binding proteins, protein kinase Cδ, actin, and the intermediate filament-associated polypeptide Gas2 (reviewed in Refs. 5 and 15). The individual proteases responsible for these cleavages *in situ* have not been conclusively identified. Recent studies have indicated that PARP can be cleaved *in vitro* by a variety of caspases including caspase-1, -2, -3, -4, -6, -7, and -8 (17, 22, 26, 32–34), although cleavage of expressed fragments of PARP by caspase-1, -2, and -4 appears to require enzyme concentrations that are stoichiometric rather than catalytic (17, 29, 33). To date, caspase-6 (previously termed Mch2) is the only caspase known to be capable of cleaving the lamins (20, 35).

In the present study, we have examined HL-60 cells undergoing apoptosis after treatment with the topoisomerase II-directed agent etoposide (reviewed in Ref. 36). These studies have focused on determining 1) the number of caspases that are activated during etoposide-induced apoptosis, 2) the subcellular distribution of these active caspases, and 3) the identity of the active caspases. In order to address these questions, a novel affinity label for active caspases was synthesized and characterized, and a new fluorogenic assay for caspase-6-like protease activity was developed.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following suppliers: etoposide, leupeptin, pepstatin, tetrapropylammonium hydroxide, PMSF, AMC, and AFC from Sigma; aprotinin from Mobay Pharmaceuticals (New York, NY); poly(A)$^+$ RNA from HL-60 cells from Clontech (Palo Alto, CA); AmpliTaq polymerase from Perkin-Elmer; Superscript RT reverse transcriptase and the Bac-to-Bac^{TM} system from Life Sciences, Inc.; rabbit anti-caspase-1 (PC84) from Calbiochem; murine monoclonal anti-caspase-2 and anti-caspase-3 from Transduction Laboratories (Lexington, KY); peroxidase-labeled secondary antibodies from KPL (Gaithersburg, MD); ^{14}C-labeled thymidine (57.8 mCi/mmol), Hybond-N membranes, and ECL enhanced chemiluminescence reagents from Amersham Corp.; *N*-hydroxysuccinimidobiotin from Pierce; *N*-(*Z*)-aspartic acid β-*t*-butyl ester and YVAD-cmk from Bachem (King of Prussia, PA); DEVD-AFC, YVAD-AFC, and DEVD-fmk from Enzyme Systems Products (Dublin, CA); and Biolytes from Bio-Rad.

Cell Culture—HL-60 cells (a kind gift from Ron Schnaar, Johns Hopkins University School of Medicine) were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A). THP.1 cells (American Type Culture Collection, Rockville, MD) were propagated in medium A containing 50 μM 2-mercaptoethanol. All cell lines were maintained at concentrations of $<1 \times 10^6$ cells/ml to ensure logarithmic growth.

Induction of Apoptosis—At the start of each experiment, nonviable cells were removed by sedimentation at 200 × *g* for 20 min on Ficoll-Hypaque step gradients (density = 1.119 g/cm^3). 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 Me_2SO) was added to HL-60 cells at a concentration of 68 μM, a high but clinically achievable concentration (37) that has previously been shown to induce apoptosis in >85% of HL-60 cells within 6 h (31).

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 phosphate-buffered saline, and resuspended in buffer C (25 mM HEPES (pH 7.5 at 4 °C), 5 mM $MgCl_2$, 1 mM EGTA supplemented immediately before use with 1 mM PMSF, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin). 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_{max} 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 M sucrose and 5 mM $MgSO_4$. The nuclei were sedimented through the 2.1 M sucrose at 80,000 × g_{max} for 1 h and resuspended in storage buffer (39) consisting of 10 mM PIPES (pH 7.4), 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 50% (w/v) glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml 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 VEID-AMC, the tetrapeptide portion (*Z*-VEID) was first synthesized from Fmoc-L-glutamic acid γ-*t*-butyl ester, Fmoc-L-isoleucine, Fmoc-L-aspartic acid β-*t*-butyl ester, and *Z*-L-valine on an acid-labile 2-chlorotrityl 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*-butyl-protected tetrapeptide was released by treatment of the peptidyl-resin with acetic acid:trifluoroethanol:dichloromethane (1:2:7) for 30 min at 20 °C. Following dilution with 50 volumes of cold H_2O, 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:*n*-propanol, and coupled to AMC through its free C-terminal end by a mixed anhydride method with isobutyl chloroformate. In brief, after gel filtration, 48 mg of protected tetrapeptide was dissolved in 2 ml of 1-methyl-2-pyrrolidinone and 50 μl of triethylamine, cooled to −5 °C, and treated with 40 μl of isobutyl chloroformate for 35 min. A solution containing 13.1 mg of AMC in 1.5 ml of 1-methyl-2-pyrrolidinone was then added dropwise, and the coupling was allowed to proceed for 18 h at 20 °C. The protected peptide-AMC conjugate was precipitated by dilution of the reaction mixture with cold H_2O, extracted with ethyl acetate, and evaporated to dryness. After treatment with trifluoroacetic acid:H_2O (19:1) for 30 min to remove the *t*-butyl protecting groups, the resulting product was purified to homogeneity by reversed phase HPLC on a Vydac Analytical C_{18} column (4.6 × 250 mm; 10 μm; Hesperia, CA) with a gradient of 0.1% trifluoroacetic acid containing 70% acetonitrile. Electrospray ionization mass spectrometry verified that the final product (1.2 mg) had the predicted molecular mass of 764.3.

The synthesis of *Z*-EK(bio)D-aomk was initiated using the methodology described by Krantz (41–43). *N*-(*Z*)-aspartic acid β-*t*-butyl 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 CH_2N_2 in Et_2O to yield the corresponding diazoketone, which was treated with a 1:1 mixture of excess glacial acetic acid and 45% HBr at 0 °C to generate the bromomethyl 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-(α-((arylacyl)oxy)methyl ketone). After removal of the *Z*- protecting group by catalytic hydrogenation (1 atmosphere, 10% Pd/C, 1 M solution of compound in ethanol) in the presence of 1.05 equivalents of HCl, the lysine residue was added using conven-

tional amino acid coupling conditions (1 equivalent of *Z*-Lys(*t*-Boc)-OH, 1.6 equivalents of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, 6 equivalents of *N*-methylmorpholine, dimethylformamide). After the *Z*- protecting group was again removed by catalytic hydrogenation, the glutamic acid residue was added using identical conditions to yield *Z*-Glu(OtBu)-Lys(*t*-Boc)-Asp(OtBu)-(α-((arylacyl)oxy)methyl ketone). The *t*-butanol and *t*-Boc protecting groups were removed with trifluoroacetic acid in methylene chloride to yield *Z*-Glu-Lys-Asp-(α-((arylacyl)oxy)methyl ketone), which was then reacted with a 2-fold molar excess of *N*-hydroxysuccinimidobiotin to derivatize the ϵ-amino group of lysine. The product *Z*-EK(bio)D-aomk was separated from unreacted aomk and excess reagents by reversed phase HPLC. Fractions containing active *Z*-EK(bio)D-aomk were identified using recombinant bacterial ICE, pooled, and concentrated.

Fluorogenic Assays—Aliquots containing 50 μg of cytosolic or nuclear protein (estimated by the bicinchoninic acid method (44)) in 50 μl of buffer C were diluted with 225 μl of freshly prepared buffer D (25 mM HEPES (pH 7.5), 0.1% (w/v) CHAPS, 10 mM DTT, 100 units/ml aprotinin, 1 mM PMSF) containing 100 μM substrate and incubated for 2 h at 37 °C. Reactions were terminated by the addition of 1.225 ml of ice-cold buffer D. Fluorescence was measured in a Sequoia-Turner fluorometer using an excitation wavelength of 360 nm and emission wavelength of 475 nm. Reagent blanks containing 50 μl of buffer C and 225 μl of buffer D were incubated at 37 °C for 2 h and then diluted with 1.225 ml of ice cold buffer D. Standards containing 0–3000 pmol of AMC or 0–1500 pmol of AFC were utilized to determine the amount of fluorochrome released. Control experiments (not shown) confirmed that the release of substrate was linear with time and with protein concentration under the conditions specified. To determine the effect of various protease inhibitors, aliquots containing 50 μg of protein were incubated with the indicated inhibitor for 5 min at 21 °C and then diluted with substrate in buffer D and assayed as described above.

IRP Expression in Sf9 Cells—Full-length cDNAs encoding P45 forms of caspase-1, -2, -3, -4, and -6 were cloned by RT-PCR from Jurkat or THP.1 cell RNA using primers flanking the respective open reading frames. The primers were designed based upon the published sequences (21, 22, 26, 45–51), and all clones were sequenced before expression. High titer recombinant baculovirus expressing caspase-1 was generated by cloning the cDNA into pVL1392 using standard methods (52). Recombinant baculoviruses expressing the other caspases were produced by transposon-mediated recombination (53) in bacteria using the Bac-to-Bac™ system according to the supplier's instructions.

Expression cultures of the caspases were produced using high titer viral stocks (>10^7 plaque-forming units/ml) infected at MOIs ranging from 2.5 to 10. At 48 h (high MOI infections) or 72 h (low MOI infections) after infection, a time when caspases are activated during virus-induced apoptosis, cells were washed twice with phosphate-buffered saline and lysed in 0.2–0.5 ml of buffer containing 20 mM HEPES (pH 7.0), 2 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml E64, and 0.1 mM PMSF. After a 10-min incubation at 4 °C, nuclei were removed by centrifugation at 12,000 × *g*. The supernatants were stored at −80 °C until treated with *Z*-EK(bio)D-aomk as described below.

Affinity Labeling—Aliquots containing the indicated amounts of nuclear or cytosolic protein were incubated for 1 h at room temperature with 1 μM *Z*-EK(bio)D-aomk (added from a 25 μM stock in Me_2SO). At the completion of the incubation, extracts were diluted with ½ volume of 3 × concentrated SDS sample buffer, heated to 95 °C for 3 min, subjected to SDS-PAGE on 16% (w/v) acrylamide gels, transferred to nitrocellulose, probed with peroxidase-labeled streptavidin, and visualized by enhanced chemiluminescence. Control experiments using purified ICE indicated that this approach could detect as little as 1 ng of caspase-1. Alternatively, samples treated with *Z*-EK(bio)D-aomk were precipitated using methanol/chloroform and resuspended in 100 μl of urea solubilization buffer (9 M urea, 4% (v/v) Nonidet P-40, 2% (v/v) β-mercaptoethanol, 20% (v/v) BioLyte 3–10 carrier ampholytes). Two-dimensional gel electrophoresis was performed using an ISO-DALT system (Hoeffer, San Francisco, CA) as described previously (54). Samples were focused on an isoelectric focusing gel for 10,200 V-h, transferred to the top of a 16% (w/v) SDS-polyacrylamide gel, subjected to SDS-PAGE at 12 mA for 16 h, transferred to nitrocellulose, and probed with streptavidin as described above.

A series of mixing experiments was performed to compare affinity-labeled proteases in HL-60 cytosol with recombinant human caspases expressed in Sf9 cells. *Z*-EK(bio)D-aomk-labeled Sf9 extracts containing caspase-3 were electrophoresed either individually or after mixing with labeled Sf9 extracts containing caspase-2 or caspase-6. A composite image was then created by merging and aligning scans from the various blots using Adobe Photoshop. The location of the known enzyme large subunits in the composite image was then overlaid upon the distribution of active caspase subunits in two-dimensional gel analysis of HL-60 cell cytosol. Caspase-3 species present in all blots were used as reference points for the alignment.

Inhibitor Competition Experiments—Aliquots containing 37 μg of cytosolic protein from etoposide-treated HL-60 cells were incubated for 1 min on ice with the tetrapeptide inhibitor YVAD-cmk dissolved in phosphate-buffered saline containing 10% Me_2SO. *Z*-EK(bio)D-aomk was then added to a final concentration of 1 μM. After a 5-min incubation at 37 °C, the samples were diluted with ½ volume of 3 × concentrated SDS sample buffer, heated to 95 °C for 3 min, subjected to SDS-PAGE, and reacted with peroxidase-coupled streptavidin as described above.

Preparation of Whole Cell Lysates for Immunoblotting—After incubation with etoposide for the indicated lengths of time, HL-60 cells were sedimented at 200 × *g* for 10 min at 4 °C, washed once with ice-cold RPMI containing 10 mM HEPES (pH 7.4 at 4 °C), and solubilized at a concentration of 3 × 10^7 cells/ml in SDS sample buffer consisting of 4 M deionized urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8 at 21 °C), 1 mM EDTA, and 5% (w/v) freshly added β-mercaptoethanol. Samples were immediately sonicated and frozen at −70 °C. Aliquots were subsequently heated to 65 °C for 20 min, subjected to SDS-PAGE on gels with 5–15% (w/v) acrylamide gradients, transferred to nitrocellulose, and probed with antibodies as described previously (55). Antibodies utilized for these experiments included murine monoclonal antibodies that recognize PARP (56), chicken polyclonal antisera raised against rat lamin B_1 (57) and rat nucleolar protein B23 (58), and the commercially available antibodies described above.

PCR—Primers were generally designed to amplify cDNA sequences encompassing the pro domain into the amino-terminal half of the respective P20 subunits, regions of the caspase cDNAs that display relatively low sequence homology. In brief, 1 μg of poly(A)$^+$ RNA from HL-60 cells was reverse transcribed with a combination of random hexamer and oligo(dT) primers using Superscript RT under standard conditions (59). One-twentieth of the cDNA product was used for each amplification. PCR reactions were performed in 50-μl volumes containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 1 μM each primer, 200 μM dNTPs, and 2.5 units of AmpliTaq. Reactions were initiated under hot start conditions and continued for a total of 30 cycles under step cycle conditions on a Perkin-Elmer PE-480 DNA thermal cycler using 92 °C for denaturation, 60 °C for annealing, and 72 °C for extension (30 s/step). The caspase-5 product was amplified for 35 cycles using a 55 °C annealing step. For each procaspase (accession number indicated in parentheses), the forward and reverse primers were as follows: procaspase-1 (X65019), GGGTACAGCGTAGATGTGAAAAAAAATCTCACTGCTTCG and GCCCATTGTGGGATGTCTCCAAGAA; procaspase-2 (U13022), GTGCACTTCACTGGAGAGAAA and GGGCAGTCTCATCTTCGGCAA; procaspase-3 (U13737), AAGGTATCCATGGAGAACACTGAAAAC and AACCACCAACCAACCATTTCT; procaspase-4 (U28014), CAGAGCACAAGTCCTCTGACA and AGGGCTGGGCTGCTTGTG; procaspase-5 (U28015), GTATCCTTCAGAGTGGATTGG and AATTCTTCACGAGGACAAAGTTTGA; procaspase-6 (U20536), CCGGCAGGTGGGGAAGAAAA and TGCAACAGAGTAACACATGAGGAAGTC; procaspase-7 (U37449), CCGCCGTGGGAACGATGGCAGATG and TGACCCATTGCTTCTCAGCTAGAATGTAC; procaspase-8 (U60519), ACAAGGAAGCCGAGTCGTATCAAGGAGAGG and TCGTTGACAGCAGTGAGGATGGATAAGATG; procaspase-10 (U60520), TAAACACTAGAAAGGAGGAGATGGAAAGGGAACT and CATGGGAGAGGATACAGCAGATGAAGCA. Control PCR reactions included 1) HL-60 cDNA template and human β-actin primers (forward, GTGGGGCGCCCCAGGCACCA; reverse, CTCCTTAATGTCACGCACGATTTC) to validate the cDNA and 2) 0.05 μg of HL-60 poly(A)$^+$ RNA template to confirm that the products observed were derived from cDNA rather than contaminating genomic DNA. Following amplification, one-tenth of the PCR reactions were electrophoresed for 160 V-h on a 3.5% composite agarose gel (2.5% NuSieve, 1% Seakem-GTG; FMC Bioproducts, Rockland, ME) containing 0.5 μg/ml ethidium bromide in 1 × TAE buffer. Products were visualized on a UV transilluminator (256 nm), excised, and sequenced.

RESULTS

Caspase Expression in HL-60 Cells—The present studies focused on HL-60 cells, a human leukemia cell line that readily undergoes apoptosis in response to a variety of chemotherapeutic agents (30, 31, 60–63). Recent studies suggesting that caspase activation plays a critical role in initiating the active

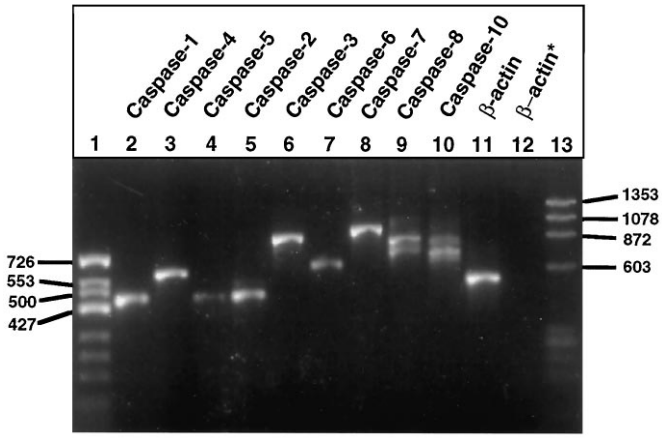

FIG. 1. **Detection of caspase transcripts in untreated HL-60 cells.** Each lane contains one-tenth the volume of the PCR reaction for the indicated target mRNA. Except for procaspase-5, which was amplified for 35 cycles, all reactions were carried out for 30 cycles. The lane marked *β-actin** is a control PCR reaction using RNA as template. The lack of a product in this lane confirms that the products observed in the other lanes are derived from cDNA rather than genomic DNA.

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 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. 2*A*). 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. 2*A*). Control experiments (Fig. 2*B*) 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. 2*C*). 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. 2*D*) 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-amino-2-heptanone (Fig. 2, compare TLCK in *panels B* and *D*).

The YVAD-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 YVAD-AFC cleavage activity after the addition of etoposide (Fig. 2*E*). 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 YVAD-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. 2*F*).

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 [^{35}S]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. 7*A*). 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. 4*A*, *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. 4*A*, *lane 6*). These bands were termed IRP_1 (relative mobility of 21.5 kDa), IRP_2 (relative mobility of 19.1 kDa), IRP_3 (relative mobility of 18.4 kDa), and IRP_4 (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. 4*B*), 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. 4*A*, *lanes 3–6*). The bands denoted IRP_2 and IRP_3 were faintly visible 2 h after the addition of etoposide, increased dramatically by 3 h, and remained relatively constant thereafter. In contrast, IRP_1 and IRP_4 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^{ϵ}-

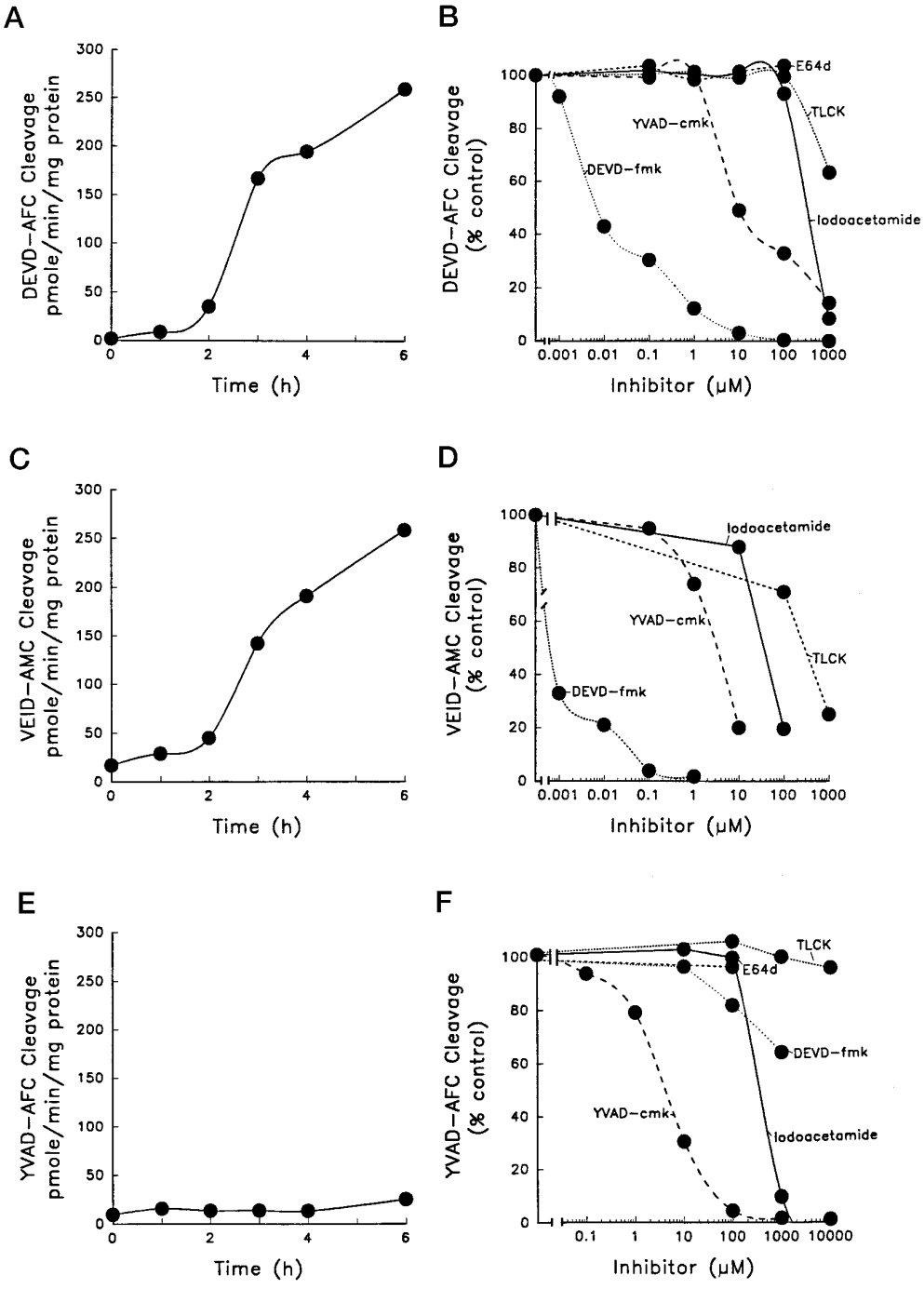

FIG. 2. **Detection of peptidase activity in cytosol from etoposide-treated HL-60 cells.** *A*, *C*, and *E*, cytosol was prepared from HL-60 cells treated with 68 μM etoposide for the indicated lengths of time. Aliquots (50 μg of protein) from the same set of extracts were incubated with DEVD-AFC (*A*), VEID-AMC (*C*), or YVAD-AFC (*E*). The amount of fluorochrome released was determined by comparison to an AFC or AMC standard curve prepared in buffer D. Results are representative of assays performed on six independently derived sets of cytosolic extracts. *B* and *D*, effect of various inhibitors on DEVD-AFC (*B*) or VEID-AMC (*D*) cleavage activity. Aliquots of a cytosolic extract (50 μg of protein) from HL-60 cells treated with 68 μM etoposide for 6 h were incubated with the indicated concentrations of the inhibitors for 5 min at 21 °C and then assayed for DEVD-AFC or VEID-AMC cleavage activity in the continued presence of the inhibitor (which was diluted 10-fold upon the addition of substrate). *F*, effect of various inhibitors on YVAD-AFC cleavage activity. Aliquots of THP.1 cytosol, which is known to contain ICE activity, were incubated with the indicated concentrations of the inhibitors for 5 min at 21 °C and then assayed for YVAD-AFC cleavage activity in the continued presence of the inhibitor (which was diluted 10-fold upon the addition of substrate).

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

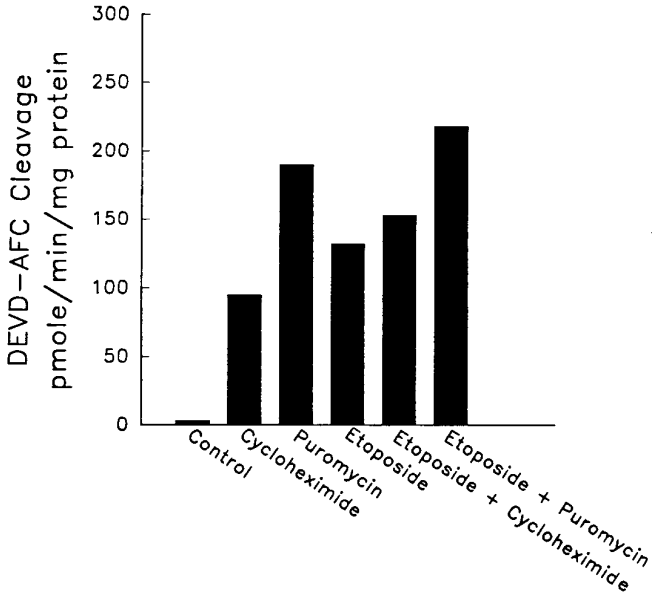

FIG. 3. **Effect of protein synthesis inhibitors on etoposide-induced activation of DEVD-AFC cleavage activity.** HL-60 cells were treated for 6 h with 70 μM cycloheximide or 100 μM puromycin alone or in combination with 68 μM etoposide. At the completion of the incubation, cytosolic extracts were prepared and assayed for DEVD-AFC cleavage activity.

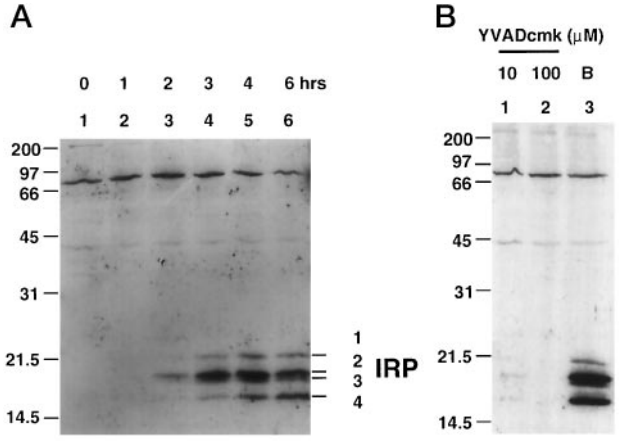

FIG. 4. **Affinity labeling with *Z*-EK(bio)D-aomk.** *A*, affinity labeling of caspases in HL-60 cytosol. Aliquots containing 37 μg of cytosolic protein prepared from HL-60 cells treated with 68 μM etoposide for the indicated lengths of time were reacted with *Z*-EK(bio)D-aomk, subjected to SDS-PAGE, and reacted with peroxidase-coupled streptavidin. Additional experiments (*e.g.* Fig. 5*B*) indicate that bands detected in 0-h samples bind streptavidin in the absence of a reaction with *Z*-EK(bio)D-aomk. *B*, inhibition of *Z*-EK(bio)D-aomk affinity labeling by the ICE tetrapeptide inhibitor YVAD-cmk. Cytosol from etoposide-treated HL-60 cells was reacted with the indicated concentration of YVAD-cmk (*lanes 1* and *2*) or solvent (*lane 3*) prior to *Z*-EK(bio)D-aomk labeling.

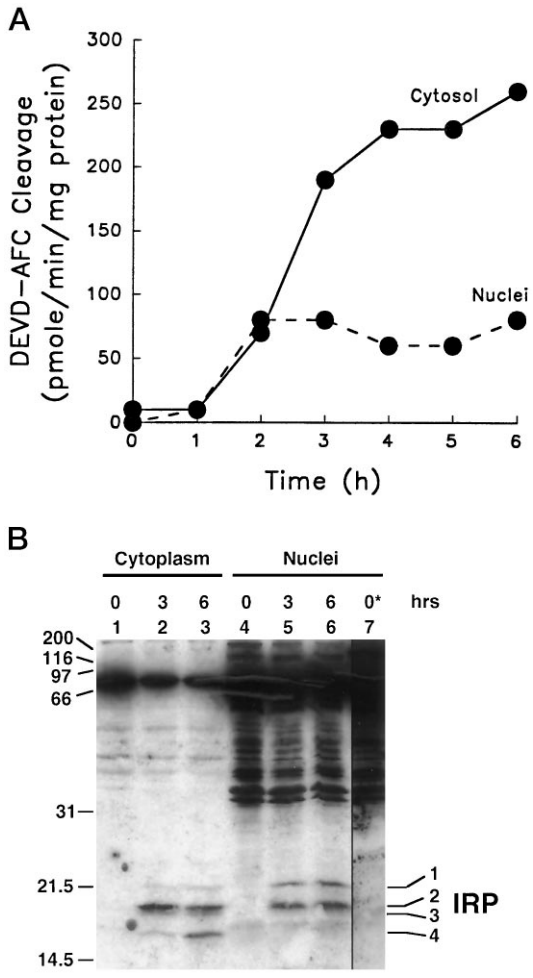

FIG. 5. **Detection of active caspases in purified nuclei.** *A*, DEVD-AFC cleavage activity in cytosol and nuclei prepared from HL-60 cells after treatment with 68 μM etoposide for the indicated lengths of time. *B*, *Z*-EK(bio)D-aomk labeling of cytosol and nuclei of cells treated with 68 μM etoposide for the indicated lengths of time. Samples containing cytosol or nuclei from 3×10^6 cells were incubated with 1 μM *Z*-EK(bio)D-aomk for 1 h at room temperature and then subjected to SDS-PAGE and blotting as described in the legend to Fig. 7. The lane marked *0** contains nuclei that were subjected to SDS-PAGE without *Z*-EK(bio)D-aomk treatment, thereby revealing the streptavidin-binding polypeptides that are normal constituents of these nuclei. In order to obtain clear bands for the active caspases in fractionated nuclei and cytosol, it was necessary to expose the ECL blot to film overnight.

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. 5*A*) 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 cells but not control cells.

Labeling with *Z*-EK(bio)D-aomk (Fig. 5*B*) confirmed the presence of active caspases in nuclei prepared 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. 5*B*, *lane 7*), including a polypeptide of M_r~ 17,000 that migrated just below the expected position of IRP_3. These streptavidin-binding polypeptides did not change during the course of etoposide treatment (Fig. 5*B*, *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 IRP_1 and IRP_2 (Fig. 5*B*, *lanes 4–6*). Interestingly, IRP_3 and IRP_4 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

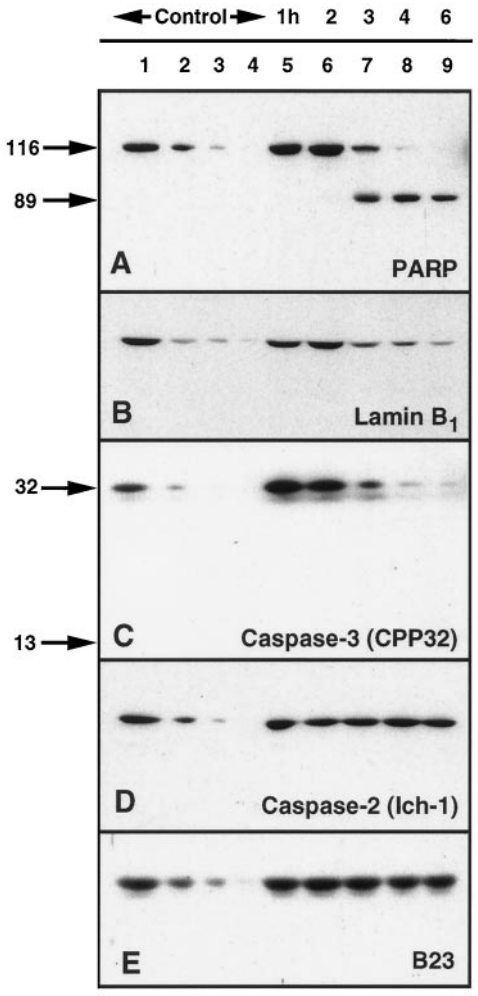

FIG. 6. **Immunoblot analysis of caspase precursors in etoposide-treated HL-60 cells.** HL-60 cells were treated with 68 μM etoposide continuously for 0, 1, 2, 3, 4, or 6 h (*lanes 1* and *5–9*, respectively). Samples containing total cellular polypeptides from 3×10^5 cells were subjected to SDS-PAGE followed by immunoblotting with antibodies that recognize PARP (*A*), lamin B_1 (*B*), procaspase-3 (*C*), procaspase-2 (*D*), or the 38-kDa nucleolar protein B23 (*E*), a polypeptide that does not undergo apoptosis-associated proteolysis (31). *Lanes 2–4*, serial 2-fold dilutions of the samples in *lane 1*.

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 B_1 as well as the nucleolar protein B23, a polypeptide that is 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. 6*A*, *lane 7*). A marked decrease in lamin B_1 content, indicative of lamin B_1 degradation (39, 66, 67), was also evident 3 h after etoposide addition (Fig. 6*B*, *lane 7*). At this time point, a decrease in procaspase-3 was evident (Fig. 6*C*, *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. 6*D*).

Despite the marked decrease in procaspase-3 signal (Fig. 6*C*), 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. 7*A*) revealed that IRP_1 and IRP_3 comigrated with two bands observed in Sf9 cells expressing caspase-6 (Fig. 7*A*, *lane 4*); IRP_2 and IRP_4 comigrated with two bands observed in Sf9 cells expressing caspase-3 (Fig. 7*A*, *lane 5*); and IRP_4 also comigrated with the band observed in Sf9 cells expressing caspase-2 (Fig. 7*A*, *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. 7*A*.

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. 7*C*, *bottom*). When apoptotic HL-60 cytosol and the recombinant caspases were compared, a number of the labeled spots comigrated (Fig. 7*C*, *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 ($IRP_{1–3}$) 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

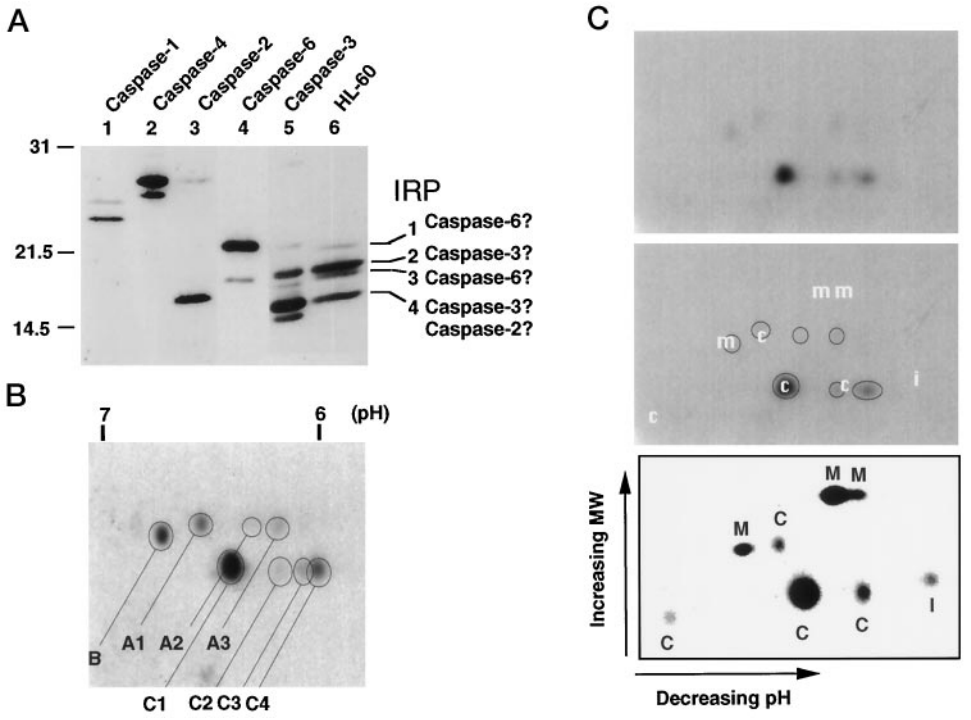

FIG. 7. **Analysis of *Z*-EK(bio)D-aomk-labeled caspases by two-dimensional gel electrophoresis.** *A*, comparison of the mobilities of cloned human caspases with the IRPs detected in apoptotic HL-60 cytosol by unidimensional SDS-PAGE. Samples containing 1 μg (*lanes 1*, *4*, and *5*), 10 μg (*lanes 2* and *3*) or 37 μg (*lane 6*) of extract protein from Sf9 cells expressing caspase-1 (*lane 1*), caspase-4 (*lane 2*), caspase-2 (*lane 3*), caspase-6 (*lane 4*), caspase-3 (*lane 5*), or apoptotic HL-60 cells (*lane 6*) were labeled with *Z*-EK(bio)D-aomk, subjected to SDS-PAGE, and detected with peroxidase-coupled streptavidin as described under "Experimental Procedures." The putative identity of the various IRP species observed in etoposide-treated HL-60 cells is shown at the *right*. *B*, indexing of the active caspases detected in HL-60 cytosol by *Z*-EK(bio)D-aomk labeling followed by two-dimensional analysis. Species labeled *A*, *B*, and *C* comigrate in the second dimension (SDS-PAGE) and may correspond to posttranslational modifications of a single subunit. *C*, two-dimensional labeling pattern of cytosol from apoptotic HL-60 cells (*top*) or Sf9 cells expressing various combinations of caspase-2, -3, and -6 (*bottom*). The *middle panel* shows a composite image obtained by merging the *upper* and *lower images* in *panel C*. *Circles* show the position of active caspases in HL-60 cytosol, and *letters* indicate the positions of active species of caspase-2 (*I*), caspase-3 (*C*), and caspase-6 (*M*) as determined in the *lower panel*. The *upper* and *lower panels* were aligned by co-electrophoresing a mixture of active caspase-3 with HL-60 cytosol (not shown). Note that the relative intensity of the spots in the *lower panel* is arbitrary; this image was adjusted to allow maximum visibility of all recombinant spots.

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. 2*A*) and VEID-AMC (Fig. 2*C*) 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. 2*E*), 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. IRP_2 and IRP_4 appear to correspond to caspase-3 (Fig. 7*B*). The appearance of these active species occurs with a time course (Fig. 4) that parallels the appearance of DEVD-AFC cleavage activity (Fig. 2*A*) and disappearance of procaspase-3 from immunoblots (Fig. 6*C*). Our demonstration that caspase-3 is enzymatically active in HL-60 apoptosis (*i.e.* covalently labels with *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_3/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 IRP_1 might correspond to a second active species of caspase-6 (Fig. 7*A*), this assignment could not be confirmed by two-dimensional isoelectric focusing/SDS-PAGE because IRP_1 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. 7*C*) 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. 5*B*). 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. 5*B*) 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. 2*A*) or *Z*-EK(bio)D-aomk-labeled polypeptides corresponding to the large subunit of active caspases in cytosol from control HL-60 cells (Fig. 4*A*). These observations argue against the possibility that caspase-3 (or any related protease) is preactivated in the cytosol of HL-60 cells and effectively rule out a model in which the activity of preactivated 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 etoposide-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 several other human cell lines) prior to the application of apoptotic stimuli (Fig. 6; see also Refs. 17, 22, 26, 27, and 45).

In support of this conclusion, RT-PCR (Fig. 1) indicated that untreated HL-60 cells contain transcripts for at least nine caspases. Although procaspase polypeptide levels might not always reflect the levels of the corresponding transcripts, it is reasonable to assume that as many as nine procaspase polypeptides are expressed in HL-60 cells. This does not mean, however, that all nine procaspases are activated during the course of apoptosis. We failed to detect an increase in caspase-1 enzymatic activity (YVAD-AFC cleavage) after etoposide treatment (Fig. 2*E*). Likewise, immunoblotting indicated that the bulk of procaspase-2 present in HL-60 cells remained intact during apoptosis (Fig. 6*D*), and two-dimensional analysis failed to detect a *Z*-EK(bio)D-aomk-labeled spot corresponding to active caspase-2 (Fig. 7*C*). This is in contrast to the extensive cleavage (Fig. 6*C*) and activation (Fig. 7*C*) of procaspase-3. The mechanism for selective apoptotic activation of some caspase precursors (*e.g.* procaspase-3) and not others (*e.g.* procaspase-2) is currently unclear and requires further investigation.

Acknowledgments—We thank Dr. Ivan Lieberburg for making this collaboration possible; Michael Power for DNA sequence confirmation of clones and constructs used in this work; Alastair Mackay and Joe Lewis for help with the two-dimensional gel analysis; and Deb Strauss for secretarial assistance.

REFERENCES

1. Sen, S., and D'Incalci, M. (1992) *FEBS Lett.* **307,** 122–127
2. Dive, C., Evans, C. A., and Whetton, A. D. (1992) *Semin. Cancer Biol.* **3,** 417–427
3. Sachs, L., and Lotem, J. (1993) *Blood* **82,** 15–21
4. Kerr, J. F., Winterford, C. M., and Harmon, B. V. (1994) *Cancer* **73,** 2013–2026
5. Kaufmann, S. H. (1996) *Mol. Med. Today* **2,** 269–313
6. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) *Int. Rev. Cytol.* **68,** 251–306
7. Kyprianou, N., English, H. F., Davidson, N. E., and Isaacs, J. T. (1991) *Cancer Res.* **51,** 162–166
8. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994) *Science* **266,** 807–810
9. Martin, D. S., Stolfi, R. L., Colofiore, J. R., Nord, L. D., and Sternberg, S. (1994) *Cancer Invest.* **12,** 296–307
10. Li, X., Gong, J., Feldman, E., Seiter, K., Traganos, F., and Darzynkiewicz, Z. (1994) *Leuk. Lymph.* **13,** 65–70
11. Miyashita, T., and Reed, J. C. (1993) *Blood* **81,** 151–157
12. Bedi, A., Barber, J. P., Bedi, G. C., el-Deiry, W. S., Sidransky, D., Vala, M. S., Akhtar, A. J., Hilton, J., and Jones, R. J. (1995) *Blood* **86,** 1148–1158
13. Earnshaw, W. C. (1995) *Cur. Opin. Cell Biol.* **7,** 337–343
14. Martin, S. J., and Green, D. R. (1995) *Cell* **82,** 349–352
15. Nicholson, D. W. (1996) *Nat. Biotechnol.* **14,** 297–301
16. Black, R. A., Kronheim, S. R., and Sleath, P. R. (1989) *FEBS Lett.* **247,** 386–390
17. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., and Lazebnik, Y. A. (1995) *Nature* **376,** 37–43
18. Sleath, P. R., Hendrickson, R. C., Kronheim, S. R., March, C. J., and Black, R. A. (1990) *J. Biol. Chem.* **265,** 14526–14528
19. Howard, A. D., Kostura, M. J., Thornberry, N., Ding, G. J., Limjuco, G., Weidner, J., Salley, J. P., Hogquist, K. A., Chaplin, D. D., and Mumford, R. A. (1991) *J. Immunol.* **147,** 2964–2969
20. Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8395–8400
21. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., and Aunins, J. (1992) *Nature* **356,** 768–774
22. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55,** 2737–2742
23. Fraser, A., and Evan, G. (1996) *Cell* **85,** 781–784
24. Ellis, H. M., and Horvitz, H. R. (1986) *Cell* **44,** 817–829
25. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) *Cell* **75,** 641–652
26. Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salveson, G., Earnshaw, W. C., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55,** 6045–6052
27. Chinnaiyan, A. M., Orth, K., O'Rourke, K., Duan, H., Poirier, G. G., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 4573–4576
28. Patel, T., Gores, G. J., and Kaufmann, S. H. (1996) *FASEB J.* **10,** 587–597
29. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) *Nature* **371,** 346–347
30. Kaufmann, S. H. (1989) *Cancer Res.* **49,** 5870–5878
31. Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. (1993) *Cancer Res.* **53,** 3976–3985
32. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salveson, G. S., and Dixit, V. M. (1995) *Cell* **81,** 801–809
33. Gu, Y., Sarnecki, C., Aldape, R. A., Livingston, D. J., and Su, M. S.-S. (1995) *J. Biol. Chem.* **270,** 18715–18718
34. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
35. Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 16443–16446
36. Froelich-Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem.* **270,** 21429–21432
37. Creaven, P. J. (1984) in *Etoposide (VP-16): Current Status and New Developments* (Issell, B. F., Muggia, F. M., and Carter, S. K., eds.) pp. 103–115, Academic Press, Inc., Orlando, FL
38. Miller, D. K., Ayala, J. M., Egger, L. A., Raju, S. M., Yamin, T. T., Ding, G. J., Gaffney, E. P., Howard, A. D., Palyha, O. C., Rolando, A. M., *et al.* (1993) *J. Biol. Chem.* **268,** 18062–18069
39. Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) *J. Cell Biol.* **123,** 7–22
40. Kaufmann, S. H., Gibson, W., and Shaper, J. H. (1983) *J. Biol. Chem.* **258,** 2710–2719
41. Krantz, A., Copp, L. J., Coles, P. J., Smith, R. A., and Heard, S. B. (1991) *Biochemistry* **30,** 4678–4687
42. Robinson, V. J., Pauls, H. W., Coles, P. J., Smith, R. A., and Krantz, A. (1992) *Bioorg. Chem.* **20,** 42–54
43. Dolle, R. E., Hoyer, D., Prasad, C. V. C., Schmidt, S. J., Helaszek, C. T., Miller,

R. E., and Ator, M. A. (1994) *J. Med. Chem.* **37,** 563–564
44. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
45. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) *J. Biol. Chem.* **269,** 30761–30764
46. Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) *Cell* **78,** 739–750
47. Faucheau, C., Diu, A., Chan, A., Blanchet, A. M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A., Rocher, C., Su, M. S., Livingston, D. J., Hercend, T., and Lalanne, J. L. (1995) *EMBO J.* **14,** 1914–1922
48. Lippke, J. A., Gu, Y., Sarnecki, C. Caron, P. R., and Su, M. S.-S. (1996) *J. Biol. Chem.* **271,** 1825–1828
49. Kamens, J., Paskind, M., Hugunin, M., Talanian, R. V., Allen, H., Banach, D., Bump, N., Kackett, M., Johnston, C. G., Li, P., Mankovich, J. A., Terranova, M., and Ghayur, T. (1995) *J. Biol. Chem.* **270,** 15250–15256
50. Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineaux, S. M., Yamin, T. T., Yu, V. L., and Nicholson, D. W. (1995) *J. Biol. Chem.* **270,** 15870–15876
51. Duan, H. J., Chinnaiyan, A. M., Hudson, P. L., Wing, J. P., He, W. W., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 1621–1625
52. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual,* W. H. Freeman Co., New York
53. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) *J. Virol.* **67,** 4566–4579
54. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021
55. Kaufmann, S. H., Ewing, C. M., and Shaper, J. H. (1987) *Anal. Biochem.* **161,** 89–95
56. Lamarre, D., Talbot, B., deMurcia, G., Laplante, C., Leduc, Y., Mazen, A., and Poirier, G. G. (1988) *Biochim. Biophys. Acta* **950,** 147–160
57. Kaufmann, S. H. (1989) *J. Biol. Chem.* **264,** 13946–13955
58. Fields, A. P., Kaufmann, S. H., and Shaper, J. H. (1986) *Exp. Cell Res.* **164,** 139–153
59. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
60. Martin, S. J., and Cotter, T. G. (1990) *Cell Tissue Kinet.* **23,** 545–559
61. Martin, S. J., Lennon, S. V., Bonham, A. M., and Cotter, T. G. (1990) *J. Immunol.* **145,** 1859–1867
62. Del Bino, G., and Darzynkiewicz, Z. (1991) *Cancer Res.* **51,** 1165–1169
63. Ray, S., Ponnathpur, V., Huang, Y., Tang, C., Mahoney, M. E., Ibrado, A. M., Bullock, G., and Bhalla, K. (1994) *Cancer Chemother. Pharmacol.* **34,** 365–371
64. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) *Cell* **85,** 803–815
65. Thornberry, N. A., Peterson, E. P., Zhao, J. J., Howard, A. D., Griffin, P. R., and Chapman, K. T. (1994) *Biochemistry* **33,** 3934–3940
66. Smith, G. K., Duch, D. S., Dev, I. K., and Kaufmann, S. H. (1992) *Cancer Res.* **52,** 4895–4903
67. Lazebnik, Y. A., Takahashi, A., Moir, R., Goldman, R., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9042–9046
68. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380,** 723–726
69. Ibrado, A. M., Huang, Y., Fang, G., and Bhalla, K. (1996) *Cell Growth & Differ.* **7,** 1087–1094
70. Alnemri, E. S., Fernandes-Alnemri, T., Pomerenke, K., Robertson, N. M., Dudley, K., DuBois, G. C., and Litwack, G. (1994) *J. Biol. Chem.* **269,** 30828–30834
71. Boudreau, N., Sympson, C. J., Werb, Z., and Bissell, M. J. (1995) *Science* **267,** 891–893
72. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salveson, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) *Cell* **87,** 171

Cell Biology and Metabolism:

Activation of Multiple Interleukin-1β Converting Enzyme Homologues in Cytosol and Nuclei of HL-60 Cells during Etoposide-induced Apoptosis

Luis M. Martins, Timothy Kottke, Peter W. Mesner, Guriqbal S. Basi, Sukanto Sinha, Normand Frigon, Jr., Eric Tatar, Jay S. Tung, Karin Bryant, Atsushi Takahashi, Phyllis A. Svingen, Benjamin J. Madden, Daniel J. McCormick, William C. Earnshaw and Scott H. Kaufmann

J. Biol. Chem. 1997, 272:7421-7430.
doi: 10.1074/jbc.272.11.7421