Selective Activation of Caspases During Apoptotic Induction in HL-60 Cells

EFFECTS OF A TETRAPEPTIDE INHIBITOR

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Apoptosis is a highly regulated biochemical process that results in the selective death of cells. Members of the caspase family of cysteine proteases play a pivotal role in the effector phase of apoptosis. We show that, in HL-60 cells, the addition of either anisomycin, a protein synthesis inhibitor, or geranylgeraniol, an intermediate in the cholesterol biosynthetic pathway, results in a rapid and en masse induction of apoptosis. The levels of actin, p42 and p44 MAPK, JNK1, JNK2, p38, and PCNA were not substantially altered during this process. Although these treatments appear to function by diverse pathways, they both result in the processing and activation of caspase-3 (CPP32b/Yama/Apopain). In contrast, no activation of caspase-1 (interleukin-1β converting enzyme (ICE)) was observed. Furthermore, we obtained ambiguous results regarding the activation of caspase-2 (Ich-1) depending on the antibody used. Pretreatment of the cells with benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (zVAD.fmk), a tetrapeptide inhibitor of caspases, prevented the induction of apoptosis for 24 h. Even after 72 h of treatment, some cells were still alive and progressing through the cell cycle, suggesting that blockage of caspase activity is able to protect cells. These results suggest that selective activation of some caspases is necessary to induce apoptosis in HL-60 cells.

The growth and development of all multicellular organisms is controlled by the selective growth and death of cells throughout the lifetime of the organism (1). The realization that cancer may arise via inhibition of a cell death pathway together with the observations that many of the anti-cancer drugs currently in clinical use function to induce cell death, irrespective of their presumptive mechanism of action, has heightened awareness of the importance of understanding the pathways leading to cell death (2, 3). Apoptosis is a term used to describe a distinctive set of morphological and biochemical changes that define a cell death pathway that has far-reaching implications in our understanding of normal development as well as many diseases (4). Apoptosis is characterized by defined and systematic morphological changes, including a decrease in cell volume and buoyant density, chromatin condensation, DNA fragmentation, cell surface blebbing and the eventual formation of membrane-bound apoptotic bodies (4). Despite the large variety of stimuli that are capable of inducing apoptosis in a variety of cell types, the highly conserved nature of the biochemical and morphological changes that occur during apoptosis suggests that common effectors must irreversibly commit the cell to death. In a previous study using two-dimensional gel electrophoresis, we showed that apoptotic cells do not display extensive proteolysis of cellular proteins implying that highly selective cleavage of specific substrates is necessary to induce an apoptotic response (5). Although a number of reports have identified agents that are capable of inducing apoptosis in a variety of tissues and cells, the mechanisms responsible for the activation of the apoptotic process and that irreversibly commit the cells to death are poorly understood.

Interest in apoptosis was stimulated when it was realized that the Caenorhabditis elegans apoptosis inducing protein, Ced-3, displayed similarity to the mammalian interleukin-1β converting enzyme (ICE/caspase-1). Furthermore, both the C. elegans and mammalian proteins are functionally interchangeable, suggesting that the pathways regulating apoptosis have been highly conserved throughout evolution (6). The ICE/Ced-3-like protease family has recently been renamed caspase, with ICE/caspase-1 (7) and mammalian proteins being functionally interchangeable (8). The enzymes all recognize aspartic acid in the P1 position, while variations in the P2 to P4 position determine the relative specificity of each individual member. The active form of these proteases in bacteria results in processing of the full-length proteins into their constituent 20 and 10 kDa subunits (9). The enzymes all recognize aspartic acid in the P1 position, while variations in the P2 to P4 position determine the relative specificity of each individual member. The active form of these enzymes is a tetramer composed of two 20 and two 10 kDa subunits (12, 13). Overexpression of these proteases in bacteria results in processing of the full-length proteins into their constituent 20 and 10 kDa subunits, suggesting that the proteases are capable of autoactivation (11). Although these proteins are thought to play pivotal roles in the induction of apoptosis, the mechanisms regulating their activation in vivo remain elusive.

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Caspase Activation During HL-60 Cell Apoptosis

This is partly due to the rapidity of the apoptotic process as well as the fact that, in most instances, the induction of apoptosis is not a synchronous event.

In view of the similarity between Ced-3 and caspase-1, a central role for caspase-1 in inducing apoptosis in mammalian cells has been postulated. However, caspase-1 knockout mice do not show a general defect in apoptosis (14, 15). Alternatively, another member of this family, caspase-3, may play a more general role in mediating apoptosis in a variety of cells and tissues as suggested by the following observations. First, of the known caspases, caspase-3 displays the highest similarity to Ced-3 (16–18). Second, caspase-3 is ubiquitously expressed in all tissues examined, whereas caspase-1 distribution is restricted primarily to monocytes and macrophages (19). Third, purified caspase-3 has been shown to cleave a variety of substrates, including poly(ADP-ribose) polymerase (16, 17), U1-70K (20, 21), and DNA-dependent protein kinase (22, 23), that undergo cleavage during apoptosis. Finally, inhibition of caspase-3 blocks apoptosis in a variety of systems, whereas caspase-1 inhibitors are much less effective (17, 24–26).

In an effort to better understand the mechanisms regulating the activation of caspases, we sought to identify conditions that would lead to a complete and synchronous induction of apoptosis as possible. Using HL-60 cells, a human promyelocytic cell line, we show that both anisomycin, a protein synthesis inhibitor, and geranylgeraniol, an intermediate in the cholesterol biosynthetic pathway, are able to induce apoptosis in approximately 80% of the cells within 2 h. Induction of apoptosis in these cells is accompanied by the processing and activation of caspase-3 and possibly also caspase-2. However, caspase-1 was not processed and consequently does not appear to play a major role in this process. The addition of benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (zVAD.fmk), a tetrapeptide inhibitor of caspases, prolonged the induction of apoptosis induced by either agent and, furthermore, enabled some cells to continue to grow even after 72 h of treatment. The relevance of these results to our understanding of the mechanisms of apoptosis will be discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents including RPMI 1640, t-methionine-free RPMI 1640, and glutamine were from Life Technologies, Inc. Dialyzed fetal bovine serum was from Hyclone Laboratories, Inc. (Logan, UT). [35S]methionine (>1000 Ci/mM) was from DuPont NEN. Hoechst 33258 was from Molecular Probes (Eugene, OR). NuSieve GTG-agarose was from FMC Corp. (Rockland, ME). Anisomycin, geranylgeraniol, propidium iodide (PI), and anti-actin antibodies were from Sigma. Monoclonal antibodies against CPP32/caspase-3 (C91720, raised against a 24.7 Kd fragment corresponding to amino acids 1-219 of human caspase-3) and I-ch1/caspase-2 (I29120, raised against a 19.5 Kd fragment corresponding to amino acids 225-401 of human caspase-2) were from Transduction Labs (Lexington, KY); and the phospho-p38 antibody was from New England Biolabs Inc. (Beverly, MA). All other antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), including the rabbit polyclonal anti-Ich-1 (20, 21)-specific antibody, which recognizes the precursor form of human caspase-2) and goat polyclonal anti-Ich-1 (20, 21)-specific antibody, which recognizes the precursor form of human caspase-2 antibodies. zVAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylcoumarin (DEVD.AFC), both dissolved in dimethyl sulfoxide, and DEVD.AFC and benzyloxycarbonyl-Tyr-Val-Ala-Asp-aminolevulinic acid-trifluoromethylcoumarin (TVAD.AFC) overlay membranes were from Enzyme Systems Products (Dublin, CA). Other chemicals were of the highest grade available.

**Cell Culture, DNA Extraction and Electrophoresis**—HL-60 human leukemia cells (ATCC CCL-240) were maintained as logarithmically growing cultures in 90% RPMI 1640, 10% heat-inactivated fetal bovine serum in a humidified incubator with 95% air, 7% CO2 at 37 °C. DNA was extracted and used in a salt precipitation procedure (Stratagene, LaJolla, CA). Briefly, this involved lysis of the cells and digestion of cellular proteins using Pronase A. Proteins were removed following the addition of NaCl, and the DNA was precipitated with ethanol. The DNA was separated on a 1.5% agarose gels in Tris-EDTA-aceate buffer and visualized by UV fluorescence after staining with ethidium bromide.

**Flow Cytometry and Protease Activity Assays**—Fluorescence-activated cell sorting (FACS) was performed as described (5). The PI fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). For protease activity assays, 10–1 µl aliquots of cell lysates prepared from control and treated cells were diluted 10-fold in assay buffer (50 mM HEPES, pH 7.5, 10% sucrose, 100 µM NaCl, 0.1% CHAPS) containing 10 µM DEVD-AFC and incubated at room temperature for up to 1 h. The release of AFC was measured in a Cytoflour 11, fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, MA) using an excitation filter of 400/30 nm and an emission filter of 508/20 nm.

**Metabolic Labeling**—Metabolic labeling of cells was conducted by incubating cells with the appropriate agent for varying lengths of time, as indicated in the figure legends, prior to incubating cells in 1000 Ci/mM) was from DuPont NEN. RPMI 1640 containing 500 µCi of [35S]methionine for the final 15 min of incubation. The cells were washed with methionine-free RPMI 1640 and lysed by boiling for 10 min following the addition of SDS-PAGE sample buffer. Samples were clarified by centrifugation for 5 min at 14,000 rpm and then analyzed in pre-cast 1 mm, 12% Tris/glycine gels, according to Krajewski et al. Following this treatment, the membranes were incubated with the appropriate horseradish peroxidase-coupled secondary antibodies at a dilution of 1:10,000 and then washed extensively with TBST and then reblocked over-night at 2.5% BSA and then incubated for 1 h. The membranes were blocked overnight in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 2.5% BSA and then incubated for 1 h with the appropriate primary antibody in 2.5% BSA/TBST at concentrations recommended by the manufacturers, followed by four washes for 15 min each with TBST. The membranes were blocked for 30 min with 2.5% BSA/TBST prior to the addition of either goat anti-rabbit or goat anti-mouse, horseradish peroxidase-coupled secondary antibodies at a dilution of 1:10,000 (Amersham Corp.). The blots were incubated for 1 h in secondary antibodies followed by four washes for 15 min each with TBST. Enhanced chemiluminescence (Amersham Corp.) was used to detect the relevant proteins following protocols suggested by the manufacturer. In some experiments, after the membranes were blocked, they were incubated with the appropriate peroxidase antibody for 1 h, followed by treatment with 3,3′-diaminobenzidine tetrahydrochloride (DAB) in order to block nonspecific binding sites according to Krajewski et al. (27). Following this treatment, the membranes were washed extensively with TBST and then reblocked overnight in TBST plus BSA prior to the addition of primary antibody. The blots were then further processed as described above.
RESULTS AND DISCUSSION

The aim of this study was to determine conditions that would lead to the rapid and en masse induction of apoptosis in HL-60 cells in order to begin to dissect the molecular mechanisms leading to the commitment point of the apoptotic process. We postulated that, although a variety of agents are capable of inducing apoptosis via distinct signaling pathways, they would ultimately converge on one or at most a limited number of “common final pathways” that commits the cell to death. Apoptosis was defined by a variety of criteria, including chromatin condensation, DNA fragmentation, DNA hypo-diploidy, increased buoyant density, and a variety of morphological parameters. A number of agents known to induce apoptosis in a variety of cells were tested for their effectiveness in HL-60 cells. These agents included protein synthesis inhibitors (such as cycloheximide and anisomycin) (28), topoisomerase inhibitors (such as camptothecin) (28, 29), protein kinase inhibitors (such as staurosporine) (30), protein phosphatase inhibitors (such as okadaic acid) (31), nuclear spindle disrupting agents (such as nocodazole) (32), and intermediates in the cholesterol biosynthetic pathway (such as geranylgeraniol) (33). Many of these agents were capable of inducing apoptosis in HL-60 cells; however, the time of induction by these agents was relatively long, on the order of 8-24 h (data not shown). Two agents were found to induce rapid and en masse apoptosis, anisomycin and geranylgeraniol (Fig. 1). These two agents, however, appear to induce apoptosis in HL-60 cells by quite distinct mechanisms (see below).

Anisomycin and Geranylgeraniol Induce Apoptosis in HL-60 Cells—Treatment of HL-60 cells with either 1 µg/ml anisomycin-
cin or 10 μM geranylgeraniol led to the progressive induction of apoptosis as judged by Hoechst 33258 staining of cells (Fig. 1A). Both anisomycin and geranylgeraniol induced approximately 80% of the cells to display condensed nuclear staining within 2 h of treatment. Generally, the cells showed a punctate staining characteristic of the condensation of chromatin that accompanies apoptosis. In agreement with previous studies, apoptotic cells stained with Hoechst 33258 were more fluorescent than non-apoptotic cells, indicating either that they display increased uptake of dye or that binding of the dye to DNA is enhanced (34, 35). Continued incubation of cells with either agent led to essentially complete induction of apoptosis at later time points (data not shown). Furthermore, continued incubation of cells in the presence of geranylgeraniol led to the appearance of ghosts in which the Hoechst fluorescence was dramatically diminished. This was partly evident after 4 h of treatment (Fig. 1A). This effect may have resulted from secondary necrosis or to permeability changes in the membrane of the apoptotic bodies resulting in the release of nuclear components. The mechanism of this effect was not investigated further in this study.

In order to quantitate the induction of apoptosis by these agents, cells were labeled with PI and analyzed by flow cytometry (FACS). A characteristic hypo-diploid DNA content peak was observed following treatment of HL-60 cells with either anisomycin or geranylgeraniol (Fig. 1A). The time course for the appearance of this hypo-diploid peak of DNA correlated with the morphological appearance of apoptosis using Hoechst staining (Fig. 1A). The induction of apoptosis by either stimulus was not cell cycle-dependent as judged by the uniform loss of cells from all stages of the cell cycle (Fig. 1A). Examination of both Hoechst staining and FACS analysis demonstrated that geranylgeraniol appeared to induce apoptosis slightly more rapidly than anisomycin. This is much more evident when the proportion of cells displaying hypo-diploid DNA was plotted against time of treatment (Fig. 1A). This effect may have resulted from secondary necrosis or to permeability changes in the membrane of the apoptotic bodies resulting in the release of nuclear components. The mechanism of this effect was not investigated further in this study.

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full-length caspase-3 protein was evident (Fig. 3). As shown in Fig. 3, both anisomycin and geranylgeraniol progressively induced the processing of caspase-3. The processing of caspase-3 correlated with the time course of induction of apoptosis (compare Fig. 3 with Fig. 1). Furthermore, a new band corresponding to caspase-3-p17 was generated in samples in which processing of the full-length form of caspase-3 was evident (Fig. 3). To confirm that the processing of caspase-3 resulted in an active form of the enzyme, we assayed for an activity capable of cleaving DEVD.AFC, a fluorogenic tetrapeptide substrate for caspase-3, using both a solution assay as well as an enzyme overlay blot. Lysates from apoptotic cells were mixed with DEVD.AFC, and the fluorescence of the released substrate was recorded using a fluorescence plate reader. Alternatively, lysates were run on a native gel to retain activity, and then a membrane impregnated with DEVD.AFC was overlaid onto the gel. In both instances, an activity was detected in lysates from cells treated with anisomycin that was not apparent in control lysates (data not shown). The time course of induction using a solution-based assay correlated with the processing of caspase-3. The sensitivity of the blot overlay method was insufficient to allow us to detect significant activation of a DEVD cleaving activity at early times (data not shown). However, at later times, an activity was detectable, and the formation of the fluorogenic substrate correlated with the migration of caspase-3 as determined by Western blot analysis of the blot (data not shown). No activity was detected when an overlay membrane containing YVAD.AFC, a tetrapeptide substrate with greater specificity for proteases with greater similarity to caspase-1, was applied to the gel (data not shown). A small amount of activity toward YVAD.AFC was detected in the solution-based assays; however, this activity was only apparent at later time points (data not shown).

Originally, using a monoclonal antibody raised against caspase-2 (Transduction Labs) we were unable to observe processing of this protease in lysates treated with either anisomycin or geranylgeraniol. However, subsequent probing of blots of the same apoptotic lysates using a goat polyclonal antibody from Santa Cruz Biotechnology, Inc. revealed that a band with similar mobility to caspase-2 was observed to decrease in intensity over time with a concomitant increase in intensity of a band at approximately p12, corresponding to the carboxy-terminal portion of caspase-2 (Fig. 3). Due to the nonspecific cross-reactivity of this antibody to other proteins, this effect was only clearly discernible when nonspecific binding sites
were destroyed by pretreating the blots with horseradish peroxidase-coupled anti-goat antibody and then destroying the horseradish peroxidase with DAB prior to incubation with the primary antibody (27). Similar results were obtained using a rabbit polyclonal antibody against caspase-2 from Santa Cruz Biotechnology, Inc. although it is less apparent due to the presence of an additional nonspecific band, which migrates close to that of the apparent full-length caspase-2 (data not shown). Apart from the bands at p48 and p12, several additional bands were observed using either of the polyclonal antibodies against caspase-2. The relationship of these bands to caspase-2 or other caspases is unknown although some of these bands were detected by both polyclonal antibodies and could represent proteolytic fragments of caspase-2. The current data, however, do not allow us to substantiate this conclusion. We have obtained essentially similar results using Fas-induced apoptosis in Jurkat T lymphoblasts. In these cells, caspase-2, detected using the Santa Cruz Biotechnology, Inc. goat polyclonal antibody (data not shown), and caspase-3\(^2\) are observed to undergo processing very rapidly following stimulation with an anti-Fas antibody. In these same lysates, no change is observed in the level of the protein detected by the Transduction Labs antibody. We were unable to discern the temporal relationship between caspase-2 and caspase-3 antibodies using immunoprecipitation and protein identification techniques have been unsuccessful. Resolution of these issues is required before a conclusion can be formed regarding the state of activation of caspase-2 in apoptotic lysates and its role in the induction of apoptosis. However, the current results suggest that conclusions drawn about the state of activation of caspase-2 obtained using the Transduction Labs anti-caspase-2 antibody warrants re-evaluation.

In contrast to the results obtained with caspase-2 and caspase-3, we were unable to detect processing of caspase-1 following treatment of cells with either anisomycin or geranylgeraniol (Fig. 3). Interestingly, a band migrating at p32 was detected in both our control and apoptotic samples when we probed for caspase-1 (Fig. 3). This band possibly reflected a partially cleaved form of caspase-1. The level of this product did not change significantly during the induction of apoptosis by either anisomycin or geranylgeraniol. Previous studies by other workers have also noted the appearance of a processed form of caspase-1 in extracts of cells (42). The relevance of this product to the induction of apoptosis is unknown at this time. The lack of involvement of caspase-1 in the induction of apoptosis is not surprising given the fact that caspase-1 knockout mice do not show a general defect in apoptosis (14, 15). Furthermore, previous studies have demonstrated that YVAD.aldehyde was ineffective in blocking apoptosis induced by a variety of stimuli.

\(^{2}\) J. D. Watts, M. Gu, A. J. Polverino, S. D. Patterson, and R. Aebersold, manuscript in preparation.
Overall, these results suggest that a proteolytic cascade involving activation of all caspases is not a common or essential feature of apoptosis. Furthermore, the results suggest that activation of at least caspase-3 and possibly also caspase-2 may be necessary to induce all the characteristic features of apoptosis. This implies either that the mechanisms leading to the activation of distinct caspases are highly specific depending on the stimulus and/or tissue or that these caspases are compartmentalized and unavailable for activation. Consequently, identification of the caspases activated during apoptotic induction in specific tissues is required in order to better understand the mechanisms underlying the activation of each of these enzymes. Furthermore, these results suggest that the development of effective therapies directed against inhibition of apoptosis is much more realistic given the fact that inhibition of one or a few caspases may be necessary to prevent the onset of inappropriate or uncontrolled apoptosis.

*zVAD.fmk Blocks Apoptosis Induced by Anisomycin or Geranylgeraniol*—In view of our results demonstrating specific activation of certain caspases, we tested the ability of specific inhibitors to protect cells against the induction of apoptosis. Previous studies have suggested that tetrapeptide inhibitors, based on the cleavage specificity of known substrates of caspases, are capable of inhibiting apoptosis in a variety of systems (17, 24–26). However, these studies failed to evaluate whether the protection afforded by these compounds was complete and enabled the cells to continue to grow. We tested the ability of *zVAD.fmk*, which has previously been shown to be a potent inhibitor of caspases (44), to inhibit apoptosis induced by anisomycin or geranylgeraniol. We preloaded cells with 100 μM *zVAD.fmk* for 1 h to allow the inhibitor to accumulate within cells and then stimulated cells with either anisomycin or geranylgeraniol for a further 1 h. The cells were washed to remove excess inducers and then resuspended in medium containing *zVAD.fmk* and monitored for their ability to continue to grow. As shown in Fig. 4, A and B, there was no change in the proportion of apoptotic cells after 24 h of treatment with *zVAD.fmk* and anisomycin or geranylgeraniol. After this time, however, a gradual increase in the proportion of apoptotic cells was evident. Although, approximately 50% of the cells treated with *zVAD.fmk* and either anisomycin or geranylgeraniol were apoptotic by 72 h, the remaining cells were still capable of...
progressing through the cell cycle (Fig. 4A). Interestingly, determination of total cell numbers during this time course revealed that the rate of cell growth was dramatically inhibited in cells treated with either zVAD.fmk alone or in combination with the 2 apoptotic inducers (Fig. 4C). These studies were technically challenging and may have underestimated the ability of zVAD.fmk to protect cells against apoptosis as we found that the cells were very sensitive to excessive experimental manipulation. In addition, we were unable to test the effect of higher concentrations of zVAD.fmk as they were toxic under our experimental conditions.

Lysates were prepared from cells treated for 1 h in the presence and absence of either zVAD.fmk, anisomycin, or geranylgeraniol and probed for caspase-3. A marked decrease in the level of full-length caspase-3 was evident in the anisomycin- and geranylgeraniol-treated samples compared with the unstimulated control (Fig. 4D). In samples treated with zVAD.fmk either alone or in the presence of anisomycin or geranylgeraniol, only a small decrease in the level of full-length caspase-3 was observed (Fig. 4D). A band migrating at approximately 20 kDa and corresponding to a partially processed form of caspase-3 was observed in these samples. Processing of caspase-3 has been shown to occur via the generation of a p20 intermediate that is subsequently further processed into the p17 form (26). Binding of zVAD.fmk to this p20 intermediate blocks its activity, in agreement with the results of others (45).

A small proportion of fully processed caspase-3 was present in samples treated with zVAD.fmk plus anisomycin or geranylgeraniol, which represents the basal rate of apoptosis that is present in these cells (compare Fig. 4, B and D). We were unable to make any conclusions regarding the state of activation of caspase-2 in these lysates as reprobing of these blots with the goat anti-caspase-2 antibody failed to give sufficient signal to detect either the full-length or cleaved products of caspase-2. One interpretation of these results is that this concentration of zVAD.fmk was able to completely block the downstream functions of caspase-3. With increasing time of incubation, the levels of zVAD.fmk may become limiting and allow apoptosis to occur. Unfortunately, we were unable to determine whether zVAD.fmk prevented the processing of caspase-3 at later times due to the decrease in cell numbers evident at these times. Consequently, the ability of zVAD.fmk to protect cells against apoptosis correlated with its ability to prevent complete processing and activation of caspase-3.

We measured the incorporation of [35S]methionine in cells treated with zVAD.fmk and either anisomycin or geranylgeraniol in order to determine the level of protein synthesis in these cells. In cells treated with either anisomycin or geranylgeraniol alone, a near complete inhibition of protein synthesis was observed 24 h following treatment (Fig. 4E). Surprisingly, pretreatment of cells with zVAD.fmk did not prevent the inhibition of protein synthesis induced by either anisomycin or geranylgeraniol (Fig. 4E). Furthermore, zVAD.fmk alone inhibited protein synthesis at later times. A low level of protein synthesis was evident in samples treated with zVAD.fmk and either of the two inducers, which probably resulted from the cells that were still cycling. This is not obvious from the figure but was apparent upon longer exposure of the gel (data not shown). The ability of zVAD.fmk to inhibit protein synthesis and its ability to slow cell growth may be interrelated effects although the mechanisms by which this occurs is unknown. We could not determine from the current results whether the cells that were still cycling following 72 h of treatment represented cells in which their apoptotic pathways were completely blocked by zVAD.fmk or whether they represented clonal variants that displayed differential sensitivity to apoptotic induc-

tion by anisomycin and geranylgeraniol. Resolution of these issues is important in formulating therapeutic strategies involving apoptosis.

In conclusion, we have demonstrated that induction of apoptosis in HL-60 cells is associated with the selective activation of some but not all caspases. The inhibition of apoptosis induced by anisomycin or geranylgeraniol in cells treated with zVAD.fmk correlated with inhibition of caspase-3 processing.

The current data together with the numerous other studies showing the involvement of caspase-3 in apoptosis induced by a variety of agents, as well as the ubiquitous tissue distribution of caspase-3 (19), strongly suggests that this protease plays an important role in apoptosis. Whether other components acting in parallel pathways are also required in addition to the activation of caspase-3 for the induction of apoptosis cannot be determined from the available evidence. Furthermore, the interrelationship between caspase-2 and caspase-3 needs to be evaluated in light of the current results. Confirmation of these hypotheses awaits the results of targeted gene knock-out studies. The nature of the signals which activates the caspases is critical to our understanding of the pathways regulating apoptosis. The current system should provide invaluable insights into the mechanisms regulating the activation of distinct caspases.

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