Characterization of Caspase Processing and Activation in HL-60 Cell Cytosol Under Cell-free Conditions

NUCLEOTIDE REQUIREMENT AND INHIBITOR PROFILE*

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The present studies compared caspase activation under cell-free conditions in vitro and in etoposide-treated HL-60 leukemia cells in situ. Immunoblotting revealed that incubation of HL-60 cytosol at 30 °C in the presence of cytochrome c and ATP (or dATP) resulted in activation of procaspases-3, -6, and -7 but not -2 and -8. Although similar selectivity was observed in intact cells, affinity labeling revealed that the active caspase species generated in vitro and in situ differed in charge and abundance. ATP and dATP levels in intact HL-60 cells were higher than required for caspase activation in vitro and did not change before caspase activation in situ. Replacement of ATP with the poorly hydrolyzable analogs 5′-adenyl methylenediphosphate, 5′-adenyl imidodiphosphate, or 5′-adenyl-O-(3-thiotriphosphate) slowed caspase activation in vitro, suggesting that ATP hydrolysis is required. Caspase activation in vitro was insensitive to phosphatase and kinase inhibitors (okadaic acid, staurosporine, and genistein) but was inhibited by Zn2+, aurantricarboxylic acid, and various protease inhibitors, including 3,4-dichloroisocoumarin, N′,p-tosyl-l-phenylalanine chloromethyl ketone, N′,p-tosyl-l-lysine chloromethyl ketone, and N-(N′-benzoylcarbonylphenylalanyl)alanine fluoromethyl ketone, each of which inhibited recombinant caspases-3, -6, -7, and -9. Experiments with anti-neoepitope antiserum confirmed that these agents inhibited caspase-9 activation. Collectively, these results suggest that caspase-9 activation requires nucleotide hydrolysis and is inhibited by agents previously thought to affect apoptosis by other means.

Apoptosis is a morphologically distinct form of physiological cell death that is widely observed in nature (1, 2). Studies performed over the past 5 years have revealed that many of the changes observed in apoptotic cells result from the action of a family of cysteine-dependent aspartate-directed intracellular proteases (now termed caspases) on their substrates (3–7). As a result, considerable attention is now focused on understanding the factors that control caspase activation and activity.

Several pathways of caspase activation have been identified. One involves ligation of certain receptors (e.g. CD95 or the type 1 tumor necrosis factor-α receptor), recruitment of adaptor proteins such as FADD/Mort1, binding of procaspases-8 and -10 to the adaptor molecules, activation of these initiator caspases, and subsequent proteolytic activation of the downstream caspases-3, -6, and -7 (7–10). This canonical pathway appears to account, at least in broadstroke, for the events initiated by ligation of a number of death receptors.

An alternative pathway of caspase activation (7, 11) appears to be involved in other apoptotic deaths (12, 13). Many proapoptotic stimuli cause mitochondria to release cytochrome c to the cytosol, where it binds to a docking protein called Apaf-1, inducing a conformational change in Apaf-1 that facilitates binding and activation of procaspase-9 (14–19). Caspase-9 then proteolytically activates caspases-3 and -7; the former activates caspase-6 (16, 18).

Previous studies have indicated that this cytochrome c/Apaf-1/caspase-9 pathway can be reconstituted in vitro by incubating cytosol from nonapoptotic cells with purified cytochrome c and dATP (13, 14, 20, 21). Although this pathway has been intensively studied, several aspects remain poorly understood. First, the role of ATP or dATP is unclear. Because mutation of the ATP-binding site abolishes the caspase activating activity of the Apaf-1 homolog ced-4 (22, 23), it has generally been assumed that the nucleoside triphosphate is hydrolyzed during caspase activation. Consistent with this possibility, ATP depletion has been shown to abrogate caspase activation and subsequent apoptotic events in damaged cells (24, 25). Srinivasa et al. (18), however, observed that a fragment of Apaf-1 can facilitate caspase-9 activation in a nucleotide-independent fashion.

More recently, Kuida et al. (26) reported that caspase activation can occur upon incubation of brain cytosol in the absence of exogenous dATP and cytochrome c. These observations raise the possibility that nucleotide hydrolysis might not be required for caspase activation. Second, because the vast majority of studies have focused upon the activation of caspase-3, it is unclear how faithfully the events produced in vitro reflect the selective activation of a subset of the available procaspases that...
is observed in situ. Finally, the effects of various inhibitors on this caspase activation pathway remain to be elucidated.

A number of inhibitors of the apoptotic process have been previously identified. Early experiments established that Zn\(^{2+}\) (27) and ATA\(^{1}\) (94) decrease thymocyte apoptosis. Although these effects were initially attributed to inhibition of apoptotic nucleases, subsequent observations have indicated that Zn\(^{2+}\) inhibits active caspases (28–30) or apoptotic events further upstream (31). A variety of additional compounds have been observed to inhibit apoptosis in intact cells. These include the caspase inhibitor ZVAD-fmk (32); agents such as TLCK, TPCK, and DCI (33–43), which are often regarded as serine protease inhibitors; and ZFA-fmk (39), which is considered a specific inhibitor of sulphydryl-dependent cathepsins. Although it is now clear that ZVAD-fmk can inhibit caspase-9 activation (15, 20) and activity (44), the possibility that the other agents inhibit caspase activation has not been previously explored.

In the present study, we have compared caspase activation in HL-60 cells in situ and in HL-60 cytosol in vitro. Earlier studies established that etoposide-induced apoptosis in HL-60 cells is accompanied by cleavage of multiple caspase substrates (34, 45, 46) and that this cleavage can be inhibited by treatment of cells with TPCK, TLCK, or Zn\(^{2+}\) (34). Subsequent reports have demonstrated that a subset of the available caspase precursors is activated during the course of apoptosis in this model system (47) and that cytochrome c release from mitochondria precedes caspase activation (15, 48). In the present study, the cohort of caspases activated by treatment of HL-60 cytosol with cytochrome c and ATP in vitro has been compared with that activated in situ during etoposide-induced apoptosis. In addition, the nucleotide requirements in vitro have been compared with the nucleotide pools available in intact cells. Finally, the cell-free system was utilized to explore the possibility that small molecule inhibitors of apoptosis, including ATA and protease inhibitors that are usually thought to inhibit other types of proteases, might act by inhibiting caspase activation.

**EXPERIMENTAL PROCEDURES**

**Materials—Reagents** were obtained from the following suppliers: horse heart cytochrome c, Tween 20, DCI, TLCK, TPCK, okadaic acid, Na\(_{2}\)VO\(_{4}\), staurosporine, genistein, pNA, nucleotides and nucleotide analogs from Sigma; adjuvants from Ribi (Hamilton, MT); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from Sigma; adjuvants from Ribi (Hamilton, MT); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA); recombinant human caspases-3, -6, and -7 from PharMingen (San Diego, CA). Other reagents were obtained as previously indicated (47, 49).

**Antibodies—Rabbit antisera that recognize caspase-3 or caspase-7 were raised by injecting female New Zealand White rabbits with the large subunit of recombinant human caspase-3 or -7. In brief, the indicated large subunit was expressed as a fusion protein with glutathione S-transferase using the pGEX-2T plasmid (Amersham Pharma-
HL-60 cytosol was treated with 500 μg/ml cytochrome c and 1 μM dATP for the indicated length of time and then harvested for immunoblotting with antibodies that recognize the large subunits of the indicated active caspases. Locations of the procaspase species (open arrows) and large subunits of active species (closed arrows) are indicated. Note that procaspases-3, -6, and -7 are cleaved, whereas procaspases-2 and -8 are not. B, detection of caspase activity after incubation of cytosol with cytochrome c and dATP in vitro. After incubation for 30 min with dATP in the absence (-) or presence (+) of cytochrome c, aliquots of cytosol were assayed for ability to cleave DEVD-pNA. C, HL-60 cells treated with 68 μM etoposide for 6 h (lane 1, 0 μM dATP, C) were processed as described in A. Procaspase-3 (6 not shown) was not reliably detected in the whole cell lysates with the available antibody. Note that a subset of caspase precursors are cleaved at this time point in situ.

Measurement of Caspase Activity—After incubation under cell-free conditions as described previously (47, 51). Labeled polypeptides were visualized using peroxidase-labeled streptavidin, and visualized using ECL-enhanced chemiluminescence reagents.

Two-dimensional analysis was performed using isoelectric focusing for the first dimension and SDS-PAGE for the second dimension as described (49). Labeled polypeptides were visualized using peroxidase-coupled streptavidin followed by SuperSignal™ ULTRA chemiluminescence reagent (Pierce). Caspases expressed in Sf9 cells (47) were subjected to high performance liquid chromatography within 60 min of the final sedimentation step using conditions that permitted separation and quantitation of ATP, dATP, ADP, and dADP as well as a number of additional nucleotide species. In brief, 60 μl of the resulting supernatant was subjected to HPLC analysis using a Beckman 125 dual pump gradient system equipped with 507e autosampler, 168 diode array detector, and IBM personal computer 350 with Beckman Gold software. A Brownlee MPLC Newguard anion exchange precolumn (3.2 × 15 mm × 7 μm) and a Whatman Partisil-10 SAX column (4.6 × 250 mm × 5 μm) prequillibrated for at least 30 min with 99% mobile phase A (7 mM NH₄H₂PO₄, pH 3.8) and 1% mobile phase B (250 mM NH₄H₂PO₄, pH 4.5, containing 500 mM KCl) were used for all analyses. Separation was accomplished using a flow rate of 2 ml/min and the following elution gradient: 0–10 min, 99% mobile phase A, 1% mobile phase B; 10–30 min, linear gradient to 95% mobile phase A, 5% mobile phase B; 30–75 min, linear gradient to 100% mobile phase B. Absorbance was measured at 259 nm. Peaks were identified by coelution with authentic nucleotides in the described solvent system as well as a separate solvent system consisting of 1% aqueous triethylamine and methanol using a C18 HPLC column (Beckman Ultrasphere ODS 4.6 × 250 mm). Known amounts of ATP, dATP, ADP, and dADP were utilized to construct standard curves, which were then employed to determine the amounts of various nucleotides in the perchloric acid extracts.

RESULTS

Caspase Activation in a Cell-free System Prepared from HL-60 Cell Cytosol—Previous studies have demonstrated that caspases can be activated when cytosol from nonapoptotic cells is incubated with cytochrome c and ATP at 30 °C (13, 14, 20, 21, 22, 26). Our initial experiments (Fig. 1) were performed to characterize this process in cytosol prepared from HL-60 human leukemia cells. Incubation of dialyzed HL-60 cytosol with >100 μM ATP and >1 μM cytochrome c led to caspase-3 activation. Processing of endogenous procaspase-3 to the active enzyme was demonstrated by immunoblotting, which showed loss of the 32-kDa precursor and appearance of a new 17-kDa polypeptide that reacts with affinity purified antibodies raised against the recombinant caspase-3 17-kDa subunit (Fig. 1A, taining 50 μg of cytosolic protein were incubated for 15 min at 37 °C with 1 μM ZEKbioY-omk. Previous experiments have revealed that p20 subunits of all caspases tested (caspases-1, -2, -3, -4, -6, -7, and -8) can be labeled with this reagent (47, 51). At the completion of the incubation, samples were diluted with 1/5 volume of 3× SDS sample buffer (9% (w/v) SDS, 0.15 M Tris-HCl (pH 6.8 at 20 °C), 6 mM EDTA, 45% (w/v) sucrose, 0.03% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol), 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 using ECL-enhanced chemiluminescence reagents.

Release of free pNA, which absorbs at 405 nm, was monitored continuously. Absorbance values were converted to picomoles using a standard curve based on free pNA. Measurements of Caspase Activity—After incubation under cell-free conditions as described above, the ability of cytosol preparations to cleave DEVD-pNA was measured as described by Datta et al. (52). Reactions were assembled in microtiter plate wells by adding 160 μl of buffer B (100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, and 0.5 mM EDTA) containing 125 μM DEVD-pNA to wells containing 50 μg of cytosolic protein in 40 μl of buffer A. Plates were incubated at 37 °C in a Molecular Dynamics (Sunnyvale, CA) Thermomax plate reader. Release of free pNA, which absorbs at 405 nm, was monitored continuously. Absorbance values were converted to picomoles using a standard curve, based on free pNA. The effect of various inhibitors on activated caspases was assessed as described previously (47). In brief, recombinant caspases-3, -6, -7, or -9 in 50 μl of buffer A were incubated for 5 min at 20–22 °C with the indicated concentration of the inhibitor and then diluted with 225 μl of freshly prepared buffer C (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. The following substrates were utilized: DEVD-AFC for caspases-3 and -7; VEID-AFC for caspase-6; and LEHD-AFC for caspase-9. Reactions were terminated by addition of 1.225 ml of ice-cold buffer C. Fluorescence was measured using an excitation wavelength of 360 nm and emission wavelength of 475 nm. Reagent blanks containing 50 μl of buffer A and 225 μl of buffer C were incubated at 37 °C for 2 h and then diluted with 1.225 ml of ice-cold buffer C. Standards containing 0–1500 pmol of AFC were utilized to determine the amount of fluorochrome released. Results are the mean of 3–5 determinations at each inhibitor concentration.

Nucleotide Measurement—After treatment with 68 μM etoposide for the indicated lengths of time, aliquots containing 1.3 × 10⁶ HL-60 cells were washed twice with ice-cold PBS and lysed by incubation for 15 min at 4 °C in 0.5 mM perchloric acid. All additional steps were performed at 4 °C. Following removal of insoluble macromolecules by sedimentation at 1600 × g for 15 min, the perchloric acid extract was neutralized with 1 M KOH containing 0.33 mM potassium phosphate (pH 7.4 at neutralization) and sedimented at 1600 × g for 15 min to remove precipitated potassium perchlorate. Aliquots of the neutralized supernatant were subjected to high performance liquid chromatography within 60 min of the final sedimentation step using conditions that permitted separation and quantitation of ATP, dATP, ADP, and dADP as well as a number of additional nucleotide species. In brief, 60 μl of the resulting supernatant was subjected to HPLC analysis using a Beckman 125 dual pump gradient system equipped with 507e autosampler, 168 diode array detector, and IBM personal computer 350 with Beckman Gold software. A Brownlee MPLC Newguard anion exchange precolumn (3.2 × 15 mm × 7 μm) and a Whatman Partisil-10 SAX column (4.6 × 250 mm × 5 μm) prequillibrated for at least 30 min with 99% mobile phase A (7 mM NH₄H₂PO₄, pH 3.8) and 1% mobile phase B (250 mM NH₄H₂PO₄, pH 4.5, containing 500 mM KCl) were used for all analyses. Separation was accomplished using a flow rate of 2 ml/min and the following elution gradient: 0–10 min, 99% mobile phase A, 1% mobile phase B; 10–30 min, linear gradient to 95% mobile phase A, 5% mobile phase B; 30–75 min, linear gradient to 100% mobile phase B. Absorbance was measured at 259 nm. Peaks were identified by coelution with authentic nucleotides in the described solvent system as well as a separate solvent system consisting of 1% aqueous triethylamine and methanol using a C18 HPLC column (Beckman Ultrasphere ODS 4.6 × 250 mm). Known amounts of ATP, dATP, ADP, and dADP were utilized to construct standard curves, which were then employed to determine the amounts of various nucleotides in the perchloric acid extracts.

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Affinity labeling of active caspase species after activation in etoposide-treated cells or in vitro. A, analysis by unidimensional SDS-PAGE. Cytosol from HL-60 cells treated with etoposide for 3 h (lane 1) or cytosol from control cells treated with 500 μg/ml cytochrome c and 1 mM dATP at 30 °C for the indicated length of time (lanes 2–7) was incubated with 1 μM ZEK(bio)D-aomk and subjected to unidimensional SDS-PAGE followed by blotting with peroxidase-coupled streptavidin. a–g refer to discrete caspase species detected by varying exposures of this blot. Results are representative of seven separate experiments. B, after affinity labeling as described for A, cytosol from HL-60 cells treated with etoposide for 5 h (upper panel) or cytosol from control cells treated with 500 μg/ml cytochrome c and 1 mM dATP at 30 °C for 5 h (middle panel) was subjected to isoelectric focusing (from left to right) followed by SDS-PAGE (from top to bottom). C3 and C6 in upper two panels refer to major species of caspases-3 and -6, respectively, previously identified by this technique (47). White arrowheads denote the mobility of recombinant caspases expressed in Sf9 cells as determined in two-dimensional gels run in parallel (see lower panel). Results are representative of four separate experiments.

Top panel; by appearance of an activity that cleaves DEVD-pNA (Fig. 1B), a preferred substrate of caspase-3; and by the appearance of polypeptides that comigrate with recombinant caspase-3 species after reaction with the affinity labeling reagent ZEK(bio)D-aomk (see below).

Previous results have established that etoposide-induced apoptosis in intact HL-60 cells is accompanied by selective activation of a subset of the procaspase species present in these cells (47, 48). In particular, caspases-3 and -7 are activated by 4 h, whereas caspases-2 and -8 are not (Fig. 1C) (37, 47, 48). To determine whether similar selectivity is observed in vitro, cytosol incubated with ATP and cytochrome c was probed with antibodies that recognize several different caspases (Fig. 1A). Processing of procaspases-3 and -7 was detected within 30–60 min of the start of the incubation. Processing of the caspase-6zymogen was also observed, albeit with slightly slower kinetics. In contrast, cleavage of procaspases-2 and -8 was not observed (Fig. 1A, lower panels), confirming that this in vitro system of caspase activation recapitulates the selectivity observed in intact cells.

In further experiments, affinity labeling with ZEK(bio)D-aomk was utilized to compare the cohort of caspases activated in vitro with those detected in cytosol of etoposide-treated HL-60 cells (Fig. 2). Although analysis by unidimensional (Fig. 2A) and two-dimensional gel electrophoresis (Fig. 2B) confirmed that active caspase species were detected after incubation of cytosol with cytochrome c and dATP in vitro, the labeling patterns were significantly different from those observed when cytosol was analyzed after caspases were activated in intact cells. Earlier analysis using unidimensional gels indicated that the pattern of active caspases in cytosol of etoposide-treated cells is relatively simple early in the course of caspase activation and becomes more complicated over time (47). In contrast, during caspase activation in vitro, the labeling pattern was most complex early in the course of the incubation and became simpler over time (cf. lanes 3 and 7 in Fig. 2A). Comparison of the species detected in cytosol after activation in vivo and in vitro (cf. lanes 1 and 3 in Fig. 2A) indicated that species a, b, d/e, and g are detected in both samples, although the relative ratios appeared to be different. In contrast, species c and f were detected only after caspase activation in vitro.

These differences between the cohort of caspases activated in vivo and in vitro were even more pronounced when active caspase species were analyzed by two-dimensional isoelectric focusing/SDS-PAGE (Fig. 2B). In particular, a major species of active caspase-6 and multiple species of active caspase-3 were detected after caspase activation in vivo (Fig. 2B, upper panel) (47). Similar labeling patterns have been observed in several cell lines after treatment of intact cells with various proapoptotic stimuli (47, 48, 54). Previous studies have demonstrated that the size and charge heterogeneity of these species reflect, at least in part, the retention or removal of the caspase prodomain and different degrees of caspase phosphorylation, respectively (49, 55, 56). Although the caspase-3 species were detectable after activation in vitro (Fig. 2B, middle panel), the major caspase-6 species was not. Instead, several acidic caspase species that were present in small amounts after caspase activ-

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2 When HL-60 cells are incubated with etoposide for longer periods, procaspase-8 also decreases (S. H. K., unpublished observations), suggesting that its activation under these circumstances is downstream of caspase-9 and caspase-3 as recently suggested (13, 21).

3 Previous results, which have demonstrated that a major active species of caspase-6 does not focus in this gel system (47), appear to account for the lack of a major caspase-6 species on the two-dimensional gels (Fig. 2B, middle panel) despite the cleavage of procaspase-6 in vitro (Fig. 1A).
The effect of poorly hydrolyzable ATP analogs on caspase activation in vitro. Lane 1, input cytosol. Lane 2–9, cytosol incubated for 5 h at 30 °C in absence (lane 2) or presence (lanes 3–9) of 500 μg/ml cytochrome c and absence (lane 3) or presence (lanes 4–9) of the indicated nucleotide at a final concentration of 1 mM. At the completion of the incubation, cytosol was subjected to SDS-PAGE followed by immunoblotting with affinity purified anti-caspase-3 antibody. Locations of procaspase-3 (500 μg/ml) were indicated. All further experiments utilized dialyzed cytosol. Additional samples confirmed that addition of cytochrome c + ATP resulted in procaspase-3 activation with kinetics indistinguishable from those illustrated in Fig. 1A. C, effect of ATPγS on caspase activation in vitro. Lane 1, input cytosol. Lanes 2–5, cytosol incubated for 5 h at 30 °C in the absence or presence of exogenous ATP (1 mM) and/or cytochrome c (500 μg/ml) as indicated. Lanes 6–9, cytosol incubated for 5 h at 30 °C in the presence of 500 μg/ml cytochrome c and 0.1, 1, 5, or 10 mM ATPγS, respectively. For this experiment, undialyzed cytosol was utilized. Activation of caspases upon addition of cytochrome c (lane 4) presumably reflects the high levels of endogenous adenine nucleotides (see Fig. 4).

To determine the influence of reaction conditions on caspase activation in vitro, we systematically varied several of the experimental parameters. During the preparation of some batches of cytosol, the dialysis step was omitted. Under these conditions, we observed batch-to-batch variation in the requirement for exogenous cytochrome c and/or ATP. Some undialyzed HL-60 cytosol preparations behaved as illustrated in Figs. 1 and 2, whereas others facilitated caspase activation in the absence of exogenous cytochrome c and ATP (data not shown) in a manner similar to extracts recently described by Kuida et al. (26). In contrast, when dialyzed cytosol was utilized, exogenous cytochrome c and ATP were required. Unless otherwise indicated, all further experiments utilized dialyzed cytosol.

Additional experiments (not shown) examined other parameters. Titration experiments revealed that quantitative cleavage of procaspase-3 in HL-60 cytosol required ≥1 μM cytochrome c, in agreement with observations made using cytosol from Xenopus oocytes (53). NaCl or KCl concentrations above 60 mM were observed to inhibit caspase-3 activation in HL-60 cytosol, a finding consistent with results obtained using cytosol from rat thymocytes (57). Finally, incubation of HL-60 cytosol with ATP and cytochrome c resulted in more rapid and complete procaspase cleavage at 30 °C than at 37 °C. All subsequent activation studies were performed at 30 °C in the absence of exogenous monovalent cations.

Nucleotide Requirements for Caspase Activation in HL-60 Cytosol—Further experiments examined the nucleotide requirements of the in vitro activation system. When 1 μM cytochrome c was present, titration experiments revealed complete processing of procaspase-3 in the presence of ≥100 μM ATP (data not shown). When other nucleotides were tested at 1 mM, dATP or dADP could substitute for ATP, whereas AMP, ADP-ribose, CMP, CTP, or UTP could not (Fig. 3A and data not shown). Further examination revealed that dATP was effective at concentrations below 10 μM (Fig. 3B, lane 5), although it inhibited procaspase-3 activation at extremely high concentrations (Fig. 3B, lane 6).

To determine whether the ATP/dATP requirement reflected the need to hydrolyze a high energy phosphate bond, ATP was replaced with the poorly hydrolyzable analogs AMPPCP, AMPPNP, and AMPPNP. Some undialyzed cytosol supplemented with 500 μg/ml cytochrome c and the following nucleotides: ATP at 1 mM (lane 4); dATP at 0.01, 0.1, 1, and 10 mM (lanes 5–8, respectively); AMPPCP at 0.01, 0.1, 1, and 10 mM (lanes 9–12, respectively); and AMPPNP at 0.01, 0.1, 1, 10, and 100 mM (lanes 13–17, respectively). All samples were treated in one experiment but applied to two separate minigels. Additional samples confirmed that addition of cytochrome c + ATP resulted in procaspase-3 activation with kinetics indistinguishable from those illustrated in Fig. 1A. C, effect of ATPγS on caspase activation in vitro. Lane 1, input cytosol. Lanes 2–5, cytosol incubated for 5 h at 30 °C in the absence or presence of exogenous ATP (1 mM) and/or cytochrome c (500 μg/ml) as indicated. Lanes 6–9, cytosol incubated for 5 h at 30 °C in the presence of 500 μg/ml cytochrome c and 0.1, 1, 5, or 10 mM ATPγS, respectively. For this experiment, undialyzed cytosol was utilized. Activation of caspases upon addition of cytochrome c (lane 4) presumably reflects the high levels of endogenous adenine nucleotides (see Fig. 4).
PNP, or ATPγS. As shown in Fig. 3B, replacement of dATP with 1 mM AMPPCP or 1–10 mM AMPPNP resulted in partial cleavage of procaspase-3 after 5 h. When compared with the quantitative cleavage of procaspase-3 in <1 h (Fig. 1A; see also legend to Fig. 3B), these results indicate that AMPPCP and AMPPNP support caspase activation relatively poorly. In further experiments, ATPγS at a wide range of concentrations did not support caspase-3 processing in the in vitro system (Fig. 3C). On the contrary, ATPγS prevented activation by endogenous nucleotides in the undialyzed cytosol used for this particular experiment (cf. lanes 4 and 6, Fig. 3C). The slowing or abrogation of the caspase activation process by these nonhydrolyzable analogs suggests that hydrolysis of the terminal phosphodiester bond in ATP plays an important role in the caspase activation mechanism.

To compare the nucleotide requirements of this reaction in vitro with the potential nucleotides available during apoptosis in situ, ATP and dATP were quantitated by HPLC (Fig. 4A) in HL-60 cells undergoing etoposide-induced apoptosis. This analysis revealed a base-line ATP concentration of 0.92 mM, a value that is within the range previously reported for this cell line (58, 59). The same analysis revealed a base-line dATP concentration of 0.17 mM. During the first 2 h after addition of etoposide, which is the period of time when caspase activation begins to occur in this cell line (47), concentrations of ATP and dATP did not change appreciably (Fig. 4B). At later time points, ATP and dATP began to decline. ADP and dADP levels decreased in a similar fashion (data not shown).

Effects of Inhibitors on Caspase Activation in Vitro—As indicated in the Introduction, a variety of small molecule inhibitors inhibit apoptosis in various model systems. To examine the possibility that some of these might be acting by inhibiting caspase activation, the effects of these compounds on caspase activation in the cell-free system were assessed.

Zn²⁺ and ATA, two classical inhibitors of programmed cell death (27, 94), inhibited activation of procaspase-3 when cytosol was incubated with cytchrome c and ATP in vitro. As illustrated in Fig. 5A, > 0.5 mM Zn²⁺ inhibited procaspase activation. Likewise, > 40 μM ATA inhibited processing of procaspase-3 under these conditions.

Recent studies have indicated that active caspases in etoposide-treated HL-60 cells are phosphoproteins (49). Additional studies have demonstrated that caspase-9 is also phosphorylated under some conditions (60). Based on these results, the effects of inhibitors of protein kinases (staurosporine and genistein) and protein phosphatases (okadaic acid and Na₂VO₄) were investigated. Staurosporine, which inhibits calmodulin-dependent kinase, cyclic AMP-dependent kinase, protein kinase G, and myosin light chain kinase in the nanomolar range and protein kinase C in the micromolar range (61), had no discernible effect on procaspase-3 activation at millimolar concentrations (Fig. 5B, lane 3). Genistein, which inhibits tyrosine kinases at low micromolar concentrations (62), was similarly unable to block procaspase-3 processing at millimolar concentrations (Fig. 5B, lane 4).

In additional experiments, okadaic acid, which inhibits protein phosphatases 1, 2A, and 2B at concentrations below 1 μM (63), had no discernible effect on procaspase-3 activation at concentrations of up to 100 μM (Fig. 5B, lane 5). Na₂VO₄, which broadly inhibits protein tyrosine phosphatases as well as alkaline phosphatase in the micromolar range (64), was similarly ineffective in blocking procaspase-3 processing at submillimolar concentrations (Fig. 5B, lanes 6 and 7). These data suggest that alterations of caspase phosphorylation are not required for activation of effector caspases such as caspase-3 in HL-60 cytosol. On the other hand, higher concentrations of Na₂VO₄, which are known to inhibit other enzymes involved in phosphate metabolism, including ATPases (65, 66), did inhibit caspase activation (Fig. 5B, lane 9), consistent with data presented above suggesting that nucleotide hydrolysis might be required for this process.

To examine the effects of various protease inhibitors on caspase activation, cytosolic extracts were incubated at 4°C for 5–10 min with each inhibitor, then warmed to 30°C for 5 h, and examined for procaspase-3 cleavage. As indicated in Fig. 5C, a variety of protease inhibitors prevented procaspase-3 activation in a dose-dependent manner. Iodoacetamide, an irreversible sulfhydryl-blocking reagent, completely prevented procaspase-3 activation at concentrations of up to 500 μM. This reaction was also inhibited by several different caspase inhibitors, including DEVD-fmk, VEID-fmk, and ZVAD-fmk. Interestingly, the methyl ester ZVAD(OMe)-fmk was at least 20–50-fold less potent than the parent compound in this reaction (cf. 4th and 5th panels, Fig. 5C).

Results in Fig. 5, C and D, also illustrate results obtained with other classes of protease inhibitors. TPCK and TLCK, two chloromethyl ketones that inhibit sulfhydryl-dependent proteases (67–69) as well as certain serine proteases (70), abolished procaspase-3 activation at all concentrations above 1 and 5 mM, respectively. DCI, which is usually considered an inhibitor of serine proteases (71), completely inhibited procaspase-3 activation at concentrations above 500 μM. In addition, ZFA-
concentration of ATA or ZnSO₄ was incubated for 5 h at 30 °C and then subjected to immunoblotting with an antiserum that recognizes caspase-3 (arrowhead). B, effects of phosphatase inhibitors on caspase activation in vitro. Lanes 1 and 2, cytosol incubated for 5 h at 30 °C in the absence (lane 1) or presence (lane 2) of exogenous ATP (1 mM) and cytochrome c (500 μg/ml). Lanes 3–9, cytosol containing ATP and cytochrome c was supplemented with 1 mM staurosporine (lane 3), 100 μM genistein (lane 4), 1 mM okadaic acid (lane 5), or 0.1, 0.5, 1, or 10 mM Na₃VO₄ (lanes 6–9, respectively), prior to incubation at 30 °C for 5 h. Arrowhead, procaspase-3. C, effects of various protease inhibitors on caspase activation. Lanes 1 and 2, cytosol incubated at 30 °C for 5 h in the absence (lane 1') or presence (lane 2') of exogenous ATP (1 mM) and cytochrome c (500 μg/ml). Lanes 1–3, cytosol containing ATP and cytochrome c was supplemented with increasing concentrations of iodoacetamide (IAA) (10, 100, and 500 μM), DEVD-fmk (1, 10, and 100 μM), VEID-fmk (1, 10, and 100 μM), ZVAD-fmk (10, 100, and 1000 nM), ZVAD(OMe)-fmk (10, 100, and 1000 nM), TPCK (0.1, 0.5, and 1 mM), TLCK (0.1, 1, and 10 mM), or DCI (0.1, 0.25, and 0.5 mM), respectively. D, effect of various concentrations of ZFA-fmk on caspase-3 activation. Cytosol was incubated at 30 °C for 5 h in the presence of 0.1, 0.5, 1, or 5 mM ZFA-fmk, respectively.

Fig. 5. Effect of selected inhibitors on caspase activation in vitro. A, effects of Zn²⁺ and ATA on caspase activation in vitro. Lane 1, unsupplemented cytosol incubated at 30 °C for 5 h. Lanes 2–10, cytosol supplemented with 1 mM ATP, 500 μg/ml cytochrome c, and the indicated concentration of ATA or ZnSO₄ was incubated for 5 h at 30 °C and then subjected to immunoblotting with an antiserum that recognizes procaspase-3 (arrowhead). B, effects of phosphatase inhibitors on caspase activation in vitro. Lanes 1 and 2, cytosol incubated for 5 h at 30 °C in the absence (lane 1) or presence (lane 2) of exogenous ATP (1 mM) and cytochrome c (500 μg/ml). Lanes 3–9, cytosol containing ATP and cytochrome c was supplemented with 1 mM staurosporine (lane 3), 100 μM genistein (lane 4), 1 mM okadaic acid (lane 5), or 0.1, 0.5, 1, or 10 mM Na₃VO₄ (lanes 6–9, respectively), prior to incubation at 30 °C for 5 h. Arrowhead, procaspase-3. C, effects of various protease inhibitors on caspase activation. Lanes 1 and 2, cytosol incubated at 30 °C for 5 h in the absence (lane 1') or presence (lane 2') of exogenous ATP (1 mM) and cytochrome c (500 μg/ml). Lanes 1–3, cytosol containing ATP and cytochrome c was supplemented with increasing concentrations of iodoacetamide (IAA) (10, 100, and 500 μM), DEVD-fmk (1, 10, and 100 μM), VEID-fmk (1, 10, and 100 μM), ZVAD-fmk (10, 100, and 1000 nM), ZVAD(OMe)-fmk (10, 100, and 1000 nM), TPCK (0.1, 0.5, and 1 mM), TLCK (0.1, 1, and 10 mM), or DCI (0.1, 0.25, and 0.5 mM), respectively. D, effect of various concentrations of ZFA-fmk on caspase-3 activation. Cytosol was incubated at 30 °C for 5 h in the presence of 0.1, 0.5, 1, or 5 mM ZFA-fmk, respectively.

**Effect of Inhibitors on Recombinant Caspase Activity**—The preceding experiments raised the possibility that Zn²⁺, ATA, DEVD-fmk, VEID-fmk, ZVAD-fmk, DCI, TPCK, TLCK, and ZFA-fmk might be exerting their effects by inhibiting caspase activation. If so, one logical target might be caspase-9, the apical caspase in the cytochrome c/Apaf-1 pathway. To assess this possibility, recombinant human caspase-9 was incubated with these agents for 5 min, diluted, and assayed for ability to cleave its preferred substrate, LEHD-AFC. Results of this analysis revealed that the fluoromethyl ketones DEVD-fmk, VEID-fmk, ZVAD-fmk, ZVAD(OMe)-fmk, and ZFA-fmk all inhibited caspase-9 activity (Fig. 6A). In agreement with the in vitro activation data, ZVAD(OMe)-fmk was at least 10-fold less potent than ZVAD-fmk. In addition, ATA and Zn²⁺, which are commonly considered to be nuclease inhibitors, inhibited caspase-9 (Fig. 6B). Finally, TPCK, TLCK, and DCI, three agents that are frequently classified as serine esterase inhibitors, also inhibited caspase-9 activity (Fig. 6B).

Because the effects of ATA, DCI, ZFA-fmk, TPCK, and TLCK were somewhat unexpected, the effects of these compounds on the catalytic activities of other purified recombinant human caspases were evaluated. As indicated in Fig. 7, all of these agents also inhibited purified caspases-3, -6, and -7 at submillimolar or millimolar concentrations. In particular, DCI (Fig. 7, closed squares) inhibited caspase activity by 50% at concentrations that ranged from 500 μM (caspase-6) to 1 mM (caspases-3 and -7). TPCK (Fig. 7, closed circles) inhibited all three caspases at 200–1000 μM. Likewise, ZFA-fmk (Fig. 7, open triangles) also inhibited the purified caspases, with an IC₅₀ in the 60 (caspase-7) to 1000 μM (caspase-3) range.

**Inhibition of Caspase-9 Activation**—To determine whether these agents diminish caspase-9 activation, we utilized an antiserum raised against a tetrapeptide epitope that is masked in the intact caspase-9 zymogen but is generated at the C terminus of the large subunit during caspase maturation (7, 18). As indicated in Fig. 8A, this caspase-9 neoeptope antiserum recognized multiple bands in a preparation of purified caspase-9, including prominent species at 30–35 kDa (the previously reported size of the large subunit) (13, 18, 20) and another species at 18,000 (thought to be a processed form of the large subunit). When applied to multiple purified caspases, this serum recognized active caspase-9 but not caspases-3, -6, or -7 (Fig. 8B, upper panel). In contrast, a corresponding caspase-3 neoeptope antiserum recognized caspase-3 but not caspases-6, -7, or -9 (Fig. 8B, lower panel).

When applied to HL-60 cytosol that had been incubated with cytochrome c and dATP for varying lengths of time (Fig. 8C), the caspase-9 serum recognized a M₉⁻ eighteen thousand species that appeared within 30 min. A band of the same molecular weight was also the major species detected when etoposide-treated HL-60 cells were examined (Fig. 8D). Binding to this species was prevented by the immunizing peptide. In both cases, a M₉⁻ sixty-five thousand cross-reactive band that did not change during the course of the incubation served to confirm the equivalent loading of the various lanes (Fig. 8, C and D, open arrowhead). These results suggest that the M₉⁻ eighteen thousand species of caspase-9 is the predominant species generated during caspase activation in HL-60 cytosol in vitro and in vivo.

To examine the effects of various inhibitors on caspase-9 activation, 0.1, 0.5, 1, or 5 mM ZFA-fmk was added to HL-60 cytosol incubated at 30 °C for 5 h. Lanes 1 and 2, cytosol incubated at 30 °C for 5 h in the absence (lane 1') or presence (lane 2') of exogenous ATP (1 mM) and cytochrome c (500 μg/ml). Lanes 1–3, cytosol containing ATP and cytochrome c was supplemented with 0.1, 0.5, 1, or 10 mM Na₃VO₄ (lanes 6–9, respectively), prior to incubation at 30 °C for 5 h. Arrowhead, procaspase-3. C, effects of various protease inhibitors on caspase activation. Lanes 1 and 2, cytosol incubated at 30 °C for 5 h in the absence (lane 1') or presence (lane 2') of exogenous ATP (1 mM) and cytochrome c (500 μg/ml). Lanes 1–3, cytosol containing ATP and cytochrome c was supplemented with increasing concentrations of iodoacetamide (IAA) (10, 100, and 500 μM), DEVD-fmk (1, 10, and 100 μM), VEID-fmk (1, 10, and 100 μM), ZVAD-fmk (10, 100, and 1000 nM), ZVAD(OMe)-fmk (10, 100, and 1000 nM), TPCK (0.1, 0.5, and 1 mM), TLCK (0.1, 1, and 10 mM), or DCI (0.1, 0.25, and 0.5 mM), respectively. D, effect of various concentrations of ZFA-fmk on caspase-3 activation. Cytosol was incubated at 30 °C for 5 h in the presence of 0.1, 0.5, 1, or 5 mM ZFA-fmk, respectively.

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P. A. Svingen and S. H. Kaufmann, unpublished observations.
activation, the same samples shown in Fig. 5D were probed with the antiserum raised against the caspase-9 neoepitope. Results of this analysis revealed that iodoacetamide, DEVD-fmk, VEID-fmk, ZVAD-fmk, ZVAD(OMe)-fmk, TPCK, TLCK, DCI, Zn$^{2+}$, and ATA inhibited the generation of the $M_r \approx 18,000$ caspase-9 species (Fig. 8E and data not shown). Given the fact that each active caspase-9 molecule can activate multiple procaspase-3 molecules, we anticipated that almost complete inhibition of caspase-9 activation and activity would be required to prevent procaspase-3 processing. Consistent with this prediction, higher concentrations of each agent were required to inhibit caspase-3 activation as compared with caspase-9 activation (cf. Figs. 5C and 8E). Collectively, these results suggest that the effects of the indicated inhibitors involve inhibition of caspase-9 activation as well as inhibition of activated caspases.

**DISCUSSION**

Recent studies from several laboratories have established that addition of cytochrome c and adenine nucleoside triphosphates to cytosol from control cells results in activation of caspase-3 through a pathway that involves Apaf-1-mediated activation of procaspase-9 (13, 16, 18, 20). In the present study, we have extended these observations by contrasting the cohorts...
of active caspases that are generated in vitro and in situ, comparing the nucleotide requirements in vitro to the adenosine nucleotide triphosphate levels available in intact cells, assessing the effects of poorly hydrolyzable ATP analogs, and determining the effects of selected inhibitors in the caspase activation process. These experiments address a number of unresolved questions about the activation of caspases under cell-free conditions.

Before embarking on these studies, we confirmed that the caspase activation process in HL-60 cytosol in vitro was similar to that observed in other model systems. Our studies demonstrated that the process generated active forms of caspases-3, -6, and -7 in vitro (Fig. 1A) but did not generate active forms of caspases-2 or -8 (Fig. 1B). This selectivity mirrors the activation process in intact HL-60 cells (Fig. 1C). Failure to activate caspase-2 (73, 74) and caspase-8 (74) has been reported in other cell-free systems, although it appears that this selectivity might be cell type-dependent (21). Our studies demonstrated that the activation process in dialyzed HL-60 cytosol was dependent upon addition of exogenous cytochrome c and dATP or ATP (Fig. 3), consistent with results observed in other laboratories (13, 14, 20, 21). A number of other nucleotides, including AMP, ADP-ribose, GMP, CTP, or UTP, could not substitute for ATP or dATP (Fig. 3A), in agreement with the results of Liu et al. (14). Finally, our studies demonstrated that the activation process was inhibited by 60–80 mM NaCl or KCl, consistent with the results obtained in rat thymocyte cytosol (57). Based on these similarities, it appears that the caspase activation process in HL-60 cytosol is similar to that observed in other cell-free systems.

Some of the results obtained using the HL-60 cell-free system raise questions about the relationship between conditions that result in caspase activation in vitro and in situ. First, as noted above, caspase activation in vitro is strongly inhibited by buffers of physiological ionic strength. Second, the activation process is more efficient in vitro at 30 °C than at 37 °C. Third, results in Fig. 2B indicate that the cohort of active caspase species detected by affinity labeling after activation in vitro differs significantly from that detected after activation in situ, possibly reflecting differences in reactions that remove the caspase postdomain and alter caspase phosphorylation. These observations raise the possibility that one or more factors that modulate caspase activation in situ at physiological ionic strength and temperature might have been lost during the cell fractionation procedure. The similarities between the results observed in HL-60 cytosol and other model systems (see above) suggest that similar concerns might apply to many of the recently described cell-free systems for caspase activation. Further studies are required to evaluate the cause of the differences between caspase activation in vitro and in vivo.

With these limitations in mind, the HL-60 system was utilized to study nucleotide requirements and inhibitor sensitivity of caspase activation. Studies employing cytosol from HeLa cells had previously suggested that dATP might be specifically required for activation of caspases (14). In particular, ATP was initially reported to be inactive in vitro, whereas dATP was reportedly active, leading to the speculation that elevations in dATP levels might occur after drug treatment and contribute to the caspase activation process (14). Consistent with this hypothesis, Wakade et al. (75) observed a 40-fold increase in dATP levels when chick embryo sympathetic neurons were induced to undergo apoptosis by treatment with 2-deoxyadenosine-5'-phosphate. However, these results were not reproduced in our experiments with HL-60 cells. Inhibition studies employing 3'-deoxyadenosine-5'-phosphate (75) consistently demonstrated that the activation process was inhibited by 60–80 mM NaCl or KCl, consistent with the results obtained in rat thymocyte cytosol (57).

FIG. 8. Effects of various protease inhibitors on caspase-9 activation in HL-60 cytosol. A, reaction between neoepitope antiserum and various caspase-9 species. Recombinant His₆-tagged human caspase-9 expressed in bacteria was purified on nickel-agarose, subjected to SDS-PAGE, and probed with anti-caspase-9 neoepitope antiserum. This antiserum recognized the major M₉ ~30–35,000 species present in this preparation, as well as a less abundant M₉ ~18,000 species. B, reaction of anti-neoepitope sera with purified caspases. Caspases-3, -6, -7, and -9 were subjected to SDS-PAGE followed by blotting with the indicated caspase neoepitope antisera. C, activation of caspase-9 in HL-60 cytosol in vitro. Dialyzed HL-60 cytosol was treated with 500 µg/ml cytochrome c and 1 mM dATP for the indicated length of time and then harvested for immunoblotting with the caspase-9 neoepitope antiserum. Open arrow indicates a M₉ ~65,000 cross-reactive cytosolic polypeptide that serves as a loading control. D, activation of caspase-9 in intact HL-60 cells. HL-60 cells treated with diluent (-) or 68 µM etoposide for 1 h (+) were washed, incubated for 14 h, and harvested for immunoblotting with the caspase-9 neoepitope antiserum. The intense M₉ ~65,000 band (open arrow) served as a loading control. Closed arrows, species detected in apoptotic HL-60 cells. **, location of procaspase-9 when the blot was reprobed with anti-procaspase-9 antibody. E, effects of various inhibitors on etoposide-induced caspase activation in HL-60 cytosol. Lanes 1 and 2, caspase incubated at 30 °C for 5 h in the absence (lane 1) or presence (lane 2) of exogenous ATP (1 mM) and cytochrome c (500 µg/ml); Lanes 3–5, cytosol containing ATP and cytochrome c was supplemented with iodoacetamide (IAA) (10, 100, and 500 µM), DEVD-fmk (1, 10, and 100 µM), VEID-fmk (1, 10, and 100 µM), ZVAD-fmk (10, 100, and 1000 nM), ZVAD(OMe)-fmk (10, 100, and 1000 nM), TPCK (0.1, 0.5, and 1 mM), TLCK (0.1, 1, and 10 µM), or DCI (0.1, 0.25, and 0.5 mM), respectively. At the completion of a 5-h incubation at 37 °C, samples were subjected to blotting with the caspase-9 neoepitope antiserum. Only the region between 15- and 25-kDa is shown.
nosome. In the present study, nucleotide levels required for caspase activation in cytosol under cell-free conditions were directly compared with nucleotide pools in the same cell line as it underwent apoptosis. Results of these studies indicated that caspase activation in HL-60 cytosol in vitro required as little as 10 μM dATP or 100 μM ATP. In intact HL-60 cells, neither dATP nor ATP increased during the induction of apoptosis by etoposide (Fig. 4B). Instead, base-line levels of both nucleotides were far above those required for caspase activation in vitro. In fact, levels of dATP observed in HL-60 cells are substantially higher than previously reported in another leukemia cell line (76), raising the interesting possibility that the exquisite sensitivity of HL-60 cells to a variety of proapoptotic agents might be related to the high base-line dATP levels.

In subsequent experiments, ATP was replaced with AMP-PCP, AMPPNP, or ATPγS. Results of these experiments help clarify the role of the nucleotide in the caspase activation process. In ATP-requiring processes that depend on ATP binding rather than hydrolysis (e.g. allosteric activation of pterussis toxin (77), the release of eukaryotic initiation factor-2 from its ternary complex (78), the binding of polyoma virus T antigen to its DNA-binding site (79), the activation of ubiquitin protein ligase (80), or release of xylose reductase from its protein chaperone (81)), one or more of these poorly hydrolyzable analogs has been observed to substitute fully for ATP. In contrast, our experiments revealed that these analogs were poor substitutes for ATP over a wide range of concentrations. In particular, AMPPCP and AMPPNP permitted only slow caspase activation (Fig. 3B), and ATPγS did not facilitate caspase activation at all (Fig. 3C). Although these compounds are frequently considered “nonhydrolyzable,” previous studies have demonstrated that various ATPases can hydrolyze these analogs at rates that range from 0.0007 to 0.25 times the corresponding rates of ATP hydrolysis (72, 82–85). The slowing of caspase activation in the presence of these poorly hydrolyzable analogs (Fig. 3, B and C) is consistent with a process that requires hydrolysis of the terminal phosphodiester bond. Likewise, the inhibition of caspase activation by millimolar concentrations of Na2VO4 (Fig. 5B), which have previously been shown to inhibit ATPases (65, 66), is consistent with the suggestion that ATP or dATP hydrolysis is involved in this process.

In further experiments, the effects of a variety of inhibitors were analyzed. A major advantage of the cell-free system is the lack of permeability barriers that have been postulated to inhibit the action of certain caspase inhibitors (86). Results obtained with the cell-free system were compared with effects observed when recombinant caspases were treated with the same inhibitors. For these experiments, we utilized antisera that recognized caspase large subunits as well as an antiserum raised against a neoeptope present in mature caspase-9 but not the zymogen. This latter antiserum recognized caspase-9 but not other caspases (Fig. 5B). In addition, this serum recognized multiple species in a preparation of purified recombinant caspase-9, including several 30–35-kDa species that correspond in molecular weight to the reported sizes of active caspase-9 species (18) as well as a unique M r ~18,000 species that appears to represent further processing of the large subunit (Fig. 8A). Interestingly, the major species detected by this antiserum after caspase activation in vitro (Fig. 5C) or in intact HL-60 cells (Fig. 5D) was the M r ~18,000 species. A similar M r ~18,000 product of procaspase-9 activation was recently observed by Susin et al. (87) but not by others (13, 18, 20, 21). Further studies are required to understand the nature and significance of the processing event that gives rise to this species. Nonetheless, this reagent allowed us to examine caspase-9 activation under cell-free conditions.

Based on recent observations that some active caspases are phosphoproteins (49), we examined the effect of broad spectrum kinase and phosphatase inhibitors on caspase activation. At concentrations that selectively inhibit kinases or phosphatases, these reagents had little if any effect on caspase activation (Fig. 5B), suggesting that phosphorylation or dephosphorylation of the effector caspases does not play a major role in this process in vitro.

In contrast, a variety of other agents that have previously been reported to diminish apoptosis in intact cells inhibited caspase activation in vitro. For example, Zn2+, which was originally introduced into the apoptosis literature as a nuclease inhibitor, inhibited caspase-9 activity (Fig. 6B) and caspase activation (Fig. 5A). Although previous studies have identified Zn2+ as a potential caspase inhibitor (28–30), the present study provides the first evidence that caspase-9 is a pertinent target of this cation. Consistent with this hypothesis, additional experiments have demonstrated that activation of caspase-9 and its downstream target caspase-3 is inhibited by Zn2+ in intact cells.

Similarly, our data raise the possibility that ATA might inhibit apoptosis by acting as a caspase inhibitor. Although ATA is known to inhibit endonucleases (88), studies performed over the past 20 years have indicated that ATA can also inhibit proteases, including serine proteases (89), the proteosome (90), calpains (91), and certain caspases (92). Our results extend these previous studies by demonstrating that ATA not only inhibits recombinant caspases-3, -6, -7, and -9 in vitro (Figs. 6 and 7) but also abrogates caspase activation under cell-free conditions (Fig. 5A).

The same analysis revealed that various caspase inhibitors prevented caspase activation (Figs. 5C and 8E). Consistent with results that were published as the present studies were nearing completion (13, 44), we observed that ZVAD-fmk inhibited caspase-9 activity (Fig. 6) and activation (Figs. 5C and 8E). ZVAD(OMe)-fmk exhibited lower potency as an inhibitor in vitro, presumably because esterases that activate this agent in intact cells were not available to deesterify it after preparation of cytosol in the presence of the serine esterase inhibitor PMSF (93). Interestingly, DEVD-fmk was only 10-fold less potent than ZVAD-fmk as an inhibitor of caspase activation (Figs. 5C and 8E) and caspase-9 activity (Fig. 6). Although it has often been argued that the anti-apoptotic effects of DEVD-fmk result from the inhibition of caspase-3, these results raise the possibility DEVD-fmk might also be acting by inhibiting caspase-9 at the 10–300 μM concentrations used in many experiments.

Caspase activation was also inhibited by protease inhibitors that are not traditionally viewed as caspase inhibitors. The chloromethyl ketones TPCK and TLCK, which have been reported to inhibit caspases-3 and -7 in crude bacterial lysates (69), inhibited caspase activation (Figs. 5C and 8E). DCI and ZFA-fmk also prevented caspase activation under cell-free conditions (Figs. 5, C and D, and 8E). Further analysis revealed that all of these reagents could inhibit purified caspases-3, -6, -7, and -9 as well (Figs. 6 and 7). Collectively, the results in Figs. 5–8 raise the possibility that these agents might be affecting apoptosis through effects on caspase activation and activity rather than effects on noncaspase proteases. In view of these results, previous studies that utilized these inhibitors to support the view that noncaspase proteases play a role in apoptosis might need to be reinterpreted.

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6 T. J. Kottke and S. H. Kaufmann, unpublished observations.
