Granule-mediated Killing: Pathways for Granzyme B–initiated Apoptosis

By Robert V. Talanian,* XiaoHe Yang,‡ Jane Turbov,‡ Prem Seth,¶ Tariq Ghayur,* Carlos A. Casiano,§ Kim Orth,‖ and Christopher J. Froelich‡

From the *BASF Bioresearch Corporation, Worcester, Massachusetts 01605; ‡Department of Medicine, Evanston Hospital, Northwestern University, Evanston, Illinois 60201; §W.M. Keck Autoimmune Disease Center, Scripps Research Institute, La Jolla, California 92037; ‖Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109; and the ¶Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Summary

We report that the serine protease granzyme B (GrB), which is crucial for granule-mediated cell killing, initiates apoptosis in target cells by first maturing caspase-10. In addition, GrB has a limited capacity to mature other caspases and to cause cell death independently of the caspases. Compared with other members, GrB in vitro most efficiently processes caspase-7 and -10. In a human cell model, full maturation of caspase-7 does not occur unless caspase-10 is present. Furthermore, GrB matured caspase-3 with less efficiency than caspase-7 or caspase-10. With the caspases fully inactivated by peptidic inhibitors, GrB induced in Jurkat cells growth arrest and, over a delayed time period, cell death. Thus, the primary mechanism by which GrB initiates cell death is activation of the caspases through caspase-10. However, under circumstances where caspase-10 is absent or dysfunctional, GrB can act through secondary mechanisms including activation of other caspases and direct cell killing by cleavage of noncaspase substrates. The redundant functions of GrB ensure the effectiveness of granule-mediated cell killing, even in target cells that lack the expression or function (e.g., by mutation or a viral serpin) of one or more of the caspases, providing the host with overlapping safeguards against aberrantly replicating, nonself or virally infected cells.

Lymphocyte granule-mediated cytotoxicity is designed to protect the host from invasion by intracellular pathogens, tumor, and nonself cells. Two distinct mechanisms encompass this phenomenon: (a) perforin (PFN)[1]–mediated necrosis of the target and (b) PFN/granzyme–induced apoptosis in which granzyme B (GrB) plays a pivotal role. Unlike PFN-induced target cell necrosis, the mechanism of lymphocyte granule-mediated apoptosis has only recently become apparent (1). An important clue to its function is the preference of GrB for cleavage of peptide bonds after Asp residues. The caspases, which are expressed as zymogens, are activated by proteolytic cleavage at specific Asp residues, and act by cleaving substrates at Asp residues as well. Except for caspase-1 (2), all caspases that have been tested as GrB substrates can be processed and activated by GrB in vitro: caspase-3 (3–5), caspase-6 (6, 7), caspase-7 (8–10), caspase-8 (11–13), caspase-9 (14), and caspase-10 (15, 16). The relative rates of processing of these enzymes by GrB is not known, nor is it clear whether GrB can access and cleave these substrates in vivo.

We have proposed that GrB is delivered to target cells by a mechanism unique to mammalian cells (17). Secreted PFN and GrB are cointernalized into endosomes of the target cell during granule-mediated cytotoxicity. PFN then permeabilizes the vesicles, delivering GrB to the cytosol. Subsequently, GrB induces cell death by activating the caspases. Caspase-1, -2, -3, -6, and -7 have been reported to undergo processing in target cells during GrB-mediated apoptosis (3, 8, 17, 18). Because many caspases can auto- or cross-activate one another, and because the relative contribution of caspase activation by caspases and by GrB is unknown, it remains unclear whether a subset of caspase(s) are directly cleaved by GrB to initiate the death pathway. Although the apparent polyspecificity of GrB toward multiple caspases complicates dissection of the pathway(s) activated by GrB, this attribute may be crucial under conditions where full activation of the caspases is hampered by the ab-

---

[1]Abbreviations used in this paper: AD, replication-deficient adenovirus type 2; GrB, granzyme B; PFN, perforin; PARP, poly-(ADP-ribose) polymerase; PI, propidium iodide; FITC–TUNEL, terminal deoxyribonucleotidyl transferase labeling of DNA strand breaks with FITC–dUTP; snRNP, small nuclear ribonucleoprotein.

sence of a specific caspase (19) or by the presence of an inhibitor that inactivates one or more members of the pathway (20, 21). Knowledge of the preferences of GrB toward the caspases would enable predictions of the caspase(s) first processed by GrB to initiate apoptosis under physiologic conditions and of downstream caspases that may be activated by the granzyme when some enzymes are disrupted. Although it is commonly accepted that GrB induces cell death only through activation of the caspases, this notion must be reconciled with the evidence that GrB rapidly translocates to the nucleus in targets treated with a combination of GrB and PFN or replication-deficient adenovirus type 2 (AD; reference 22) (Pinkoski, M., A. Caputo, P. Seth, C.J. Froelich, and R.C. Bleackley, manuscript submitted for publication); and (Trapani, J., P. Jans, M. Smyth, C.J. Froelich, V. Sutton, and D. Jans, manuscript submitted for publication). These results argue that GrB induces cell death by an intranuclear process separate from or in addition to the caspases.

We report experiments designed to define the mechanism(s) of GrB-induced apoptosis. The catalytic efficiencies of GrB against all 10 known caspases was measured. We developed a protocol for fully blocking caspase activity in whole cells. This system was used to demonstrate the kinetics of caspase protein cleavage due only to GrB activity (and not to caspase auto- or cross-activation), and to reveal that GrB can induce both growth arrest and cell death in target cells in the absence of caspase activity.

Materials and Methods

Cell lines. Jurkat cells were maintained in RPMI-1640, 10% heat-inactivated FCS supplemented with 2 mM l-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin. MCF7w and MCF7i, breast cancer cells reported to be deficient in capases -3 and -10 (16, 19), were provided by Drs. D. Boothman (University of Wisconsin, Madison, WI) and K. Tomaselli (IDUN, Inc. San Diego, CA), respectively.

Reagents. Human GrB was purified to homogeneity from a human NK cell line (YT cells; reference 23). Titration with the GrB-specific protease inhibitor, anti-GraB, an antichymotrypsin engineered to react specifically with GrB (17), showed that ~80% of the serine protease is present in its active form (data not shown). A nonreplicating strain of AD was cultured and isolated as described (24). The peptidic inhibitors and substrates z-DEVD-fmk, z-VAD-fmk, and Ac-DEVD-afc were supplied by Kamiya Biomedical (Spokane, WA). Human sera containing highly specific autoantibodies to poly-(ADP-ribose) polymerase (PARP), small nuclear ribonucleoprotein (snRNP) and lamin B were from the serum bank of the W.M. Keck Autoimmune Disease Center of The Scripps Research Institute (La Jolla, CA; reference 25).

Caspase Cleavage by GrB. Caspases-1–9 and 10/b were encoded on vectors under the control of a T7 RNA polymerase promoter (12, 16, 26–28). [^{35}S]Methionine–labeled proteins were prepared from these vectors using a T7-coupled reticulocyte lysate transcription translation (TnT) system (Promega, Madison, WI). Cleavage assayed consisted of 75 μl of TnT reaction mix and 75 μl of reaction buffer (100 mM Hepes, pH 7.5, 20% glycerol, 0.5 mM EDTA, 5 mM DTT) containing purified GrB at a final concentration of 5.2 nM. Incubations were at room temperature, and 10 μl aliquots were removed at various times between

0 and 30 min, and stopped by diluting with 75 μl of a buffer containing SDS and heating to 90°C for 5 min. Aliquots (7.5 μl) were separated by SDS-PAGE using 10–20% Tris–tricine gels (Integrated Separation Systems, Natick, MA). Dried gels were imaged, and bands corresponding to caspases were quantitated using a GS-250 Molecular Imager and Molecular Imaging Screen CS (BioRad, Hercules, CA). Apparent V_{max}/K_m values were obtained by plotting substrate band intensity versus time and fitting to an exponential decay curve (where $k_{obs} = V_{max}/K_m$) as described (29). Reported k_{cat}/K_m values were obtained by dividing k_{obs} by enzyme concentration corrected for fractional activity as described above, and are means of assays performed in triplicate.

Target Cells. Jurkat cells were treated with GrB and AD as described (17); unless indicated, cells (10^6/ml) were mixed with GrB (1 μg/ml; 30 nM) and AD (100 PFU) in 1-ml microfuge tubes containing RPMI supplemented with 0.5% BSA. Target cells were pretreated with z-DEVD-fmk and/or z-VAD-fmk (100 μM) except as indicated. The peptides were dissolved in Me$_2$SO and used at <0.5% (vol/vol). Cell number and viability were determined by Trypan Blue dye exclusion and conventional light microscopy. To minimize in vitro caspase processing in cell lysates for immunoblotting, 5 min before the end of the assay target cells were washed with PBS to remove excess GrB, transferred to a fresh tube, and lysed in buffer containing the GrB-specific antiprotease, anti-GraB (17).

Terminal Deoxyribonucleotidyl Transferase Labeling of DNA Strand Breaks with FITC–dUTP, Propidium Iodide Reactivity, and Hoescht 33342 Staining. Cell death was measured by terminal deoxyribonucleotidyl transferase catalyzed labeling of DNA strand breaks with FITC–dUTP (FITC–TUNEL; reference 30) and/or propidium iodide (PI) staining followed by flow cytometry. Data acquisition consisted of 5,000 events/analysis on a Coulter Epic V. For Hoescht staining, cells were fixed with 0.5% paraformaldehyde for 15 min, cytospun to microscope slides, and stained with Hoescht 33342 (1 μg/ml). Cells were visualized with a Zeiss Fluorescent microscope.

Western Blotting of Caspases. Processing of caspase-3, -6, and -7 was measured as described (4, 6, 17, 31). Treated cells (10^6/ml) were lysed, resolved by SDS-PAGE (10%), and transferred to nitrocellulose. Anti–caspase-3, -6, and -7 rabbit antisera were used at dilutions of 1:1,000 followed by incubation with anti–rabbit Ig–horseradish peroxidase (Amersham, Arlington Heights, IL) at a dilution of 1:10,000. The signal was visualized with the ECL kit (Amersham).

Western Blotting for PARP, snRNP, and Lamin B. Harvested target cells were resuspended at 10^7 cells/ml in lysis buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and the COMPLETE protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Lysates were heated at 100°C for 5 min, passed several times through a 27-gauge needle to shear DNA, and stored at −70°C until use. Samples containing total protein from ~10^6 cells were applied to individual lanes in 12% polyacrylamide–SDS gels. After electrophoresis under reducing conditions, proteins were transferred to nitrocellulose at 220 mA for 4–5 h. Nitrocellulose strips corresponding to the individual lanes of gels were blocked for 30 min in PBS containing 0.05% Tween-20 (PBST) and 5% nonfat dried milk, probed for 45 min with the appropriate human antibody diluted from 1:100 to 1:400 in the same buffer (25), washed for 1 h in several changes of PBST with gentle shaking, and probed for 30 min with a peroxidase-coupled secondary antibody (Zymed Laboratories, South San Francisco, CA). Bound antibody was detected with the ECL kit (Amersham).

Protease Assay. Proteolytic activity in cell lysates was measured using the fluorogenic substrate Ac-DEVD-afc as described (32).

Results

Comparison of Caspases as GrB Substrates. Caspase-1, -2, -3, -6, and -7 have been reported to undergo processing in target cells during GrB-mediated apoptosis (3, 8, 17, 18). It is not known whether GrB processes each of these proteases directly or whether some are matured by a subset of the caspases that are the direct GrB substrates. To compare the caspases as GrB substrates, all 10 known family members were expressed in an in vitro transcription–translation system, and tested as GrB substrates. By monitoring the decrease in caspase substrate parent band intensity, a single value potentially including GrB cleavage at more than one site, plus autoactivation by matured caspase, was obtained. Caspase-7 and -10 were clearly preferred GrB substrates, displaying observed k_{cat}/K_m values of 533,000 and 325,000 $M^{-1}s^{-1}$, respectively. Under conditions that resulted in ~90% cleavage of caspase-10 by GrB after 30 min, processing of caspase -1, -2, -3, -4, -5, -6, -8, and -9 was not observed.

GrB displays a preference for peptide substrates in vitro consistent with cleavage of caspase-7 and -10 at IxxD motifs (IQAD[198]–S and IEAD[372]–A, respectively), which separate the pSmall and pLarge subunits (Talanian, R.V., unpublished results). Caspase-3 contains a similar sequence in the analogous position (IETD[174]–S), so it seemed likely that this enzyme would be cleaved by GrB preferentially with respect to the other caspases. When sixfold higher concentrations of GrB was added to in vitro translated caspase-3, processing could be detected at 30 min (Fig. 1 *a*). In contrast, virtually all caspase-7 and most of caspase-10 were processed within 5 min. Cleavage of another nonpreferred GrB substrate, caspase-6, was not observed until 120 min (Fig. 1 *d*). Although caspase-7 and -10 are clearly preferred GrB substrates, the results suggest that caspase-3 and others can be matured directly by GrB under circumstances where caspase-7 and/or -10 are absent.

Inhibition of Caspase Activation during GrB-mediated Apoptosis. To allow the assessment of caspase activation by GrB in cells independently of caspase auto- and trans-activation, we explored the use of irreversible peptidic ligands for full caspase inhibition. Caspase activation in GrB/AD-treated cells was measured by cleavage of the peptidic substrate Ac-DEVD-afc, a readout of caspase-3–like proteolytic activity that is typical of lysates of cells undergoing apoptosis. On addition of GrB/AD, we observed a transient induction of caspase-3–like activity that was fully inhibited by z-DEVD-fmk at 100 μM (Fig. 2 *a*). To test more stringently for full inhibition of all caspases, we evaluated cell lysates for GrB/AD-induced cleavage of PARP, snRNP, and lamin B to the signature apoptotic fragments of 85, 40, and 45 kD, respectively. Because z-DEVD-fmk at 100 μM failed to block PARP cleavage completely (data not shown), we used a combination of z-DEVD-fmk and z-VAD-fmk each at 100 μM. The two inhibitors completely prevented
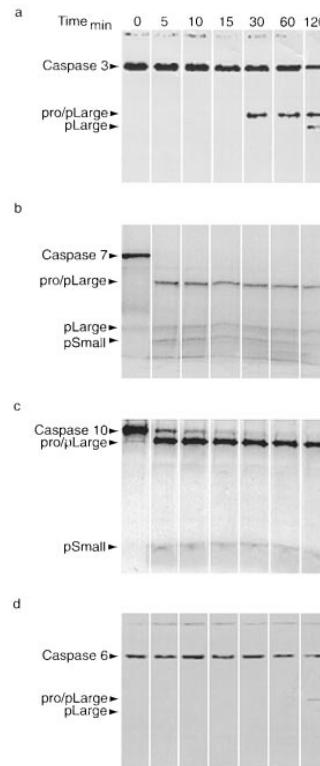

Figure 1. Comparison of kinetics of proteolysis in vitro that GrB manifests against caspase-3, -6, -7 and -10. The [35S]methionine caspases were generated and isolated as described (28). In a total volume of 320 μl, translated caspases were mixed with GrB (30 nM) for the times indicated and analyzed by SDS-PAGE and autoradiography. (*a*) Kinetics of [35S]–caspase-3 (~250 ng) processing; (*b*) kinetics of [35S]–caspase-7 (~300 ng) processing; (*c*) kinetics of [35S]–caspase-10 processing (~300 ng); and (*d*) kinetics of [35S]–caspase-6 processing (~250 ng).

cleavage of all three proteins up to 18 h after GrB/AD application (Fig. 2 *b*). We conclude that these inhibitors at 100 μM each give full caspase inhibition.

To confirm direct caspase inhibition by the peptidic ligands in cells, caspase-3 and -7 were examined by immunoblot analysis of cells treated with z-DEVD-fmk and GrB (Fig. 3). Inhibitor treatment resulted in a shift of the pLarge subunit of each enzyme to higher molecular weight, consistent with the location of the putative catalytic nucleophiles (Cys163 and Cys186 for caspase-3 and -7, respectively) on the pLarge subunits (10, 33). Caspase-3 also displayed a shifted pro/pLarge fragment (Fig. 3 *b*), suggesting that cleavage at that site (ESMD[28]–S) is autocatalytic. The results demonstrate that z-DEVD-fmk inactivates caspase-3 and -7 in cells directly, and suggest that the combination of z-DEVD-fmk and z-VAD-fmk gives similar inactivation of the other caspases as well.

Caspase-3 and -7 Are Processed Directly by GrB in Cells with Inactivated Caspases. Using z-DEVD-fmk and z-VAD-fmk to block auto- and trans-caspase activation, we used immunoblotting to examine caspase processing directly by GrB in Jurkat cells. Owing to the lack of a suitable antibody to detect processed forms of caspase-10, our efforts focused on caspase-3 and -7. In the absence of inhibitors, GrB/AD resulted in rapid maturation of caspase-7 characterized by removal of the propeptide by cleavage at DSVD[23]–A and apparently slower cleavage between the pLarge and pSmall subunits at IQAD[198]–S (Fig. 4 *a*). With addition of the inhibitors, caspase-7 was cleaved only between the pLarge and pSmall subunits (Fig. 4 *a*). The re-
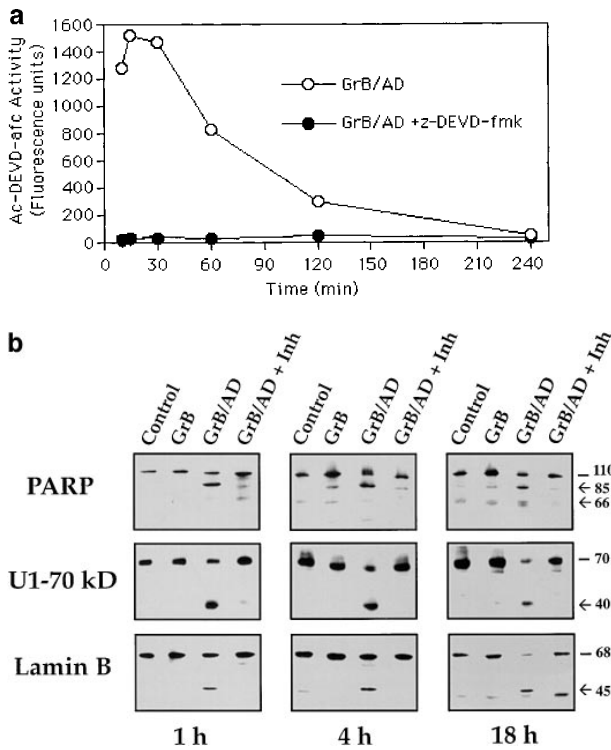
Figure 2. Blockade of caspase proteolytic activities with oligopeptide inhibitors during GrB-induced apoptosis: effect on Ac-DEVD-afc fluorogenic activity and cleavage of death substrates, PARP, snRNP, and lamin B. (a) z-DEVD-fmk (100 μM) inhibits Ac-DEVD-afc cleavage during GrB/AD-mediated apoptosis. Target cells were pretreated with z-DEVD-fmk for 15 min. After exposure to GrB/AD for the times indicated, cells were withdrawn for measurement of fluorogenic Ac-DEVD-afc activity. (b) Generation of the apoptotic fragments of PARP, snRNP, and lamin B is completely inhibited by the combination of z-DEVD-fmk and z-VAD-fmk. Total Jurkat cell lysates obtained from control cells and cells treated for 1, 4, and 18 h with either GrB alone, GrB/AD, or GrB/AD plus z-DEVD-fmk and z-VAD-fmk. The lysates were electrophoresed in 12% SDS–polyacrylamide gels and proteins were transferred to nitrocellulose. Individual lanes (containing protein corresponding to ~10^6 cells) were reacted with specific human autoantibodies to PARP, snRNP, and lamin B. Representative blots are shown. Intact proteins are indicated by lines, whereas proteolytic fragments are indicated by arrows. Numbers to the right represent relative molecular weights.

sults show that GrB initiates caspase-7 activation by cleavage between the pLarge and pSmall subunits. Pro-region removal, which is rapid compared with GrB-mediated cleavage between the pLarge and pSmall subunits, is conducted by and requires active caspases.

Caspase-3 processing in Jurkat cells by GrB/AD can be detected in 15–30 min (Fig. 4 b; reference 17). The onset of proteolysis (60 min) was slower and the quantity matured was reduced compared with caspase-7 (Fig. 4 b). Like caspase-7, the inhibitors prevented removal of the pro-region, showing that cleavage at this site is also caspase dependent and that GrB initiates caspase-3 maturation by cleavage at the IxxD motif between its large and small subunits. The results also provide evidence that GrB can process a less preferred substrate such as caspase-3 directly in cells, at correspondingly lower efficiency.
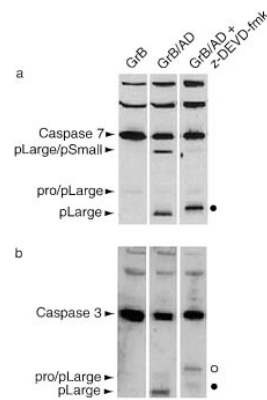

Figure 3. Complex formation between large subunits of caspase-7 and -3 and z-DEVD-fmk in Jurkat cells subjected to GrB-induced apoptosis. Jurkat cells were treated with z-DEVD-fmk (40 μM) for 30 min followed by GrB/AD for 1 h. Lysates were immunoblotted from 10–20% gradient gels and reacted with the polyclonal Abs against (a) caspase-7 and (b) caspase-3. Inhibitor treatment resulted in a shift of the large subunits to higher molecular weight (*closed circle to the right*), demonstrating that the inhibitor reacted directly with the caspase large subunits. Caspase-3 similarly displayed a shifted pro/pLarge fragment (*open circle to the right*).

GrB Activates the Caspases through Caspase-10. The processing pattern of caspase-7 in cells suggests that its pro-region is cleaved either by another caspase or autocatalytically. Because caspase-10 is also a preferred substrate for GrB, it is a likely candidate for the completion of caspase-7 maturation. We find that the breast carcinoma cell lines MCF7w and MCF7i, which are deficient in caspase-3 and -10 (16, 19), are also deficient in GrB/AD-induced caspase-7 pro-region removal (Fig. 5). In these cells, GrB cleaves caspase-7 between the pLarge and pSmall subunits as expected. The resulting species cannot remove its pro-region (in *cis* or *trans*), demonstrating that caspase-7 pro-region cleavage requires the action of other caspase(s). We propose that this occurs through the preferred GrB substrate caspase-10, and thus that caspase activation initiated by GrB occurs primarily through caspase-10.

Caspase Inhibitors Do Not Prevent GrB-mediated Cell Death. We have observed extremely rapid nuclear translocation of endocytosed GrB into the majority of target cells after treatment with PFN or AD. We reasoned that GrB might catalyze intranuclear proteolysis directly and cause cell death independently of caspase action. Using the irreversible caspase inhibitors, we examined the cells for morphologic evidence of cell death. Similar to target cells treated only with z-DEVD-fmk (17), the two caspase in-
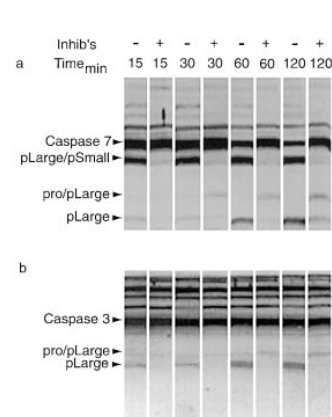

Figure 4. GrB can directly process caspase-7 and -3 in Jurkat cells with inhibited caspases: effect on pattern and rate of processing. Lysates obtained from Jurkat cells treated for 15, 30, 60, and 120 min with either GrB/AD or GrB/AD plus z-DEVD-fmk and z-VAD-fmk. The lysates were electrophoresed in 12% SDS–polyacrylamide gels and caspases were detected by immunoblotting. Individual lanes (containing protein corresponding to ~10^6 cells) were reacted with (a) anti–caspase-7 Ab or (b) anti–caspase-3 Ab.
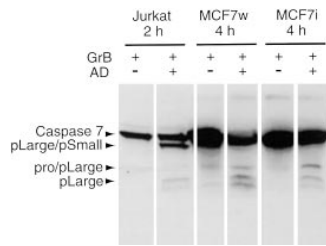
Figure 5. GrB directly processes caspase-7 in cells lacking caspase-10. MCF cells (10^6/ml) were allowed to adhere to 24-well culture plates overnight and then treated with GrB/AD as described in Materials and Methods. After 4 h, cells were harvested by trypsinization, and lysates were subjected to immunoblotting for caspase-7. For comparison, the processing of caspase-7 in Jurkat cells exposed to GrB/AD for 2 h is also shown.

hibitors blocked DNA fragmentation (TUNEL) but only a minor portion of the cells expressed condensed nuclei and PI staining at 4 h ($<15\%$; data not shown). Longer-term effects of intranuclear GrB was examined by PI and Hoescht over a 4-d period. In these experiments, fresh inhibitors were added to the target cells at 24 h. Compared with controls, cells treated with the caspase inhibitors and GrB/AD were present in reduced numbers and completely failed to proliferate throughout a 96-h period (Fig. 6 *a*). Morphologic analysis by PI and Hoescht stain showed two populations: an increased percentage of dying cells (Fig. 6, *b* and *c*) whose nuclei became progressively more condensed throughout the culture period (40% at 96 h), and a second group with normal sized nuclei and intact plasma membrane (60%) (Fig. 7 *c*). These results suggest that GrB induces dimorphic changes in the targets in which one subset has died and the other is in growth arrest. The mechanisms that result in these outcomes do not involve proteolysis of substrates usually associated with apoptotic cell death mediated by activated caspases (see Fig. 2 *b*).

Discussion

We recently proposed that granule-mediated apoptosis mimics a pathway used by viruses to enter nucleated cells (17). In this model, apoptosis is induced in target cells through the delivery of GrB by PFN. GrB and PFN are internalized into a coated vesicle, and during fusion with an early endosome, PFN, by an endosomolytic mechanism, releases GrB to the cytosol. Consistent with this model, the replication-deficient type 2 AD can substitute for PFN (17). Cytosolic delivery of GrB by AD offers two advantages to study the processing of caspases in target cells undergoing apoptosis. First, target cells vary markedly in their susceptibility to the apoptotic action of PFN and granzymes. Although this is often attributed to variable effects of the granzymes, the membranolytic activity of PFN is also highly variable (34). AD, on the other hand, when properly engineered, can be used to deliver GrB in a highly reproducible fashion. Second, unlike AD, PFN may lyse a proportion of the target cells, exposing cytosol components to the proteolytic action of the granzymes. Consequently, target cells treated with GrB/PFN contain a mixture of products that reflect GrB-mediated cleavage of proteins in whole cells as well as cellular extracts. In the present study, we took advantage of the GrB/AD model to determine the mechanism(s) by which GrB initiates apoptosis in target cells, asking which caspase(s) are activated directly by GrB, and whether GrB can effect apoptosis independently of the caspases.

Measuring the catalytic efficiencies expressed by GrB against the known caspases showed that GrB has a substantial preference for caspase-7 and -10. Therefore, either caspase may be cleaved by GrB to initiate cell death. We find that caspase-7 pro-region removal after GrB cleavage between the pLarge and pSmall subunits is deficient in cell lines lacking caspase-3 and -10, suggesting that full caspase activation requires one or both of those enzymes. Caspase-10 is probably situated near the cytoplasmic aspect of the plasma
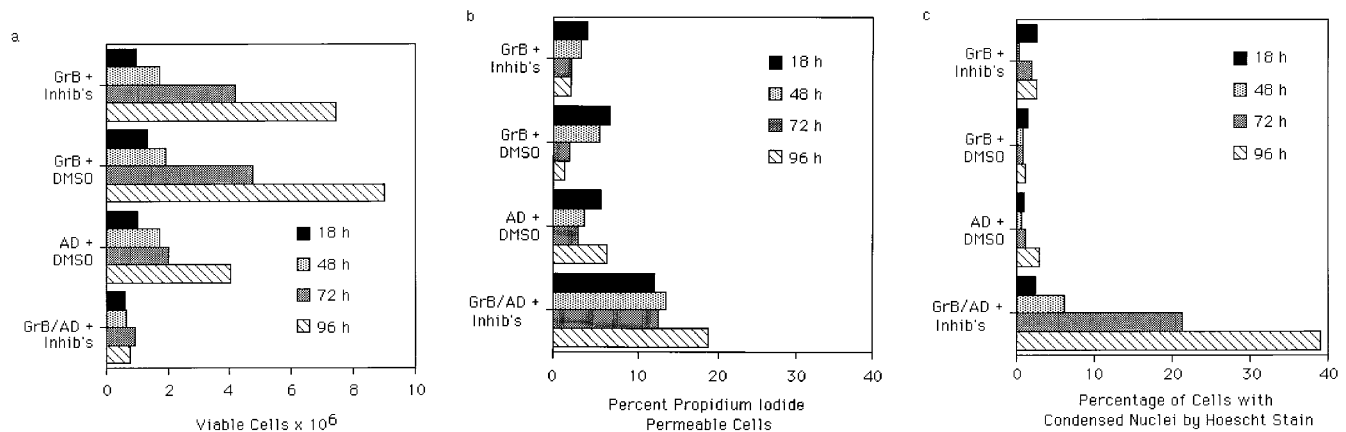

Figure 6. GrB/AD treatment produces growth arrest and cell death in Jurkat cells with inhibited caspases. Target cells (10^6/ml) in RMPI, 0.5% BSA were added to 24-well plates and pretreated with caspase inhibitors (100 μM) for 30 min followed by GrB/AD. At 18 h, 0.5 ml of the media was removed and replaced with RMPI, 10% FCS plus fresh inhibitors. (*a*) Total number of viable (i.e., PI impermeable) cells; (*b*) percentage of cells that developed permeability to PI staining; and (*c*) percentage of cells with condensed nuclei. Control cells treated with AD alone at the same multiciplicity of infection were not significantly growth impaired (data not shown).
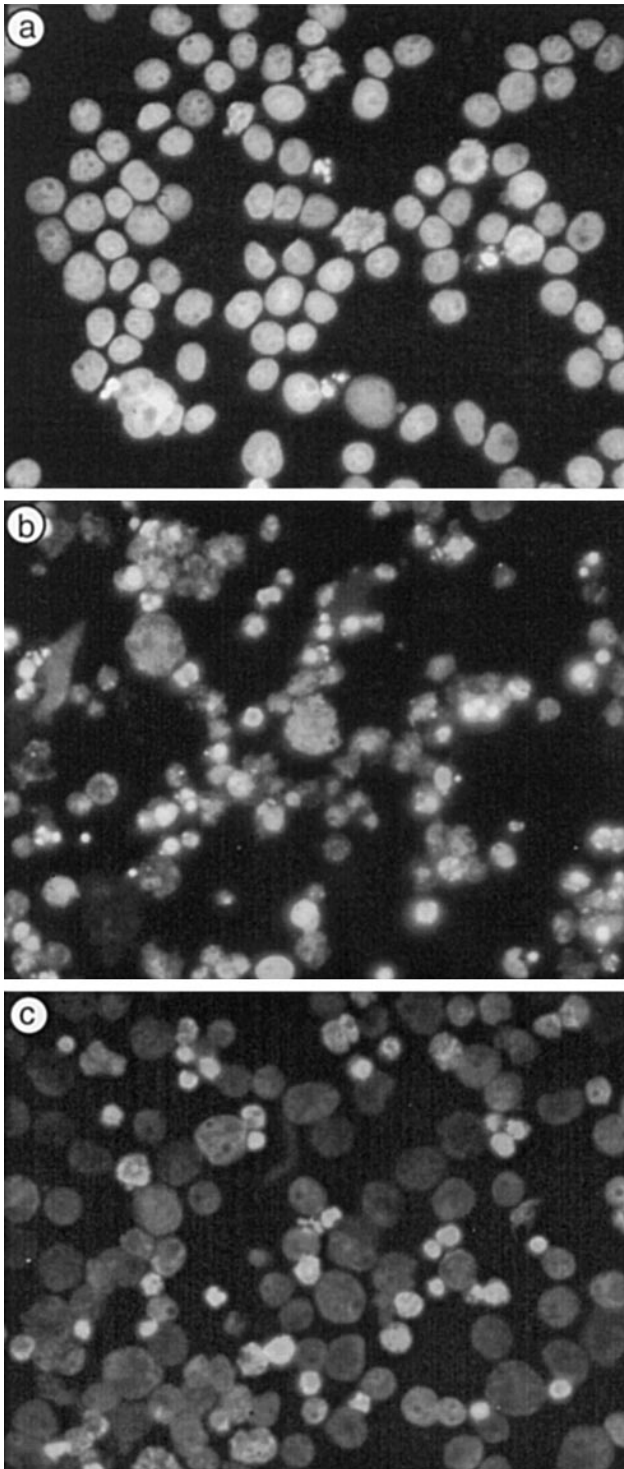
Figure 8. Model for caspase activation in whole cells undergoing GrB-mediated apoptosis. The dominant pathway (*closed arrows*) describes the effect of GrB in cells that contain a full complement of known caspases. In this case, we propose that GrB initially activates caspase-10, which then matures caspase-3 and -7. These caspases then mature the other members of the family. The open arrows (secondary pathway) signify how GrB can directly activate other caspases under conditions where caspase-10 is disrupted by a mutation or a viral serpin (e.g., CrmA; reference 40). The gray arrow suggests a pathway in which intranuclear GrB can induce cell death independently of the caspases.

Figure 7. Morphology during GrB-mediated death in Jurkat cells with inhibited caspases. Target cells (10^6/ml) in RMPI, 0.5% BSA were added to 24-well plates and pretreated with caspase inhibitors (100 μM) for 30 min followed by GrB/AD. At 24 h, 0.5 ml of the media was removed and replaced with RMPI, 10% FCS plus fresh inhibitors. Photomicrograph displays cells at 96 h after Hoescht stain and imaging at 100× magnification. (*a*) Media; (*b*) GrB/AD; and (*c*) GrB/AD treated with caspase inhibitors. Cells treated with AD alone at the multiplicity of infection used here did not impair growth (data not shown).
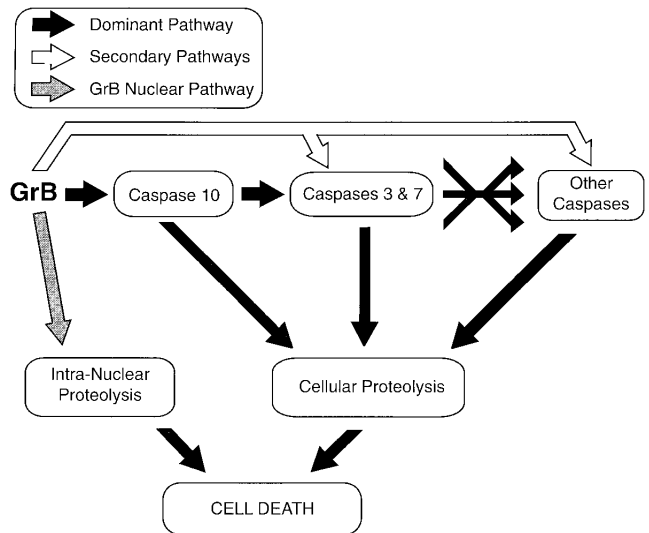
membrane (12), making it accessible to GrB released from the early endosomal compartment. Thus, caspase-10 may be the more important GrB target for initiation of apoptosis. This represents a proposed functional role for caspase-10, and suggests a novel model for GrB-initiated apoptosis in cells (Fig. 8).

The subsequent sequence by which caspases are activated is suggested by the pattern in which caspase-6 and -7 are cleaved in whole cells. Both are first cleaved free of the propeptide, represented by sequences TETD[23]–A and DSVD[23]–A, respectively (17). The former matches the substrate specificity of caspase-6 itself (29), and might be largely autocatalytic. The latter matches well the preferences of caspase-3 and -7 (29). Thus, in vivo, caspase-10 may activate caspase-3 by cleaving the latter between the pLarge and pSmall subunits (IETD[174]–S). The activation of caspase-7 plus presumably other caspases is then followed by rapid auto- and cross-activation in an explosive process resulting in full activation of the caspases (Fig. 8). We note that the participation of undiscovered caspases or other proteases in this process is neither ruled out by our data nor particularly unlikely.

Although GrB most efficiently elicits apoptosis in target cells by initiating caspase activation through caspase-10, there is sufficient evidence to propose also that GrB can still activate caspases through caspase-7 or through less preferred substrates such as caspase-3 if the target cells lack functional caspase-10 (see Fig. 8, *secondary pathway*). Owing to mutation, inhibitory viral serpins, or lack of expression

in a given tissue or stage of differentiation, any particular caspase may be nonfunctional; therefore, consistent with its dominant role in tumor surveillance and viral clearance, GrB has the redundant capacity to initiate caspase activation despite the absence of specific caspases.

Reflecting another level of redundancy in its apoptogenic potential, GrB apparently has the capacity to activate cell death independently of the caspases. Caspases cleave substrates after Asp residues, and are also activated by cleavage after Asp. Because GrB also has a preference for cleavage of proteins after Asp residues, in principle GrB can induce apoptosis both by activating caspases and by cleaving substrates also recognized by the caspases. We find that in the absence of caspase activation, cleavage of cellular substrates considered critical for the induction of apoptosis (PARP, snRNP, and Lamin B) did not occur (Fig. 2 *b*), but GrB still caused cell death. In our model system for granzyme delivery, GrB enters the cytosol and nucleus of all target cells. In the presence of the inhibitors, intranuclear delivery of GrB resulted in two distinct responses: the target cells either die or experience growth arrest. Therefore, our data reveal an unforseen biologic role for GrB during granule-mediated cytotoxicity. In another system that examined the role of caspases during CTL-mediated apoptosis, the results showed caspase inhibitors blocked DNA fragmentation but not cell death (35). These results are consistent with our previous observation that z-DEVD-fmk (40 µM) blocked only DNA cleavage (17) and with the data reported here. Using CTLs to identify the granule components and the pathways that these proteins activate to cause cell death is not possible without specific inhibitors. Our experimental system extends these studies by clearly demonstrating that GrB alone is sufficient to induce cell death in target cells. The substrates directly cleaved by GrB to induce this response have not been identified, and call into question the significance of several so-called universal markers of apoptosis such as PARP cleavage.

The finding that target cells underwent growth arrest was unexpected. GrB is reported to rapidly induce both cyclin A/cdc 2 and cyclin A/Cdk 2 kinase activities (36). Although these activities are temporally related to the apoptotic response in GrB-treated cells, the biologic significance of these findings has remained enigmatic. Based on evidence that cells with increased cdc2 kinase activity are more susceptible to apoptotic stresses (37, 38) and that inhibition of cdc2 kinase activation by Wee 1 kinase inhibits GrB-induced apoptosis (39), we predict that efficient induction of granule-mediated cell death results from the interplay of these two pathways triggered by GrB: activation of cytosolic caspases and induction of intranuclear cyclin A–kinase complexes.

The evolution of cytopathic virus–host interactions has led viruses to adopt strategies that prevent or delay the death of the host until productive replication has occurred. The cytokine response modifier A (CrmA) of the cowpox virus (20) as well as the description of a new family of viral inhibitors (21) exemplify this strategy. Additional viral as well as tumor-associated inhibitors that inactivate apoptotic proteases will undoubtedly be discovered. Furthermore, MCF7 cells express minimal caspase-3 and -10 (16, 19). Despite the absence of these caspases, microinjection of GrB results in rapid apoptosis (Pinkoski, M., A. Caputo, P. Seth, C.J. Froelich, and R.C. Bleackley, manuscript submitted for publication). Cytotoxic cells have evolved a family of serine proteases that are delivered to pathogenic cells ensuring apoptotic cell death by activating distinct but interwoven pathways. The ability of GrB to induce cell death in the presence of a partially or completely inactivated caspase pathway typifies the robustness of this important host defense system.

Address correspondence to Dr. Christopher J. Froelich, Evanston Hospital, Research Department, WH Building, Rm B624, 2650 Ridge Ave., Evanston, IL 60201. Phone: 847-570-2348; FAX: 847-570-1253; E-mail: granzyme@merle.acns.nwu.edu

References

1. Atkinson, E.A., and R.C. Bleackley. 1995. Mechanisms of lysis by cytotoxic T cells. *Crit. Rev. Immunol.* 15:359–384.
2. Darmon, A.J., N. Ehrman, A. Caputo, J. Fujinaga, and R.C. Bleackley. 1994. The cytotoxic T cell proteinase granzyme B does not activate interleukin-1β–converting enzyme. *J. Biol. Chem.* 269:32043–32046.
3. Darmon, A.J., D.W. Nicholson, and R.C. Bleackley. 1995. Activation of the apoptotic protease CPP32 by cytotoxic

T-cell–derived granzyme B. *Nature (Lond.).* 377:446–448.

4. Quan, L.T., M. Tewari, K. O'Rourke, V.M. Dixit, S.J. Snipas, G.G. Poirier, C. Ray, D.J. Pickup, and G.S. Salvesen. 1996. Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. *Proc. Natl. Acad. Sci. USA.* 93:1972–1976.

5. Martin, S.J., G.P. Amarante-Mendes, L.F. Shi, T.H. Chuang, C.A. Casiano, G.A. O'Brien, P. Fitzgerald, E.M. Tan, G.M. Bokoch, A.H. Greenberg, and D.R. Green. 1996. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2407–2416.

6. Orth, K., A.M. Chinnaiyan, M. Garg, C.J. Froelich, and V.M. Dixit. 1996. The CED-3/ICE–like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.* 271:16443–16446.

7. Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri. 1995. *Mch2*, a new member of the apoptotic *Ced-3/Ice* cysteine protease gene family. *Cancer Res.* 55:2737–2742.

8. Chinnaiyan, A.M., K. Orth, W.L. Hanna, H.J. Duan, G.G. Poirier, C.J. Froelich, and V.M. Dixit. 1996. Cytotoxic T cell–derived granzyme B activates the apoptotic protease ICE–LAP3. *Curr. Biol.* 6:897–899.

9. Gu, Y., C. Sarnecki, M.A. Fleming, J.A. Lippke, R.C. Bleackley, and M.S.S. Su. 1996. Processing and activation of CMH-1 by granzyme B. *J. Biol. Chem.* 271:10816–10820.

10. Fernandes-Alnemri, T., A. Takahashi, R. Armstrong, J. Krebs, L. Fritz, K.J. Tomaselli, L. Wang, Z. Yu, C.M. Croce, G. Salveson, et al. 1995. Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* 55:6045–6052.

11. Boldin, M.P., T.M. Goncharov, Y.V. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT1/FADD–interacting protease, in Fas/APO-1– and TNF receptor–induced cell death. *Cell.* 85:803–815.

12. Muzio, M., A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, C. Scaffid, J.D. Bretz, M. Zhang, J. Ni, R. Gentz, et al. 1996. FLICE, a novel FADD-homologous ICE/CED-3–like protease, is recruited to the CD-95 (FAS/Apo-1) death-inducing signaling complex (DISC). *Cell.* 86:817–821.

13. Srinivasula, S.M., M. Ahmad, T. Fernandes-Alnemri, G. Litwack, and E.S. Alnemri. 1996. Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE–like cysteine proteases. *Proc. Natl. Acad. Sci. USA.* 93:14486–14491.

14. Duan, H.J., K. Orth, A.M. Chinnaiyan, G.G. Poirier, C.J. Froelich, W.-W. He, and V.M. Dixit. 1996. ICE–LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J. Biol. Chem.* 171:16720–16724.

15. Fernandes-Alnemri, T., R.C. Armstrong, J. Krebs, S.M. Srinivasula, L. Wang, F. Bullrich, L.C. Fritz, J.A. Trapani, K.J. Tomaselli, G. Litwack, and E.S. Alnemri. 1996. *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA.* 93:7464–7469.

16. Vincenz, C., and V.M. Dixit. 1997. Fas-associated–death domain protein interleukin-1β–converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J. Biol. Chem.* 272:6578–

6583.

17. Froelich, C.J., K. Orth, J. Turbov, P. Seth, B.M. Babior, R.A. Gottlieb, G.M. Shah, R.C. Bleackley, V.M. Dixit, and W.L. Hanna. 1996. New paradigm for lymphocyte granule mediated cytotoxicity: targets bind and internalize granzyme B but a endosomolytic agent is necessary for cytosolic delivery and apoptosis. *J. Biol. Chem.* 271:29073–29081.

18. Shi, L.F., G. Chen, G. MacDonald, L. Bergeron, H.L. Li, M. Miura, R.J. Rotello, D.K. Miller, P. Li, T. Seshadri, J.Y. Yuan, and A.H. Greenberg. 1996. Activation of an interleukin 1 converting enzyme-dependent apoptosis pathway by granzyme B. *Proc. Natl. Acad. Sci. USA.* 93:11002–11007.

19. Krajewska, M., H.G. Wang, S. Krajewski, J.M. Zapata, A. Shabaik, R. Gascoyne, and J.C. Reed. 1997. Immunohistochemical analysis of *in vivo* patterns of expression of CPP32 (Caspase-3), a cell death protease. *Cancer Res.* 57:1605–1613.

20. Komiyama, T., C.A. Ray, D.J. Pickup, A.D. Howard, N.A. Thornberry, E.P. Peterson, and G. Salvesen. 1994. Inhibition of interleukin-1β converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J. Biol. Chem.* 269:19331–19337.

21. Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meinl, F. Neipel, C. Mattmann, K. Burns, J.L. Bodmer, M. Schröter, et al. 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature (Lond.).* 386:517–521.

22. Shi, L., S. Mai, S. Israels, K.A. Browne, J.A. Trapani, and A.H. Greenberg. 1997. Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *J. Exp. Med.* 185:855–866.

23. Hanna, W.L., X. Zhang, J. Turbov, U. Winkler, D. Hudig, and C.J. Froelich. 1993. Rapid purification of cationic granule proteases: application to human granzymes. *Protein Purif. Exp.* 4:398–402.

24. Seth, P. 1994. A simple and efficient method of protein delivery into cells using adenovirus. *Biochem. Biophys. Res. Commun.* 203:582–587.

25. Casiano, C.A., S.J. Martin, D.R. Green, and E.M. Tan. 1996. Selective cleavage of nuclear autoantigens during CD95 (Fas/APO-1)–mediated T cell apoptosis. *J. Exp. Med.* 184:765–770.

26. Hugunin, M., L.J. Quintal, J.A. Mankovich, and T. Ghayur. 1996. Protease activity of *in vitro* transcribed and translated *Caenorhabditis elegans* cell death gene (*ced-3*) product. *J. Biol. Chem.* 271:3517–3522.

27. Ghayur, T., M. Hugunin, R.V. Talanian, S. Ratnofsky, C. Quinlan, Y. Emoto, P. Pandey, R. Datta, Y.Y. Huang, S. Kharbanda, et al. 1996. Proteolytic activation of protein kinase C d by an ICE/CED 3–like protease induces characteristics of apoptosis. *J. Exp. Med.* 184:2399–2404.

28. Orth, K., K. O'Rourke, G.S. Salvesen, and V.M. Dixit. 1996. Molecular ordering of apoptotic mammalian Ced-3/ICE proteases. *J. Biol. Chem.* 171:16443–16446.

29. Talanian, R.V., C. Quinlan, S. Trautz, M.C. Hackett, J.A. Mankovich, D. Banach, T. Ghayur, K.D. Brady, and W.W. Wong. 1997. Substrate specificities of caspase family proteases. *J. Biol. Chem.* 272:9677–9682.

30. Gorczyca, W., J. Gong, and Z. Darzynkiewicz. 1993. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 53:1945–1951.

31. Duan, H.J., A.M. Chinnaiyan, P.L. Hudson, J.P. Wing, W.W. He, and V.M. Dixit. 1996. ICE–LAP3, a novel mam-

malian homologue of the *Caenorhabditis elegans* cell death protein ced-3 is activated during fas- and tumor necrosis factor–induced apoptosis. *J. Biol. Chem.* 271:1621–1625.

32. Enari, M., R.V. Talanian, W.W. Wong, and S. Nagata. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature (Lond.).* 380: 723–726.

33. Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1b–converting enzyme. *J. Biol. Chem.* 269: 30761–30764.

34. Müller, C., and J. Tschopp. 1994. Resistance of CTL to perforin-mediated lysis: evidence for a lymphocyte membrane protein interacting with perforin. *J. Immunol.* 153:2470–2478.

35. Sarin, A., M.S. Williams, M.A. Alexander-Miller, J.A. Berzofsky, C. M. Zacharchuk, and P.A. Henkart. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/

Ced-3 family proteases. *Immunity.* 6:209–215.

36. Shi, L.F., G. Chen, D.L. He, D.G. Bosc, D.W. Litchfield, and A.H. Greenberg. 1996. Granzyme B induces apoptosis and cyclin A–associated cyclin-dependent kinase activity in all stages of the cell cycle. *J. Immunol.* 157:2381–2385.

37. Ongkeko, W., D.J.P. Ferguson, A.L. Harris, and C. Norbury. 1995. Inactivation of Cdc2 increases the level of apoptosis induced by DNA damage. *J. Cell Sci.* 108:2897–2904.

38. Schröter, M., M.C. Peitsch, and J. Tschopp. 1996. Increased $p34^{cdc2}$-dependent kinase activity during apoptosis: a possible activation mechanism of DNase I leading to DNA breakdown. *Eur. J. Cell Biol.* 69:143–150.

39. Chen, G., L. Shi, D.W. Litchfield, and A.H. Greenberg. 1995. Rescue from granzyme B–induced apoptosis by *Wee1* kinase. *J. Exp. Med.* 181:2295–2300.

40. Zhou, Q., S. Snipas, K. Orth, M. Muzio, V.M. Dixit, and G.S. Salvesen. 1997. Target protease specificity of the viral serpin CrmA: analysis of five caspases. *J. Biol. Chem.* 272: 7797–7800.