New Paradigm for Lymphocyte Granule-mediated Cytotoxicity

TARGET CELLS BIND AND INTERNALIZE GRANZYME B, BUT AN ENDOsomolytic AGENT IS NECESSARY FOR CYTOSOLIC DELIVERY AND SUBSEQUENT APOPTOSIS*

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The granule secretory pathway is critical to immune surveillance against aberrant cells (1). The most prominent components of cytotoxic granules are perforin (PFP)¹ and a family of serine proteases termed granzymes (2, 3). Abnormal cells may be killed instantly by PFP alone or survive the initial onslaught to die an apoptotic death (1, 4). The crucial importance of GrB and PFP in rapid granule-mediated apoptosis has been demonstrated by gene ablation experiments in mice (5–7). Nevertheless, precisely how PFP and GrB initiate apoptosis has remained enigmatic. Shi et al. (8, 9) reported that simultaneous incubation of tumor cells with GrB and sublytic PFP resulted in DNA fragmentation in murine targets. These results suggested that PFP generated pores large enough for soluble granzyme to diffuse to the cytosol or that membrane damage could result in uptake of soluble granzyme into a vesicular pathway. We have demonstrated that human granzyme A, a trypsin-like protease in cytotoxic granules, induces interleukin (IL)-6 and IL-8 production in diverse cell types, presumably by interacting with cell surface binding sites (10, 11). We show here that GrB binds and enters the target cell independently of PFP. The pore-forming protein, however, functions by facilitating egress of bound GrB to the cytosol, where the protease induces apoptosis in an unassisted manner. In support of this concept, we show that a replication-deficient adenovirus (AD) can efficiently transport GrB into targets that bind both the granzyme and virus, resulting in apoptosis.

MATERIALS AND METHODS

Cell Lines

Jurkat cells were maintained in RPMI 1640, 10% heat-inactivated fetal calf serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin.

Reagents

Human GrB was purified to homogeneity (12). Human PFP was extracted in 1 M NaCl from YT granules and purified by ion metal affinity chromatography using imidazole gradient in 10% betaine (sheep RBC assay (13)) were concentrated by Centricon ultrafiltration and stored at 4°C in 2 mM EDTA and 0.1% fatty acid free bovine serum albumin until use. A nonreplicating strain of adenovirus 2 was cultured and isolated as described (14). Carbobenzyo-DEVD-fluoromethylketone (DEVDFMK) (15) was supplied by Kamiya Bio-chemical Co. (Spokane WA).

Apoptosis Assays

FITC-TUNEL and Propidium Iodide Reactivity—Target cells (10⁶/ml) were pulsed with isolated GrB (in RPMI, 0.05% bovine serum albumin) in microcentrifuge tubes for designated time. After four

transferase labeling of DNA strand breaks with FITC-dUTP, DEVD-FMK, carbobenzyo-DEVD-fluoromethylketone.

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Lymphocyte granule-mediated apoptosis is postulated to entail the formation of membrane pores by perforin. Then soluble granzyme reaches the cytosol either through these pores or by reparative pinocytosis. We demonstrate here that Jurkat cells bind and internalize granzyme B via high affinity binding sites without toxic consequence. Apoptosis occurs, however, if sublytic perforin is added to targets washed free of soluble granzyme B. We suggest that granule-mediated apoptosis mimics viral strategies for cellular entry. Accordingly, co-internalization of granzyme B with adenovirus, a virus that escapes endosomes to reach the cytosol, also induced apoptosis. Poly(A) ribose polymerase cleavage and processing of CPP32, ICE-LAP3, and Mch2 were detected at 30 min, while cytosolic acidification and DNA fragmentation occurred at 60 min. Annexin V binding and membrane permeabilization arose at 4 h. The concurrent activation of the Ced-3 proteases differed from the rate at which each cytostatic protease is cleaved in vitro by granzyme B. Thus, granzyme B may not directly process these proteases in whole cells but rather may function by activating a more proximal enzyme. These results indicate that adenovirus-mediated delivery of granzyme B is suitable for elucidating biochemical events that accompany granule-mediated apoptosis.
Granzyme B-mediated Apoptosis in Whole Cells

washes, targets were resuspended in RPMI supplemented with 2 mM CaCl2 and PFF were added dropwise to the mixture. Sublytic concentration of PFF defined as less than 10% propidium iodide staining in PFF-treated controls. Alternately, target cells were treated with isolated GrB and adenovirus for the times indicated. Death was measured by terminal deoxyribonucleotidyl transferase-catalyzed labeling of DNA strand breaks with FITC-UDTP (FITC-TUNEL) (16) and/or propidium iodide (PI) staining followed by flow cytometry. Data acquisition consisted of 10,000 events analysis on the Coulter Epics V; histograms show cell count on the y axis and log scale of fluorescence intensity on the x axis. The original histograms were scanned with an HP III scanner interfaced with a Macintosh LC475 computer and converted to PICT files for display.

FITC-Annexin V Binding—Inversion of phosphatidylserine on apoptotic plasma membrane was determined by FITC-annexin V binding and flow cytometry (Kamiya) (17).

Analysis of CPP32, ICE-LAP3, and McIh2 Activation in Cell Lysates by Western Blotting—Detection of CPP32, ICE-LAP3, and McIh2 activation was performed as described previously (18–20). Treated cells were lysed and resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Anti-CPP32, anti-ICE-LAP3, and McIh2 rabbit antiserum were used at a dilution of 1:1000 followed by incubation with anti-rabbit Ig-horseradish peroxidase (Amersham Corp.) at a dilution of 1:10,000. Signal was visualized with the ECL kit (Amersham).

Analysis of PARP Proteolysis in Cell Extracts by Western Blotting—After treatment, cells were suspended in loading buffer, and Western blotting of whole PARP and fragments was performed as described previously (21). Total cell extracts were resolved on 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. PARP and its fragments were identified by probing with mAb C-2-10 (1:10,000) followed by peroxidase-conjugated anti-mouse IgG (1:2,500) and detection by the ECL kit (DuPont NEN). Used to detect full human PARP and its proteolytic fragments, anti-PARP monoclonal antibody (1:10,000) followed by peroxidase-conjugated anti-mouse IgG (1:2,500) was used to detect full human PARP. Blotting of whole PARP and fragments was performed as described previously (23).

Radioligand Binding Assay
GrB was labeled by the IODO-BEAD technique and separated from free 125I by cation exchange fast protein liquid chromatography. Active labeled protease (0.12 μg/μl) was determined by reduced SDS-polyacrylamide gel electrophoresis, Coomassie stain, densitometry, and measurement of esterolytic activity. More than 97% of 125I was incorporated into GrB. The binding assay was performed with Jurkat cells (106/ml) in microcentrifuge tubes preblocked with 1% bovine serum albumin at the concentrations and times indicated. Four wash steps with PBS (1 ml) were necessary to ensure removal of unbound radiolabel. The cells were transferred to fresh blocked tubes, treated with trypsin (100 μg/106/ml) for 10 min at room temperature, and washed with phosphate-buffered 0.5 M NaCl (1 ml). With this approach, coefficients of variation did not exceed 12%. cpm released by trypsin treatment represents membrane-associated GrB. cpm of pelleted cells, obtained by cutting the tip of the tubes, represents internalized granzyme.

RESULTS
To determine whether soluble GrB was essential for apoptosis, Jurkat cells were analyzed after sequential treatment with granzyme and PFF. Since single- and double-stranded DNA breaks occur in apoptotic Jurkat cells (24), FITC-TUNEL/flow cytometry was used to detect both forms of DNA damage (16). Jurkat cells (human T cell lymphoma) pulsed with GrB (30 nM) for 1 h, washed, and then exposed to PFP for 4 h underwent apoptosis (Fig. 1A). Under these conditions, catalytically inactive GrB did not induce cell death (data not shown). To determine the time after pulse when Jurkat cells remained susceptible to apoptosis, the cells were treated with GrB (60 nM) for 15 min, washed, rested for 1, 2, and 3 h, washed again to remove any released GrB, and then exposed to PFP. Three hours after the removal of soluble GrB, the Jurkat cells still underwent DNA strand breakage when exposed to sublytic PFP (Fig. 1B). Next, FITC-TUNEL reactivity and PI staining were performed in a kinetic study to assess the onset of nuclear and membrane damage. DNA strand breakage, as well as increased PI staining, were not observed until 4 h after the addition of PFP (Fig. 1C). These results demonstrate that the addition of sublytic PFP to targets pulsed and washed free of soluble GrB is sufficient for apoptosis.

Preincubation of targets with GrB would be sufficient for the protease to bind and enter the target by receptor-dependent endocytosis or, due to intrinsic cationicity, by nonspecific adsorptive endocytosis. Therefore, experiments were designed to determine the number of binding sites and to quantify the amount of active granzyme internalized by the Jurkat cells (Fig. 2). Specific binding sites were measured with proteolytically active 125I-GrB bound in the presence and absence of a...
FIG. 2. Granzyme B binding to Jurkat is specific and saturable. A, Jurkat cells have specific binding sites for GrB. Specific binding of GrB to Jurkat cells (10⁶/100 μl) was determined by measuring the membrane-associated cpm in the presence and absence of a 150-fold excess of unlabeled GrB. After adding either medium or unlabeled GrB for 30 min at 4°C, cells were washed and incubated at room temperature with radiolabeled GrB for 1 h. cpm released by trypsin treatment represents bound or membrane-associated GrB. cpm of pelleted cells, obtained by cutting the tip of the microcentrifuge tubes, represents endocytosed granzyme (see "Materials and Methods"). Specific binding (●—●), total binding (○—○), and nonspecific binding in the presence of a 150-fold excess of GrB (□—□) are shown. Data represent one of four experiments. B, kinetics of binding and endocytosis of 125I-GrB to Jurkat cells at 37°C. Jurkat cells were incubated with 125I-GrB (60 nM) for the times indicated at 37°C (mean ± S.D., n = 2). Membrane-bound GrB (●—●) and internalized GrB (○—○) are shown. The quantity of GrB that bound to the cell membrane over time was similar at 4°C, and incubation of cells at 4°C greatly reduced internalization (data not shown). C, inhibition of the endocytosis of 125I-GrB by unlabeled GrB. The experiment was performed as described in B. The internalization of GrB by Jurkat cells (10⁷/100 μl) was determined by measuring internalized cpm in the presence and absence of 150-fold excess of unlabeled granzyme. After adding either medium or unlabeled GrB for 30 min at 4°C, cells were incubated at room temperature with radiolabeled GrB. Internalized 125I-GrB in the absence of unlabeled GrB (●—●) and internalized 125I-GrB in the presence of a 150-fold excess of GrB (○—○) are shown. D, correlation of bound and internalized GrB in targets with the percentage of FITC-TUNEL-positive cells after a pulse-chase incubation with GrB. Jurkat cells were pulsed with 125I-GrB (60 nM) for 15 min, washed, and cultured as described in the Fig. 1B legend, except cells were at 10⁶/100 μl. Bound and internalized GrB was determined at the defined times as described in Fig. 2 with the following changes. Cells were washed 3 times with RPMI, 0.5% bovine serum albumin and then with PBS. Bound and internalized GrB are reported as mol/10⁶ target cells. For comparison, apoptosis (percentage of FITC-TUNEL-positive cells (●—●)) at these time points is shown for data typical for Fig. 1B.

150-fold excess of unlabeled protease. Granzyme binding and internalization were time- and concentration-dependent as well as specific and saturable (Kₘ = 10 nM) (Fig. 2, A and B). The calculated number of binding sites was 3 × 10⁴/cell. Unlabeled protease blocked uptake of 125I-GrB, indicating that the identified binding sites participate in granzyme internalization (Fig. 2C). A concentration-dependent increase in apoptosis was noted when targets were pulsed with the granzyme at values ranging from 3 to 90 nM for 1 h and then treated with PFP (data not shown).

As described above, the addition of PFP to targets 1, 2, and

3 h after a GrB pulse resulted in apoptosis (Fig. 1B). If cell death depends on the presence of membrane-bound GrB, such binding should be detectable at these time points. Radiolabeling experiments showed that membrane-associated GrB fell by approximately 50% over the 3 h, while the level of FITC-TUNEL-positive cells remained constant (Fig. 2D). This suggests that sufficient membrane-bound granzyme was present for an apoptotic response after treatment with PFP. The total cell-associated GrB (membrane plus internalized) also decreased over time, indicating that GrB was rereleased to the medium from an endosomal pathway. Supernatants obtained by washing the cells immediately prior to the addition of PFP at each time point demonstrated externalization of the radiolabel (data not shown). Importantly, despite a final wash step to remove the rereleased GrB, the target cells were still susceptible to apoptosis. Therefore, it is unlikely that PFP-mediated pinocytosis of rereleased GrB is responsible for the observed cell death. The more plausible explanation is that PFP contributes to apoptosis by facilitating entry of the membrane-bound GrB to the cytosol.

If binding of GrB to the target cell membrane is an essential step that precedes entry to the cytosol, identifying an agent that could substitute for PFP should also induce apoptosis. Tactics used by viruses to penetrate nucleated cells involve adherence to membrane receptors followed by internalization and release from endosomes by membrane fusion or viral protease-mediated damage (25, 26). We speculated that granule-mediated apoptosis mimics these viral strategies. AD can deliver protein to the cytoplasm if the cell has specific binding sites for both the virus and designated protein ligand (14). Jurkat cells were preincubated with GrB for 1 h, washed and then exposed to AD (200 pfu/cell). Apoptotic cells were readily detectable at 2 h (Fig. 3), while treatment with AD alone was harmless. Importantly, there was a concentration-dependent increase in apoptosis at values comparable with the calculated Kₘ for the specific binding of GrB to the targets. A minimum of 50 pfu/cell of AD was sufficient to induce apoptosis with GrB (data not shown). The observation that AD delivers GrB to the cytosol, resulting in apoptosis, offers additional support for the existence of a specific binding site for the granzyme on Jurkat cells (14).

Using this new model of cytotoxic cell granule-mediated apoptosis, we then performed experiments designed to detect the onset of plasma membrane, nuclear, and cytosolic changes associated with apoptosis by measuring PI staining, FITC-annexin binding, FITC-TUNEL reactivity, and intracellular pH. Treatment of Jurkat cells with GrB (60 nM) and AD (200 pfu/cell) resulted in extremely rapid, reproducible induction of
apoptosis. TUNEL reactivity was first noted at 60 min and reached a plateau at 120 min (Fig. 4A). Although the percentage of cells with DNA strand breaks did not increase at later time points, fluorescence intensified, reflecting the increased DNA fragmentation per cell. Coinciding with these nuclear changes, acidification of the cell cytosol was also first observed at 60 min (Fig. 4B). Plasma membrane alterations, on the other hand, occurred relatively late. Significant increases in membrane permeability and phosphatidylserine externalization were not observed until 4 h after the onset of DNA fragmentation (Fig. 4A).

GrB has been reported to mediate proteolytic activation of CPP32, ICE-LAP3, and Mch2 in vitro (18, 20, 27). Therefore, we determined whether in this whole cell system the Ced-3-like proteases were processed to their active form using antibodies raised against the large subunits of CPP32, ICE-LAP3, and Mch2. The appearance of the large subunit, indicative of a processed active protease, was first detected at 30 min for each death protease (Fig. 5A–C; lane 3). Interestingly, the pattern of cleavage of ICE-LAP3 differed in vivo and in vitro. GrB directly cleaves ICE-LAP3 between the large and small subunits in vitro, yielding the detectable prolarge subunit (27, 28). A relatively small amount of this intermediate was detected in extracts of cells incubated with GrB, regardless of the presence or absence of AD (Fig. 5B, lanes 1–7). However, induction of apoptosis was associated with an initial cleavage of ICE-LAP3 between the propeptide domain and the large subunit producing the Δ-pro intermediate (Fig. 5B, lanes 1–5), which is the same intermediate observed in anti-Apo-1-mediated programmed cell death. These results suggest that GrB does not directly cleave ICE-LAP3 in vivo, but rather that processing of ICE-LAP3 occurs through an intermediate ICE-like protease.

GrB is postulated to activate Ced-3-like cysteine proteases, which in turn cleave structural and regulatory proteins, producing an apoptotic morphology. One common biochemical characteristic observed during the induction of numerous forms of apoptosis is cleavage of PARP to the signature 89-kDa fragment (29). Exposure of targets to GrB/AD also resulted in PARP degradation to the 89-kDa fragment. Evidence of PARP cleavage was first noted at 30 min and continued to increase at 60 min (Fig. 6A).

The oligopeptide inhibitor DEVD-FMK binds with high affinity to CPP32 and inhibits apoptosis in many systems (15, 30). Experiments were performed to determine if the inhibitor also abrogated apoptosis measured by FITC-TUNEL and PARP degradation in GrB/AD-treated cells. Jurkat cells were treated with DEVDFMK (40 μM), a concentration that completely inhibits anti-Apo-1-mediated apoptosis. After exposure to GrB and AD, PARP cleavage and DNA fragmentation were assessed at 60 and 120 min, respectively. Although DNA strand breakage was completely prevented by DEVDFMK (Fig. 6B), PARP proteolysis was only partially reduced (Fig. 6A, lane 13). Importantly, when the cells were visualized by Hoechst stain, nuclear condensation was still detectable in DEVDFMK-treated cells despite the absence of DNA strand breakage (Fig. 6C).

**DISCUSSION**

The goals of this study were 1) to determine if membrane bound GrB could induce apoptosis in the presence of sublytic concentrations of PFP, 2) to determine whether the delivery of GrB into the cytosol of targets in the absence of PFP is sufficient to induce apoptosis, and 3) to provide a comprehensive description of the biophysical events that occur in this form of cell death.

The presence of specific binding sites for GrB on Jurkat cells endows specificity to a cytotoxic process that has been considered indiscriminate. Furthermore, we have shown for the first time that AD-mediated transfection of GrB protein is sufficient to cause cell death, implying that the sole function of PFP is to effect cytoplasmic delivery of the granzyme. We propose that granule-mediated apoptosis represents the vertebrate counterpart of strategies utilized by viruses to enter nucleated cells. After interaction with specific binding sites, GrB is endocytosed with plasma membrane that contains sublytic pores generated by PFP. The altered structural integrity of the vesicle then results in the escape of GrB to the cytosol, where the protease cleaves nuclear and cytosolic substrates activating the death pathway.

Replication-deficient AD has been used previously to introduce protein ligands into cells (14). Following interaction with their respective binding sites, the protein and AD are co-internalized into the endosomal compartment. The acidic environment then facilitates egress of AD and protein to the cytosol. Therefore, to enable the effective delivery of GrB, the target cell...
must have binding sites for both the protease and AD. U937 cells have binding sites for GrB but not for AD; therefore, unlike the results observed with Jurkat, U937 are not damaged by exposure to AD and granzyme (data not shown). Incubation of U937 with GrB and another virus, vesicular stomatitis virus, however, resulted in apoptosis. Taken together, GrB can be delivered to the cytosol of any cell that has binding sites for the granzyme and the designated virus. More importantly, GrB could induce apoptosis in cells undergoing infection with cytopathic viruses without the assistance of PFP. These results suggest that GrB may have an unforeseen role in host defense against viral infections.

We have detailed here for the first time the relative order in which biophysical changes of apoptosis occur in whole cells undergoing granule-mediated cell death. CPP32, ICE-LAP3, and Mch2 activation and PARP proteolysis were detectable within 30 min. The onset of DNA strand cleavage and cytosolic acidification were both apparent at 60 min. Annexin V binding and increased membrane permeability, on the other hand, lagged behind these changes occurring at 4 h. The biological relevance of the GrB/AD model is supported by the observation that similar biochemical changes were observed when Jurkat cells were exposed to GrB and PFP. Under both conditions, extracts of Jurkat cells contained the 89-kDa apoptotic fragment of PARP, and ICE-LAP3 displayed a similar pattern of proteolysis. Taken together, the results indicate that AD effectively replaces PFP to study granule-mediated apoptosis in whole cells.

A recent study has examined annexin V binding to targets exposed to a variety of apoptotic stimuli and found that the externalization of the phosphatidylserine leaflet preceded the onset of morphologic changes in the nucleus. Compared with the onset of intracellular biochemical changes measured here, annexin binding is a relatively late apoptotic event. The discrepancy between these studies may reflect the fact that the delivery of GrB bypasses the usual transmembrane signaling step observed in other apoptotic systems (i.e. Apo-1, tumor necrosis factor) and that a sensitive technique was used to measure nuclear damage (FITC-TUNEL).

Another insight into the apoptotic process mediated by GrB was the observed coincident appearance of cytosolic acidifica-

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3 G. Hommel-Berrey, M. R. Bochan, A. H. Montel, W. Goebel, C. J. Froelich, and Z. Brahmi, submitted for publication.

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tion and DNA strand breakage. Treatment of Jurkat cells with anti-Apo-1 antibody, cycloheximide, or short wavelength UV light resulted in acidification of the cytosol prior to the onset of apoptotic morphology (23). Acidification of the cytosol may serve as a global switch that facilitates the activity of enzymes that dismantle the cell during apoptosis (e.g., DNase II). Cytoplasmic alkalization in cells treated with anti-APO-1 antibody retards the apoptotic response, and inhibition of the ICE/Ced-3 pathway prevents acidification (32). It will be instructive to learn whether alkalization also overcomes GrB-induced apoptosis and to identify potential links between the ICE/Ced-3 pathway and biochemical steps that result in this particular apoptotic response.

GrB has been postulated to trigger the death cascade by directly activating the Ced-3-like family of cysteine proteases. Although isolated GrB is able to cleave the zymogens to their active forms in cell-free systems (18, 20, 27, 33, 34), the evidence that Ced-3-like proteases are activated in targets treated with GrB is largely untested (27, 35). Further, it is not known whether GrB directly or indirectly activates these proteases. In our model of granule-mediated apoptosis, the results demonstrate that delivery of GrB is coupled to the apparent proteolytic activation of CPP32, ICE-LAP3, and Mch2. Penetration of AD to the cytosol occurs with a half-time of 15 min (36); therefore, the detection of biochemical changes at 30 min indicates that the GrB efficiently initiates the apoptotic cascade after entering the cytosol. The concurrent activation of the three Ced-3-like proteases in GrB/AD-treated Jurkat cells differs, however, from the results obtained when isolated proteases are incubated with the granzyme. First, ICE-LAP3 was processed to the active large and small subunits in 10 min in vitro, whereas CPP32 and Mch2 were processed in 1 and 3 h, respectively.

Second, ICE-LAP3 was processed through different intermediates in vivo and in vitro. Finally, the observed processing of ICE-LAP3 is not unique to GrB/AD-treated target cells because a similar pattern was observed in Jurkat cells exposed to GrB and PFP (27) and to Apo-1 antibody (37). Therefore, although GrB, based on its preference for Asp residues, would be predicted to cleave all of these ICE/Ced-3-like proteases in vivo, the results suggest that GrB preferentially cleaves a protease upstream, which then activates the ICE/Ced-3 proteases.

Taken together, we postulate that the rapid induction of apoptosis by GrB would necessitate that the granzyme most efficiently activate the death protease situated at the apex of the cascade. In this regard, we predict that GrB will preferentially activate the FADD-like ICE (FLICE, MACH), the apical protease identified in the ICE/Ced-3 cascade (38–40). Inasmuch as the recently described FLICE 1/Mch4 is the point of convergence of the Apo-1 and tumor necrosis factor receptor death pathways, it is very tempting to speculate that GrB would also operate at this juncture and thereby provide an economical confluence of three distinct forms of immune-mediated apoptosis. In support of this concept, GrB has been reported to activate FLICE 1 in vitro (38, 40). Nevertheless, the apparent polyspecificity of GrB toward multiple Ced-ICE/3-proteases also warrants comment. In this context, a transformed cell might contain a mutation or acquire a viral inhibitor that incapacitates a proximal apoptotic protease. Despite the perturbation, GrB could induce apoptosis by activating more distal proteases. Thus, GrB appears to represent an excellent defense to prevent apoptosis of transformed cells.

The oligopeptide inhibitor, DEVD, blocks apoptosis induced by multiple stimuli (15, 30). Although DEVD-FMK (40 μM) prevented DNA strand breakage in GrB/AD-treated Jurkat cells at 2 h, both cleavage of PARP to the signature 89-kDa apoptotic fragment and nuclear condensation were not blocked. Anti-Apo-1-mediated apoptosis, on the other hand, was completely inhibited by this concentration of DEVD-FMK (data not shown). Every member of the Ced-3-like subfamily undoubtedly cleaves a particular protein substrate with unique efficiency. Activated CPP32 and ICE-LAP3 rapidly cleave PARP in cell-free experiments, while Mch2 cleaves lamin, resulting in nuclear condensation (20, 41). The Kₐ of DEVD-FMK for CPP32 is less than 1 nM (15).

Based on the amino acid sequence in the substrate binding pocket, it can be predicted that the inhibitor has decreasing affinity for ICE-LAP3 and Mch2, respectively. Therefore, the ability of DEVD-FMK to inhibit DNA strand breakage but not nuclear condensation and PARP cleavage suggests that Mch2 may remain active in the GrB/AD-treated cells. These unexpected results suggest GrB/AD-induced apoptosis is an ideal in vivo system for addressing the specific effector functions of Ced-3-like proteases.

In summary, the results show that PFP is not necessary for GrB to interact and enter the target cell. The pore-forming protein is absolutely essential, however, for the protease to reach the cytoplasm and induce apoptosis. In lieu of PFP, AD effectively delivers GrB to Jurkat cells inducing apoptosis. This experimental system will be useful to dissect the role of GrB as well as other granzymes in granule-mediated apoptosis. Finally, these studies raise the possibility that chimeras consisting of GrB and a molecule that targets the protease specifically to tumor cells could provide an alternate approach for the treatment of malignancies.

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Granzyme B-mediated Apoptosis in Whole Cells

29079

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