

Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis

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Granzyme B (GrB), acting similar to an apical caspase, efficiently activates a proteolytic cascade after intracellular delivery by perforin. Studies here were designed to learn whether the physiologic effector, GrB–serglycin, initiates apoptosis primarily through caspase-3 or through BH3-only proteins with subsequent mitochondrial permeabilization and apoptosis. Using four separate cell lines that were either genetically lacking the zymogen or rendered deficient in active caspase-3, we measured apoptotic indices within whole cells (active caspase-3, mitochondrial depolarization [$\Delta\Psi_m$] and TUNEL). Adhering to these conditions, the following were observed in targets after GrB delivery: (a) procaspase-3–deficient cells fail to display a reduced $\Delta\Psi_m$ and DNA

fragmentation; (b) Bax/Bak is required for optimal $\Delta\Psi_m$ reduction, caspase-3 activation, and DNA fragmentation, whereas BID cleavage is undetected by immunoblot; (c) Bcl-2 inhibits GrB-mediated apoptosis (reduced $\Delta\Psi_m$ and TUNEL reactivity) by blocking oligomerization of caspase-3; and (d) in procaspase-3–deficient cells a mitochondrial-independent pathway was identified which involved procaspase-7 activation, PARP cleavage, and nuclear condensation. The data therefore support the existence of a fully implemented apoptotic pathway initiated by GrB, propagated by caspase-3, and perpetuated by a mitochondrial amplification loop but also emphasize the presence of an ancillary caspase-dependent, mitochondria-independent pathway.

Introduction

Cytotoxic T lymphocytes and natural killer (NK)* cells induce apoptosis in target cells by two mechanisms: engagement of the Fas (CD95) receptor on target cells and delivery of the cytotoxic granule constituents. Ligation of the Fas receptor recruits a signaling complex to the inner leaflet of the target cell membrane resulting in the processing of procaspase-8. The granule secretion pathway appears to require the direct intracellular delivery of a family of granule-associated serine proteases, granzymes, which activate caspase-dependent and -independent death programs to ensure target cell demise.

Among the granule proteases, granzyme B (GrB) serves as a model to understand how intracellular delivery of a protease causes cell death (Barry and Bleackley, 2002). GrB shares substrate specificity with caspases for cleavage after aspartate

residues (Poe et al., 1991) and has been reported to process numerous caspases *in vitro* including caspase-3, -6, -7, -8 and -10 (Darmon et al., 1995; Chinnaiyan et al., 1996; Duan et al., 1996; Talanian et al., 1997). The results have led to the notion that the protease initiates death by processing any number of caspases *in vivo* (Medema et al., 1997; Barry et al., 2000). We have learned, however, that GrB, due to the constraints of accessibility and rates of proteolysis proceeds efficiently *in vivo* to first process caspase-3, which along with GrB then matures caspase-7 (Yang et al., 1998). On this basis, we have come to view GrB as apical caspase-like in function. We have nonetheless speculated that GrB might also have the capacity to initiate alternate death pathways if the caspase cascade is paralyzed, for example, by viral inhibitors (Talanian et al., 1997). Supporting this concept, GrB appears to process certain caspase substrates including PARP, NuMA, DNA-PK (Andrade et al., 1998), and DFF45/inhibitor of caspase-activated deoxyribonuclease (ICAD) (Thomas et al., 2000; Sharif-Askari et al., 2001) and thus might cause cell death independently of the caspases. In contradistinction to the activation of a proteolytic cascade by an intracellularly delivered protease, GrB has been reported to induce death through a mitochondria-

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*Abbreviations used in this paper: AD, adenovirus; cyt c, cytochrome c; GrB, granzyme B; ICAD, inhibitor of caspase-activated deoxyribonuclease; MEF, murine embryonic fibroblast; NK, natural killer; PFN, perforin; PFU, plaque-forming unit; SG, serglycin; WT, wild type.

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centered pathway by cleaving the BH3-only proapoptotic Bcl-2 family member, Bid. Three groups have reported data linking rapid Bid proteolysis with mitochondrial permeabilization (MacDonald et al., 1999; Heibein et al., 2000; Sutton et al., 2000; Alimonti et al., 2001; Pinkoski et al., 2001), suggesting the granzyme induces death primarily through this pathway.

Mitochondrial membrane permeabilization may be mediated by cytosolic factors such as Bax/Bak which insert in the outer membrane. Alternately, changes in the mitochondrial permeability transition pore complex (Bernardi, 1999; Crompton, 2000b) may allow the release of intramembranous proteins and/or loss of membrane potential across the inner membrane ($\Delta\Psi_m$) (Bernardi, 1999; Crompton, 2000a; Loeffler and Kroemer, 2000). The outer mitochondrial membrane, responding to proapoptotic signals, becomes permeabilized and releases factors such as cytochrome c (cyt c), apoptosis-inducing factor (Joza et al., 2001; Ye et al., 2002), and most recently the serine protease HtrA2/Omi (Suzuki et al., 2001) and endonuclease G (Hengartner, 2001; Li et al., 2001). Among these paradigms, GrB-generated truncated Bid is predicted to oligomerize with proapoptotic family members, Bax and/or Bak, forming large ion channels in the outer mitochondrial membranes. The released cyt c then coalesces into an apoptosome with Apaf-1 and dATP facilitating activation of caspase-9, which in turn processes caspase-3 (Li et al., 1997; Zou et al., 1997). GrB also has been predicted to induce apoptosis by directly perturbing mitochondrial integrity through a Bid-independent pathway (Alimonti et al., 2001). Finally, using lines deficient in Bid, Bax, and/or Bak, investigators have shown GrB readily induces mitochondrial depolarization independently of cyt c release, permeability transition, and caspase activation (Thomas et al., 2001). Together the results imply the granzyme has a multifaceted potential to ensure target cell death by initiating several pathways: (a) caspase driven, (b) BH3-only protein driven, (c) directly "damaging" mitochondria, and (d) cleavage of crucial structural and regulatory proteins.

We have reported that GrB is exclusively secreted from cytotoxic cells as a macromolecular complex bound to chondroitin sulfate proteoglycan, serglycin (SG) (Metkar et al., 2002), and have provided the biophysical basis for this observation (Raja et al., 2002). The use of free GrB could lead potentially to anomalous binding of the granzyme to anionic membranes on the target cell surface and cytosolic proteins. As a consequence, we thought it would be instructive to reassess how the granzyme initiates death after intracellular delivery of the macromolecular complexes. Using techniques that examine apoptotic events *in situ*, we report here that GrB-SG initiates death predominantly through caspase-3, and mitochondria secondarily amplify this process.

Results

Adventitious processing of Bid in GrB-treated targets

GrB has been reported to cleave Bid initiating a mitochondrial apoptotic pathway. The experimental design which served as the basis for these observations involved immuno-

blotting cytosolic extracts from solubilized targets. Targets exposed to GrB contain protease localized to the endosomes and plasma membrane. Solubilization liberates sequestered granzyme, allowing adventitious cleavage of cytosolic/nuclear substrates which might otherwise be inaccessible during granzyme delivery by either perforin (PFN) or endosomolytic agents (Froelich et al., 1996a). Our intent was to confirm that GrB-mediated cleavage of Bid indeed occurred after intracellular delivery of the granzyme rather than during solubilization. Therefore, we evaluated the processing of this key proapoptotic component under conditions designed to inhibit the granzyme during solubilization (see Materials and methods) and, in parallel, examined whether the caspases might contribute to Bid cleavage. As shown in Fig. 1 A, the delivery of GrB-SG complexes to MCF-7_{wt} and MCF-7_{casp-3} cells did not produce the anticipated cleavage of Bid despite the assurance that adenovirus (AD) was capable of endosomolysis (see Materials and methods) and caspase-3 cleavage was observed by immunoblotting in MCF-7_{casp-3} cells (Fig. 1 B).

We then examined whether the removal of the inhibitor during target cell solubilization might result in BID cleavage (Yang et al., 1998). MCF-7_{vec} cells were exposed to GrB-SG complexes for 30 min at 37°C. The cells were washed, incubated in the presence or absence of inhibitor (IETD-CHO, 250 μ M) for 20 min, and then solubilized with buffer in the presence or absence of additional inhibitor. Blockade of GrB activity in the lysates exposed to IETD-CHO was verified using the chromogenic substrate, IETD-pNA (unpublished data). The lysates were prepared for electrophoresis and subsequent blotting followed by reaction with anti-Bid and anti-caspase-7 antibodies. The latter was chosen because it is not substantially processed in MCF-7_{wt} cells during short term assays (Yang et al., 1998) but is rapidly cleaved *in vitro* and thus might be processed despite efforts to minimize proteolysis during the solubilization step. Generation of lysates without the granzyme inhibitor resulted in almost an immediate and complete processing of both caspase-7 and Bid (Fig. 1 C). A similar outcome was also noted for Jurkat cells (unpublished data). Bid appears to be available for processing by GrB during target cell solubilization but remains sequestered from the granzyme in whole cells when proteolysis of the BH3 protein is evaluated by immunoblotting. To learn whether the inaccessibility of Bid is a general attribute of MCF-7 cells undergoing apoptosis, we then evaluated processing of the BH3 protein during death receptor-induced apoptosis. The MCF-7_{wt} and MCF-7_{casp-3} cells were treated with recombinant Trail and processing of Bid was evaluated. Unlike GrB-mediated killing, engagement of the TRAIL receptor resulted in the partial disappearance of Bid (Fig. 1 D), indicating that Bid is accessible to proteolysis during death receptor-mediated apoptosis.

Observing prominent adventitious processing in GrB-treated cells after solubilization, we speculated that a similar outcome might occur when lysates are generated after whole cell cytotoxicity assays. Using the NK-lymphoma cell line YT, which lacks detectable Bid as a surrogate effector and Jurkat cells as target, the cells were mixed at a 1:1 ratio

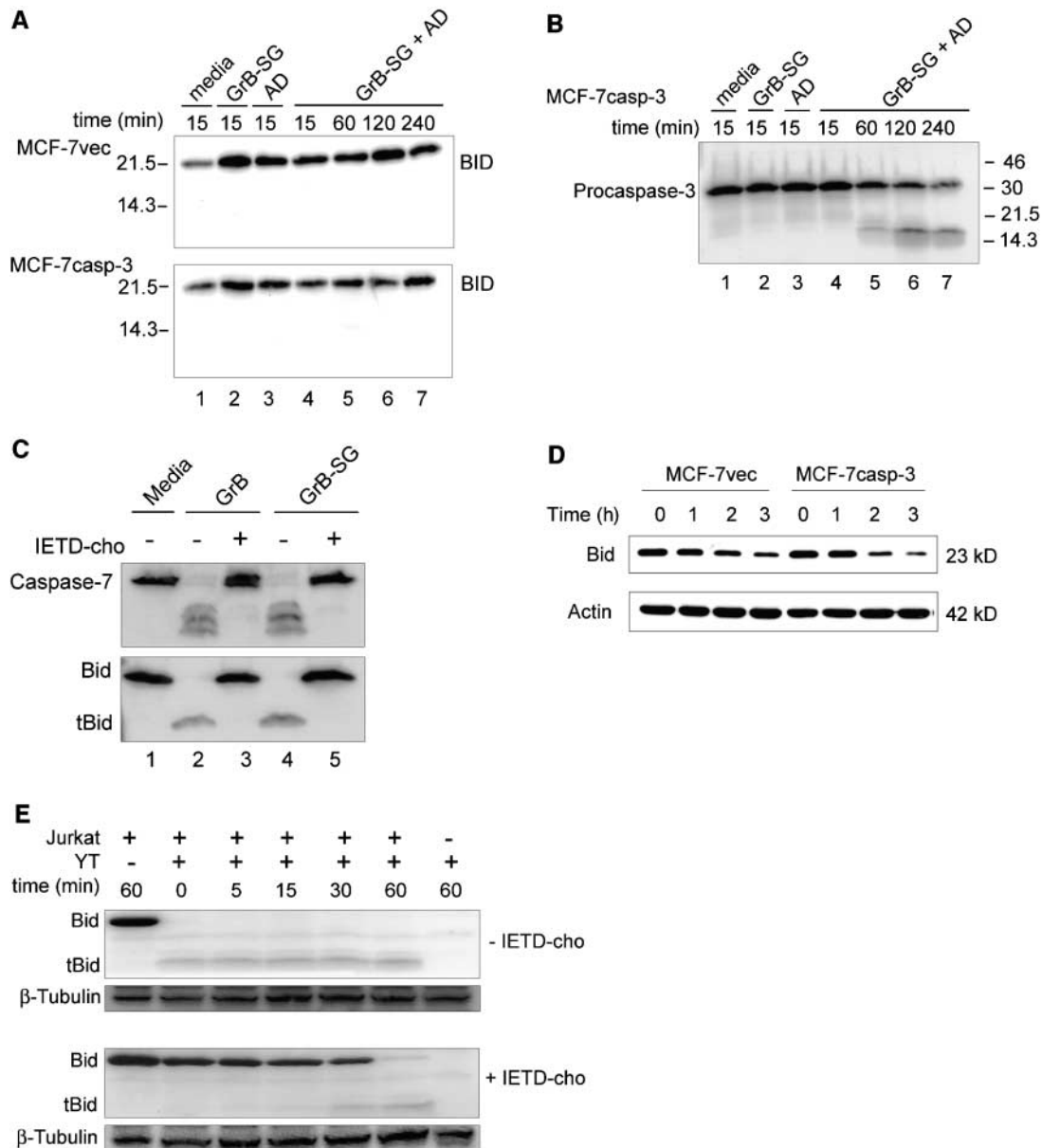


Figure 1. Processing of Bid after GrB delivery. (A) Bid is not cleaved in either MCF-7_{Vec} or MCF-7_{Casp-3} cells after GrB-SG delivery. MCF-7_{Vec} (top) or MCF-7_{Casp-3} cells (bottom) were treated as shown and probed for Bid cleavage. Cells were incubated as follows: media (lane 1), GrB-SG (lane 2), AD (lanes 3), or GrB-SG + AD for 15 min (lanes 4), 60 min (lanes 5), 120 min (lanes 6), or 240 min (lanes 7). Afterwards, cells were solubilized in the presence of IETD-cho as describe in Materials and methods, and the postnuclear lysates were run on 15% SDS-PAGE gels. Results are representative of one of three independent experiments. (B) Procaspase-3 is cleaved in MCF-7_{Casp-3} cells after GrB delivery. Cells were in media, GrB-SG, or AD for 15 min, or targets underwent delivery of GrB-SG for 15, 60, 120, or 240 min. The cells were solubilized in the presence of IETD-cho as described in Materials and methods and processed for immunoblotting with anti-caspase-3 antibody. Results are representative of one of three independent experiments. (C) MCF-7_{Vec} cells were incubated with media (lane 1), GrB (lanes 2 and 3), or GrB-SG (lanes 4 and 5) for 30 min at 37°C to allow endocytosis of the granzyme. The cells were then washed three times and incubated with PBS (lanes 2 and 4) or 250 μ M IETD-cho (lanes 3 and 5) for 20 min (37°C). Thereafter, cells in lanes 3 and 5 were solubilized in lysis buffer containing IETD-cho (250 μ M). The postnuclear lysates (10 μ g) were run on 15% SDS-PAGE gels, transferred, and probed with either anti-caspase-7 (top) or anti-Bid (bottom) antibodies. (D) Bid is cleaved after death receptor ligation in both MCF-7_{Vec} or MCF-7_{Casp-3} cells. Cells were treated with 200 ng/ml TRAIL for the times shown (1–3 h). Lysates (20 μ g) were separated by SDS-PAGE and immunoblotted with anti-Bid or antiactin antibody. (E) A model for artifactual processing of Bid during effector target cytotoxicity. One million YT NK lymphoma cells were used as effectors with Jurkat serving as targets (E:T = 1:1). The effectors and targets were mixed with either PBS (top) or IETD-cho (250 μ M) (bottom) as described in the legend to Fig. 2 B and then lysed in either the absence (top) or presence (bottom) of additional IETD-cho (250 μ M). The lysate was then allowed to stand for 0, 5, 15, 30, and 60 min at 37°C after which Laemmli buffer was added. The lysates (10 μ g) were then heated, loaded on a 15% SDS-PAGE, blotted, and probed with anti-Bid antibody. The first lane shows a Jurkat lysate control, whereas the last is the YT control. β -Tubulin served as a loading control.

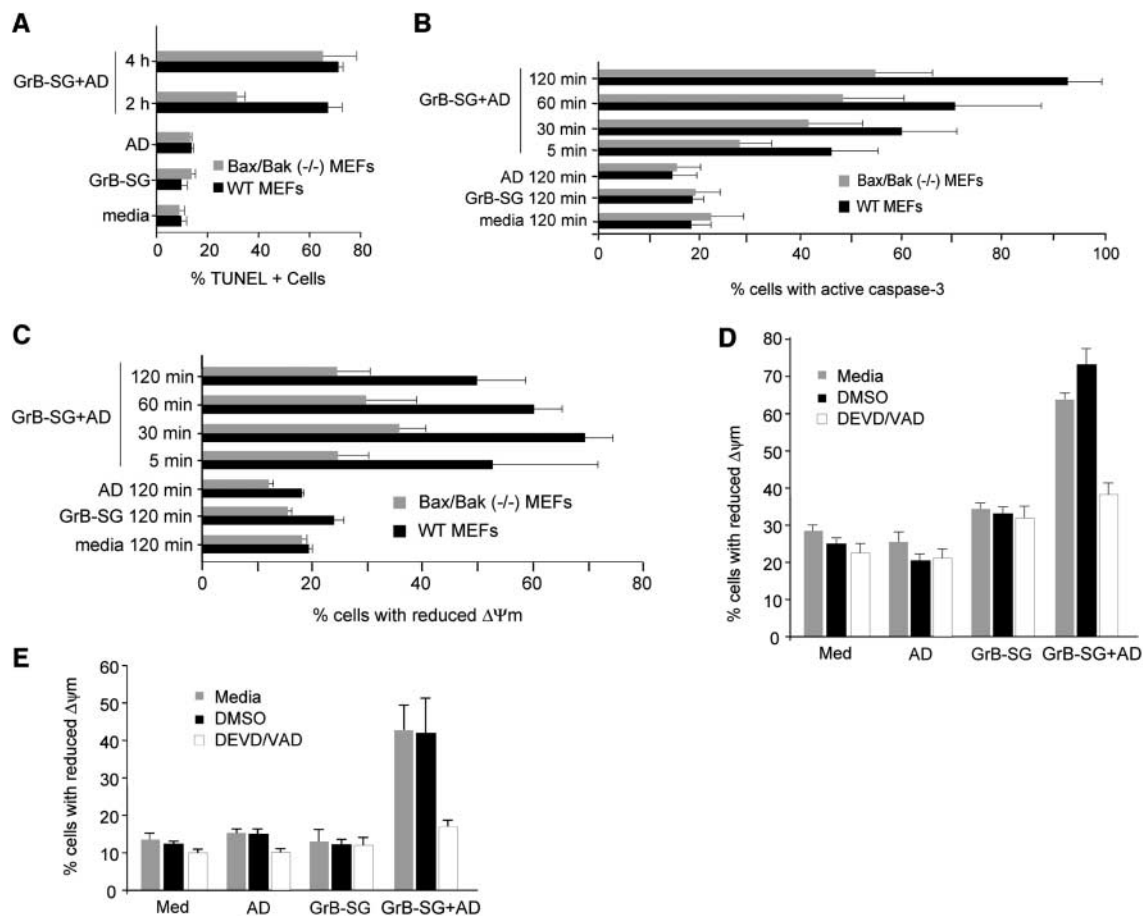


Figure 2. Bax and Bak are crucial for GrB-SG-associated mitochondrial depolarization. (A) WT and DKO (*Bax* × *Bak*^{-/-}) MEFs show comparable TUNEL staining during GrB-mediated apoptosis. Cells were treated with GrB-SG at 2 μ g/ml and AD for 4 h at 37°C and analyzed for TUNEL reactivity. Results represent mean values from three experiments. Similar results were obtained for delivery of free GrB (unpublished data). (B) DKO MEFs show a reduced elevation of caspase-3 activity compared with WT MEFs. Cells were treated as described in the legend to Fig. 2 A for 5–120 min, and the percentage of cells with active caspase-3 were measured using FAM-DEVD-FMK on a flow cytometer. Results are representative of the mean \pm SE of three experiments. (C) DKO MEFs show less mitochondrial potential loss than WT MEFs. Cells were treated as described in the legend to Fig. 2 A, and the percentage of loss in mitochondrial potential was determined with CMX-ROS dye and flow cytometry. Results are representative of mean \pm SE of three experiments. (D) DEVD-fmk and ZVAD-fmk inhibit mitochondrial potential loss in WT MEFs. WT MEFs were preincubated with media, DMSO, or DEVD-fmk and ZVAD-fmk (100 μ M) for 30 min at 37°C and then treated with GrB or GrB-SG + AD for 1 h, and the percentage of cells with a loss in mitochondrial potential was measured using the CMX-ROS dye and flow cytometry. Results are representative of the mean \pm SE of three experiments. (E) DEVD-fmk and ZVAD-fmk completely inhibit mitochondrial potential loss in DKO MEFs. DKO MEFs were preincubated with media, DMSO, or DEVD-fmk and ZVAD-fmk (100 μ M) for 30 min at 37°C and then treated with GrB or GrB-SG + AD for 4 h at 37°C followed by determination of cells with loss in mitochondrial potential. The percentage of cells with $\Delta\Psi_m$ is shown as the mean \pm SE of three experiments.

and solubilized in the presence and absence of IETD-cho. After incubation for various times (0–60 min), the lysates were blotted and probed with anti-Bid antisera. In the absence of the inhibitor, there was complete processing of Bid at the earliest measurable time (Fig. 1 E). In presence of IETD-cho, disappearance of the 22-kD Bid was observed but only after 60 min, a time when the effect of the inhibitor likely is exhausted. Together the results indicate that precautions are necessary to distinguish intracellular and postlytic proteolysis (i.e., adventitious processing) during GrB-induced apoptosis assays if treated targets require solubilization. In addition, the reported cleavage of Bid during GrB-mediated cell death appears to be due to adventitious proteolysis, suggesting the mechanism(s) responsible for granzyme-mediated mitochondrial disruption remains largely unexplained.

Bax/Bak (-/-) MEFs undergo less mitochondrial potential loss after GrB-SG delivery than WT MEFs

Although we failed to identify processed Bid in MCF-7 (*vec/casp3*) cells, mitochondrial disruption via a GrB-initiated mechanism could still occur through a Bax/Bak-dependent pathway (Wei et al., 2001) or independently of BH3 domain proteins (Alimonti et al., 2001; Thomas et al., 2001). To investigate whether Bax and Bak contributed to a granzyme-initiated apoptotic cascade, double negative (DKO) *Bax* × *Bak*^{-/-} murine embryonic fibroblasts (MEFs) were studied for their sensitivity to granzyme-mediated mitochondrial disruption and cell death.

To avoid adventitious processing and the difficulties inherent to interpreting the significance of cleaved proteins observed by immunoblot, we limited our examination for signs of cell death to intact cells. Furthermore, we observed

that the granzyme variably detached the MEFs and thereby might obscure the results by causing anoikis. To minimize this confounding variable, MEFs were proteolytically detached for the described treatment periods. Focusing first on the nuclear aspect of the apoptotic response, the Bax/Bak (-/-) MEFs showed a reduced level of DNA fragmentation by TUNEL staining at 2 h which was then comparable to wild type (WT) at 4 h (Fig. 2 A). After determining that Bax/Bak (-/-) and WT MEFs contained comparable levels of activatable procaspase-3 (unpublished data), kinetics of intracellular active caspase-3 formation and mitochondrial potential loss ($\Delta\Psi_m$) were investigated. The Bax/Bak (-/-) MEFs showed lower levels of active caspase-3 throughout the kinetic analysis (Fig. 2 B). Finally, the rate and level of mitochondrial potential loss was less in DKO than WT cells being imperceptible in one experiment (Fig. 2 C).

Previous investigations have suggested that a blockade in caspase activation does not prevent GrB-mediated mitochondrial disruption in a variety of cell lines and Bax/Bak (-/-) cells (Heibein et al., 2000; Sutton et al., 2000; Alimonti et al., 2001; Thomas et al., 2001). Efficacy of caspase inhibition has either relied on showing the designated inhibitor blocks cell death by another stimulus (e.g., death receptor ligation) (Sutton et al., 2000) or minimizes activation of protease activity measured spectrophotometrically in crude lysates (Thomas et al., 2001). The former approach may be invalid due to differences in the level of active caspase achieved in each experimental system, whereas the latter approach tends to underestimate the level of active caspase due to the nonspecific inhibitory effects of the complex protein content of the lysate (unpublished data). To circumvent the liabilities of these approaches, we used intact GrB-treated cells and the PhiPhiLux assay to determine the molar concentration of inhibitors that block caspase-3 activation in situ. For the MEFs, both the relatively caspase-3 specific inhibitor, DEVD-fmk, and the broad spectrum inhibitor, VAD-fmk (100 μ M), were necessary to ensure reproducible blockade (unpublished data). We then investigated whether caspases contributed to mitochondrial potential loss in the WT and Bax/Bak (-/-) MEFs undergoing granzyme delivery (Fig. 2, D and E). When either WT or Bax/Bak (-/-) MEFs were pretreated with these inhibitors and then subjected to granzyme delivery with AD, mitochondrial potential loss was no longer apparent (Fig. 2, D and E) and apoptosis, as defined by DNA strand breakage, was absent (unpublished data). The results therefore suggest that caspases mediate the mitochondrial depolarization during GrB-mediated apoptosis and this process is divisible into a BH3/Bax/Bak-dependent and BH3-independent pathway.

Caspase-3 is essential for mitochondrial potential loss and DNA fragmentation

The preceding results suggest that prevention of GrB-mediated caspase-3 activation protects against mitochondrial disruption and nuclear signs of apoptosis. However, due to the conflicting data surrounding this observation the results warranted verification in cells that absolutely lacked procaspase-3. To directly examine the role of caspase-3 in GrB-mediated mitochondrial dysfunction, we evaluated alter-

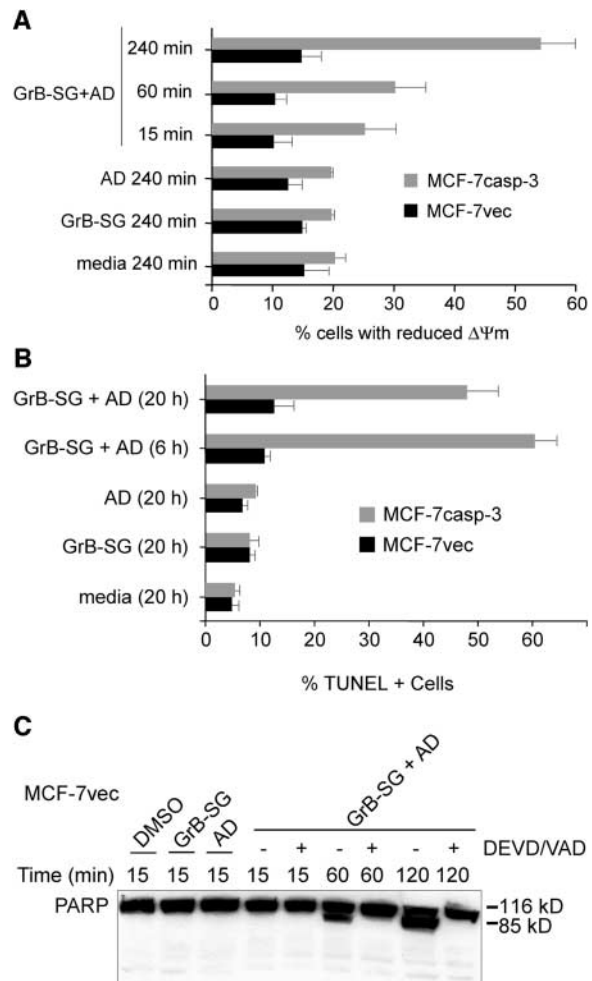


Figure 3. Caspase-3 is required for mitochondrial potential loss and DNA fragmentation. (A) Mitochondrial potential loss does not occur in MCF-7 cells lacking procaspase-3. MCF-7_{vec} and MCF-7_{casp-3} cells were treated with GrB-SG and AD for the indicated times, and mitochondrial potential loss was analyzed by CMX-ROS staining. Results are representative of the mean \pm SE of three experiments. Similar results were obtained for delivery of free GrB (unpublished data). (B) MCF-7 cells lacking caspase-3 do not undergo DNA fragmentation after GrB delivery. MCF-7_{vec}- or MCF-7_{casp-3}-transfected cells were treated as indicated for either 6 or 20 h at 37°C and analyzed for TUNEL reactivity. Results are representative of the mean \pm SE of three experiments. Similar results were obtained for delivery of free GrB (unpublished data). (C) PARP is cleaved in caspase-3-deficient cells after GrB-SG delivery. MCF-7_{vec} cells were subject to GrB-SG delivery in the presence and absence of ZVAD-fmk/DEVD-fmk (100 μ M) for the times indicated followed by solubilization and immunoblotting with anti-PARP antibody.

ations in MCF-7 cells lacking procaspase-3 (MCF-7_{vec}) and a stable transfectant expressing the zymogen (MCF-7_{casp-3}) (Yang et al., 1998). Despite the apparent absence of Bid cleavage in MCF_{casp-3} cells (Fig. 1 A), mitochondrial potential loss was clearly observed at 15 min in these cells, whereas $\Delta\Psi_m$ remained intact in MCF-7_{vec} cells lacking procaspase-3 (Fig. 3 A). The MCF-7_{vec} cells also failed to develop TUNEL reactivity for upwards to 20 h after GrB delivery, whereas MCF-7_{casp-3} cells displayed substantial DNA fragmentation at 4 h (Fig. 3 B). As expected, intracellular active

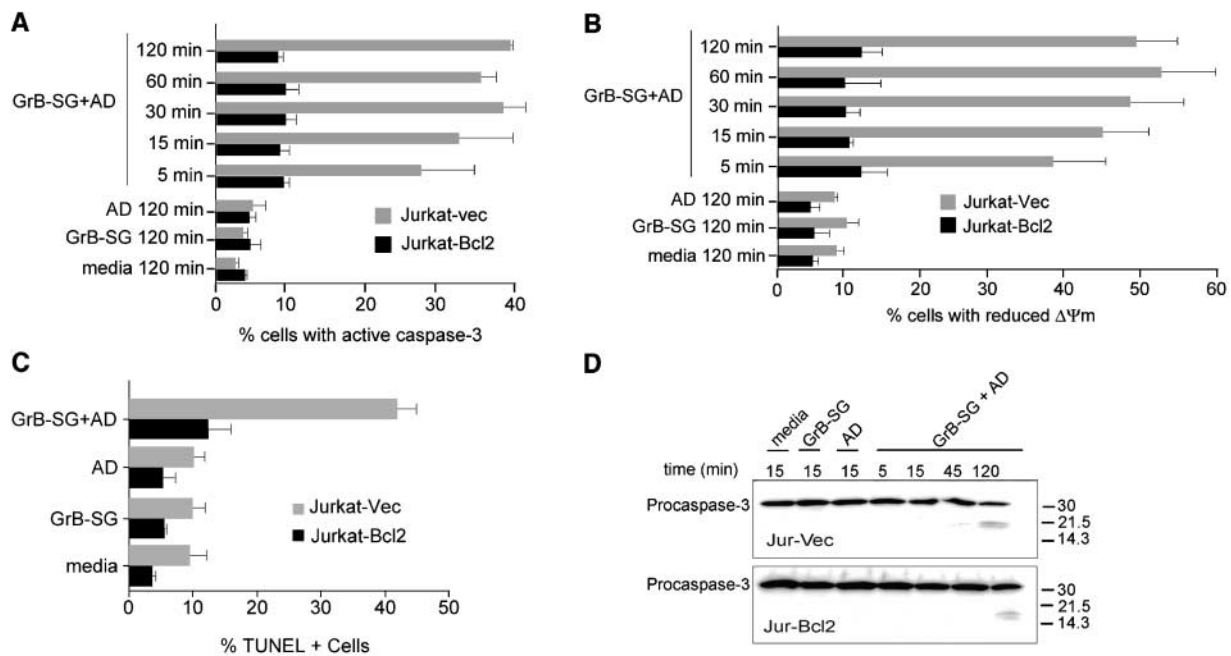


Figure 4. Resistance of Bcl-2-transfected Jurkat cells to GrB-SG is due to a block in caspase-3 activation. (A) Caspase-3 activation is blocked in Bcl-2-transfected Jurkat cells. Jurkat_{vec} or Jurkat_{Bcl-2} were treated with GrB-SG and AD for the indicated times (5–120 min), and the percentage cells with active caspase-3 enumerated by flow cytometry. Results are representative of the mean \pm SE of three experiments. Similar results were obtained for delivery of free GrB (unpublished data). (B) Mitochondrial potential loss is substantially inhibited in Jurkat cells overexpressing Bcl-2. Jurkat_{vec} or Jurkat_{Bcl-2} were treated with GrB-SG and AD for the indicated times (5–120 min), and the percentage of cells with reduction in mitochondrial potential was enumerated. Results are representative of the mean \pm SE of three experiments. Similar results were obtained for delivery of free GrB (unpublished data). (C) DNA fragmentation is significantly inhibited in Jurkat cells overexpressing Bcl-2. Cells were treated as described in Fig. 4 A for 4 h, fixed, and stained for TUNEL. The percentage of cells with increased TUNEL reactivity are shown. Results are representative of the mean \pm SE of three experiments. Similar results were obtained for delivery of free GrB (unpublished data). (D) Cleavage of caspase-3 in cells overexpressing Bcl-2. Jurkat_{vec} or Jurkat_{Bcl-2} were treated with media (lane 1), GrB-SG (lane 2), AD (lane 3), or GrB-SG + AD for 5 min (lane 4), 15 min (lane 5), 45 min (lane 6), or 120 min (lane 7) and lysed in the presence of IETD-cho. The postnuclear lysates were run on 15% SDS-PAGE gels and probed for caspase-3. Results are representative of one of two independent experiments.

caspase-3 was discernible from analysis of lysates within 15 min of GrB delivery in MCF_{casp-3} (unpublished data), whereas processed procaspase-3 was observed later by immunoblotting (60 min) (see above and Fig. 1 B).

The combined results suggest that active caspase-3 is responsible for mitochondrial potential loss during GrB-mediated apoptosis. However, the data do not exclude a role for caspase-7. On the basis of immunoblot analysis, caspase-3 acts jointly with GrB to rapidly process caspase-7 after granzyme delivery (Yang et al., 1998). Therefore, the absence of caspase-3 in MCF-7_{vec} cells could preclude effective activation of caspase-7 and thereby prevent the initiation of molecular events disrupting mitochondria. To determine the potential contribution of caspase-7, we devised the following strategy. As described above, MCF-7_{vec} cells fail to demonstrate a reduction in $\Delta\Psi_m$ after granzyme delivery. We asked whether active caspase-7 might be present in MCF-7_{vec} cells and if detected would indicate the caspase does not contribute to the detected mitochondrial disruption (Lassus et al., 2002). In the absence of a specific fluorogenic substrate for caspase-7, this issue was addressed indirectly by determining whether PARP, a substrate processed by caspase-7 (Germain et al., 1999), was cleaved to its signature apoptotic fragment in cells lacking procaspase-3. Indeed, PARP appeared to be processed in MCF-7_{vec} cells, an effect inhibited by VAD-

fmk/DEVD-fmk (Fig. 3 C). The observation thus suggests that sufficient procaspase-7 may be directly activated by GrB to cleave PARP but the caspase does not appear to contribute to mitochondrial potential loss.

Bcl-2 suppresses GrB-SG-mediated caspase-3 activation followed by mitochondrial membrane potential loss and DNA fragmentation

Among the findings reported in cells undergoing apoptosis in the presence of purified GrB, perhaps the most consistent observation is the capacity of Bcl-2 to prevent cell death. The results here show that GrB first activates caspase-3 and this or a related family member then contributes to the loss in mitochondrial membrane potential and DNA fragmentation. Since Bcl-2 has been reported to minimize activation of caspase-3 (Krebs et al., 1999), we asked whether Bcl-2 might inhibit GrB-associated mitochondrial depolarization by preventing the activation of caspase-3. Using a Jurkat cell line as a model, we examined how overexpression of Bcl-2 influenced mitochondrial membrane potential, intracellular caspase activation, and DNA fragmentation after granzyme delivery. As a prelude to these studies, we documented that recombinant Bcl-2 does not directly inhibit the enzymatic activity of either soluble GrB or active caspase-3 (unpublished data). Bcl-2-transfected Jurkat cells (Jurkat_{Bcl-2}) were

then found to show a substantial inhibition of caspase-3 activity compared with the control (Fig. 4 A); a result paralleled by the caspase-3 fluorogenic assay performed on cell lysates (unpublished data). In comparison, mitochondrial potential loss and DNA fragmentation were also markedly reduced in Jurkat_{Bcl-2} cells (Fig. 4, B and C). Finally, despite the virtual absence of activated caspase-3 in Bcl-2–transfected cells, processed procaspase-3 could be detected by immunoblotting (Fig. 4 D). Together the results reinforce the concept that mitochondria are undisturbed after granzyme delivery if the level of active caspase-3 is minimized and, Bcl-2, at least during GrB-induced killing, acts by preventing the activation of caspase-3.

Discussion

The studies performed here were designed to learn whether GrB–SG complexes initiate cell death pathways through the caspases as well as through the recently reported capacity of granzyme to cleave Bid with subsequent mitochondrial permeabilization. To this end, we used three separate cell lines that were either genetically lacking or rendered caspase-3 deficient and focused on measurements of apoptotic indices within whole cells (active caspase-3 levels, changes in $\Delta\Psi_m$ and TUNEL). Finally, when immunoblotting was necessary we used approaches that minimized adventitious proteolysis in solubilized granzyme-loaded cells. Adhering to these conditions, we observed the following in cells undergoing granzyme delivery: (a) Bid cleavage is not readily detected by immunoblotting; (b) the majority of the mitochondrial depolarization appears dependent on the presence of Bax/Bak, but DNA fragmentation may occur in the absence of the cell death amplification loop; (c) procaspase-3–deficient MCF-7 cells do not display a reduction in $\Delta\Psi_m$ or evidence of DNA fragmentation; and (d) Bcl-2 appears to inhibit GrB-mediated apoptosis, as measured by reduced $\Delta\Psi_m$ and TUNEL reactivity, by blocking activation of caspase-3. Finally, the majority of the data was generated with GrB–SG complexes as death effectors. Similar results were obtained for all experiments with free GrB (unpublished data), eliminating the possibility that the data was skewed by usage of the complexes.

Several laboratories have reported that GrB apparently cleaves Bid in situ; an event associated with the reduction in $\Delta\Psi_m$ and cyt C release. When performed with targets treated with caspase inhibitors, the combined result of Bid cleavage and persistent mitochondrial disruption has been interpreted to indicate the granzyme initiates death through a mitochondria-centered, caspase-independent pathway. We emphasize that the reliability of these interpretations are hampered by the lack of safeguards to block GrB-associated adventitious proteolysis which could yield spurious immunoblot and cyt c release data and to ensure caspases are inactivated in GrB-treated cells.

Solubilization of targets exposed to GrB release sequestered granzyme allowing cleavage of susceptible substrates. Among the proteins processed by GrB perhaps the two most readily cleaved are Bid and procaspase-7. We first recognized this problem of adventitious proteolysis during studies performed to delineate the sequence of caspases processed after

delivery of free GrB (Yang et al., 1998). In the absence of controls exposed to GrB alone and a strategy to inactivating the granzyme immediately before and during solubilization, immunoblot data that compared rates of processing of caspase-3 and -7 would be interpreted to indicate that caspase-7 is processed first after GrB delivery. Therefore, it is not surprising that Bid has been considered a key substrate through which GrB apparently activates a mitochondrial death pathway and that homogenates from GrB-treated cells contain released cyt c. Notably, a similar outcome may occur during whole cell cytotoxicity assays (Thomas et al., 2001) (Fig. 1 E), and the use of PFN at permeabilizing concentrations also could produce anomalous proteolysis due to diffusion of the granzyme into necrotic but otherwise intact cells.

Despite the lack of proof that Bid is a dominant initiator of a mitochondrial death pathway, available data indicate that mitochondria play a crucial role in GrB-mediated apoptosis. Unlike cytochrome release assays, which are hampered by the lack of safeguards against adventitious proteolysis, support for GrB-dependent disruption of mitochondrial integrity is based more reliably on studies which examine changes in mitochondrial membrane potential and the detection of released cyt c in whole cells by imaging techniques (Pinkoski et al., 2001; Thomas et al., 2001). What has been uncertain is whether mitochondrial depolarization occurs primarily via BH3-only/Bax/Bak pathway and whether the granzyme directly mediates these effects or participates indirectly via one or more of the caspases.

Studies in Bax/Bak MEFs have shown that almost every described form of cell death requires the presence of these proteins (Wei et al., 2001). Therefore, existence of Bax/Bak-dependent and -independent pathways during GrB-mediated apoptosis is most reliably assessed with Bax/Bak (–/–) MEFs. In comparison to a previous report (Thomas et al., 2001), we observed that the predominate reduction of $\Delta\Psi_m$ occurred in WT, and only a minor alteration was observed in Bax/Bak (–/–) MEFs. The disparate observations may be due to differences in the techniques used to deliver the granzyme and the confounding effect GrB-mediated detachment may have on mitochondrial depolarization.

Until now, caspases have not been considered crucial for GrB-mediated mitochondrial disruption in a variety of cell lines (Heibein et al., 2000; Sutton et al., 2000; Alimonti et al., 2001; Thomas et al., 2001). We suggest the major reason for this discrepancy rests on whether caspases were completely inhibited in the respective studies. Unlike the forms of cells death where caspases undergo controlled autoactivation via protein–protein interactions, the cytosolic delivery of GrB presents the pool of procaspases with a renewable activator that is only exhausted once the granzyme is blocked by cognate inhibitors (e.g., PI-9). This, we suspect, is the basis for the greater difficulty encountered in blocking the caspase cascade in GrB-treated cells (Talanian et al., 1997). Although the study of cells deficient in key caspases (see below) offers a more satisfactory approach, we believed it was crucial to examine cells which lacked Bax/Bak and active caspase-3 to determine whether the granzyme directly disrupts mitochondrial integrity. We had relied previously on measuring caspase activity colorimetrically in lysates after preincubation with the designated inhibitors (Talanian et

al., 1997). However, the level of activated caspase-3 in solubilized cells is reduced in the presence of cell lysates (unpublished data), resulting in an underestimation of the active protease intracellularly. To circumvent this problem, we verified the effectiveness of the caspase inhibitors in whole cells using the PhiPhiLux assay (Komoriya et al., 2000). Relying on the combination of DEVD-fmk and VAD-fmk, mitochondrial depolarization was blocked in WT cells, suggesting caspases rather than GrB were responsible.

To decisively establish that caspases play a central role in mitochondrial depolarization during GrB-mediated apoptosis, we then showed that $\Delta\Psi_m$ only occurred in targets containing procaspase-3 (e.g., MCF-7_{casp3} cells). Notably, similar results were obtained with procaspase-3 (-/-) MEFs (unpublished data), indicating the observation is not cell type specific. Coupled with the observation that $\Delta\Psi_m$ was more reduced in WT than DKO MEFs, the results indicate that the predominant GrB death pathway starts with intracellular delivery of the granzyme, continues with activation of procaspase-3, and is followed by caspase-mediated engagement of a BH3-only/Bax/Bak pathway. The BH3-only protein that is cleaved by caspase-3 is unclear. In other systems, caspase-3 has been reported to process BID, amplifying the death process (Slee et al., 2000). As observed for caspase-7 where the active protease was undetected by blotting but seemed apparent by PARP cleavage, more sensitive assays may reveal that BID is processed to amplify GrB-mediated apoptosis. However, at present the mechanism underlying GrB-initiated, caspase-3-mediated engagement of the Bax/Bak pathway may be implemented by BID or other BH3-only proteins. Consistent with data presented here that Bid may not be critical for GrB induced apoptosis, it has been shown recently that the GrB cleavage site in Bid is not conserved among different species which are known to have granzyme-laden cytotoxic T lymphocytes (Coultas et al., 2002).

Since caspase-3 and -7 are rapidly processed during GrB-induced apoptosis (Yang et al., 1998), it was unclear whether both proteases contribute to the observed reduction in $\Delta\Psi_m$. Despite our inability to detect processed caspase-7 by immunoblotting in MCF-7_{vec} cells during a 4-h assay (Yang et al., 1998), the results suggests GrB can directly activate this caspase after intracellular delivery. This interpretation is supported by evidence that PARP is similarly cut by both caspase-3 and -7 (Germain et al., 1999) and by data here, which indicates PARP cleavage in MCF-7_{vec} cells is inhibited by DEVD-fmk (Fig. 3 E). Therefore, although processed caspase-7 was not apparent in procaspase-3-deficient cells by immunoblotting, GrB appears to access and activate this procaspase. Furthermore, sufficient active caspase-7 is generated to cleave PARP and yet a reduction in $\Delta\Psi_m$ does not occur. Together, the results suggest that caspase-7 does not disrupt mitochondrial function. Furthermore, MCF-7_{vec} cells do not show signs of DNA fragmentation by TUNEL upwards to 20 h after GrB delivery (unpublished data), but Hoechst staining reveals that most MCF-7_{vec} cells contained condensed nonfragmented nuclei (unpublished data). Consistent with a recent observation (Marsden et al., 2002), caspase-7 directly activated by GrB may be sufficient to induce apoptosis and thus represent a unique death pathway which is entirely independent of mitochondria.

The WT MEFs clearly produced higher levels of active caspase-3 than DKO MEFs. This observation may provide clues as to how mitochondria amplify GrB-mediated apoptosis. Since the cell-associated procaspase-3 does not appear to be completely activated in DKO cells, a portion of the zymogen may be sequestered from the granzyme. Procaspase-3 has been reported to reside in mitochondrial (Mancini et al., 1998) and cytosolic compartments. Therefore, with mitochondrial permeabilization the zymogen leaves the mitochondria and becomes accessible to processing by the granzyme. However, recent evidence casts doubts on the localization of procaspase-3 to mitochondria (van Loo et al., 2002). Therefore, coupled with the observation that cytosolic procaspase-3 is compartmentalized to free and membrane-bound fractions (Krebs et al., 1999), a plausible alternative entails the generation of an amplification loop where caspase-9 rather than GrB activates the membrane-associated procaspase-3 zymogen.

Using Bcl-2-transfected cells, we verify previous studies which showed that the antiapoptotic protein protects targets from GrB-mediated death (Sutton et al., 1997; Pinkoski et al., 2001). However, the results here establish that Bcl-2 acts at the most proximal aspect of the granzyme-mediated death pathway by inhibiting the activation of caspase-3. Despite the marked reduction in active caspase-3 within cells, the processed subunits were identified by immunoblot (Fig. 4 D), an observation we made previously in targets undergoing GrB-induced apoptosis in the presence of DEVD-fmk (Talanian et al., 1997). However, isolated Bcl-2 does not directly inhibit the proteolytic activity of caspase-3 (unpublished data). Together, the results suggest that Bcl-2 interferes with oligomerization of the caspase into active heterodimers. We thus identify a novel mechanism through which the potent antiapoptotic protein prevents cell death (Cory and Adams, 2002) akin to the described Bcl-2-associated sequestration of BH3-only domain proteins (Cheng et al., 2001).

Combining three separate strategies to ablate procaspase-3 function with three assays that focus on intracellular events in whole cells, the results indicate that GrB initiates death by activating procaspase-3 rather than by modifying BH3-only proteins (Heibin et al., 2000; Sutton et al., 2000; Wang et al., 2001). Thereafter, caspase-3 permeabilizes mitochondria primarily in a BH3-dependent fashion. The release of mitochondrial products then amplify the death process, ensuring efficient execution of the cell. This pathway appears to represent the optimal sequence for cell death. However, the results clearly indicate that mitochondria are not essential for the granzyme-mediated killing. Independently of BH3 proteins, caspase-3 induces DNA fragmentation presumably through cleavage of ICAD (Wolf et al., 1999). If caspase-3 is lacking, DNA fragmentation is conspicuously absent, suggesting a pathway where the granzyme directly cleaves ICAD is not operative in situ (Thomas et al., 2000). Finally, in the absence of procaspase-3, activated caspase-7 produces mitochondrial independent nuclear condensation, emphasizing the crucial role the caspases play in GrB-mediated apoptosis (Fig. 5).

There has been a resurgence in the notion that caspases act as initiators and mitochondria as the amplifier of cell death (Slee et al., 2000; Guo et al., 2002; Lassus et al., 2002; Marsden et al., 2002). GrB originally served as an important

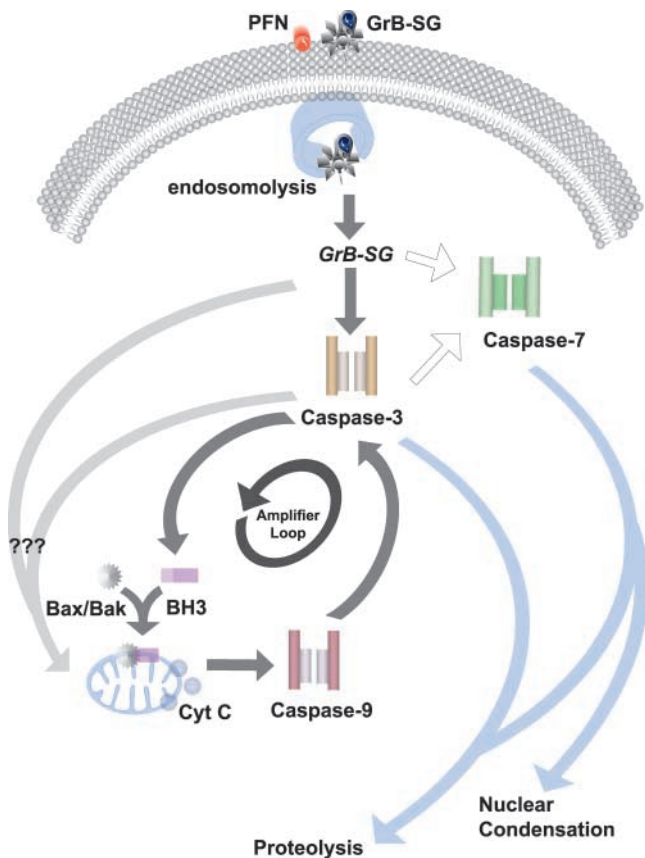


Figure 5. **Predominant and ancillary pathways for GrB-mediated apoptosis.** After endocytosis, GrB–SG complexes are released to the cytosol where a portion of the intracellular stores of procaspase-3 is activated (dark gray arrows). The activated caspase then in a BH3-only/Bax/Bak–dependent manner permeabilizes mitochondria, releasing cyt c and generating the caspase-9 apoptosome. Thereafter, caspase-9 completes the amplification loop (black arrow) by cleaving additional procaspase-3. Light gray arrows describe hypothetical pathways where GrB–SG and caspase-3 perturb mitochondria in a BH3-independent manner. Finally, white arrows describe sequential synergistic activation of procaspase-7 by GrB–SG and caspase-3, whereas light blue arrows describe nuclear condensation specifically by caspase-7 and cellular proteolysis by the caspases.

tool to characterize the enzymology of caspases. Performing whole cell studies, we have learned from granzyme once again that caspases influence mitochondrial integrity, thereby augmenting the many facets of cell death.

Materials and methods

Cell lines

Jurkat cells were maintained in RPMI-1640 with 10% FBS (Atlanta Biologicals). WT MCF-7, lacking caspase 3, (MCF-7_{wt}), caspase-3–transfected cells (MCF-7_{casp-3}), and vector controls (MCF-7_{vec}) were maintained (Yang et al., 1998). WT and double negative (Bax, Bak) murine embryonic fibroblasts (Bax/Bak [–/–] MEFs) were generated and grown as described (Wei et al., 2001). Jurkat cells overexpressing either empty vector or Bcl-2, Jurkat_{vec} or Jurkat_{Bcl-2} were provided by Marcus Peter (University of Chicago, Chicago, IL) (Stegh et al., 2002).

Reagents

L-glutamine, EDTA, glycerol, PFA, 3-aminopropyltriethoxy-silane, and propidium iodide were purchased from Sigma-Aldrich. RPMI 1640 was from GIBCO BRL. GELCODE blue stain reagent and Hoechst 33342 were from Pierce Chemical Co. and Molecular Probes, respectively. HRP rat anti-

mouse kappa mAb was from Accurate Chemical, and ready gels, silver stain kit, Immunoblot and Sequiblot-PVDF membranes were purchased from Bio-Rad Laboratories. ECL Western blot system and Hyperfilm ECL and HRP-tagged anti–mouse and –rabbit secondary antibodies were from Amersham Biosciences. Human GrB and PFN were isolated as described (Hanna et al., 1993; Froelich et al., 1996b). Human serglycin (SG) was isolated from supernatants of YT cells (Raja et al., 2002). The GrB inhibitor Ac-IETD-cho was from Alexis Biochemicals. Z-DEVD-fmk and Z-VAD-fmk were from Calbiochem. Anti-caspase-3 and -7, anti-PARP, and anti-cyt c were from BD Pharmingen, and anti-Bid was either provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX) or obtained from Cell Signaling Technology. Antiactin antibody is from ICN. Recombinant TRAIL was expressed as the cDNA fragment (aa 114–281) in pET-23d plasmid.

Generation and analysis of GrB–SG complexes

GrB–SG complexes were prepared as reported previously (Metkar et al., 2002). The concentrations of GrB retentates were assessed by ELISA (Spaeny-Dekking et al., 1998). GrB enzymatic activity was measured using Boc-Ala-Asp-thiobenzyl ester (BAADT) or Ac-Ile-Glu-Thr-Asp-pNA (IETD-paranitroanilide; Calbiochem). Cleavage was monitored at 405 nm.

Protein delivery for induction of cell death

AD was employed at a plaque-forming unit (PFU) ranging from 500–1,000 per cell. The endosomolytic activity of the AD against the various target cells was determined as follows: AD, containing the β -galactosidase construct, was added to the designated target cell at increasing PFU/cell. The cells were stained 18 h later for β -galactosidase expression. The PFU that resulted in >80% positive target cells was used. GrB and GrB–SG were used at 1 and 2 μ g/ml, respectively, for the experiments. Cells (10^6) were treated for times indicated in the presence and absence of designated inhibitors and then processed for assays described below. Viability of treated cells were comparable to media controls based on similar light scatter characteristic and acquisition rates by flow cytometry.

Immunoblotting

Detection of processed caspase-3 and -7 and Bid was performed as reported previously (Froelich et al., 1996a) with modifications described here. 15 min before the completion of the assay, IETD-cho (250–500 μ M) was added to targets. The cells were then incubated in solubilization buffer (1% NP-40, 1 mM EDTA, 50 mM Tris-HCl, 165 mM NaCl) containing additional IETD-cho. Thereafter, debris was cleared by centrifugation, and the postnuclear lysate was immediately frozen (-80°C) with aliquots held to measure protein content. Each lot of IETD-CHO was evaluated for the capacity to completely inactivate GrB in the lysates by measuring residual GrB activity with the chromogenic substrate, IETD-pNA. For immunoblotting, postnuclear lysates of varying protein content were thawed, mixed with Laemmli buffer, heated, resolved by SDS-PAGE (10–15%), and transferred to Immunoblot PVDF membranes (Bio-Rad Laboratories). Signal was visualized with the ECL kit (Amersham Biosciences).

Apoptosis assays

Hoechst assay. Cells were fixed in 4% PFA and dried on microscope slides. For analysis, cells were hydrated, stained with Hoechst 33342 (10 μ g/ml), and mounted with a drop of 50% glycerol in PBS. For each sample, 200 cells were counted to determine the percentage of cells with condensed and/or fragmented nuclei.

FITC-TUNEL. Target cells (10^6 /ml) were treated with the appropriate reagents in microfuge tubes for the required time and death was measured by terminal deoxynucleotidyl transferase–catalyzed labeling of DNA strand breaks with FITC-dUTP followed by flow cytometry (TUNEL).

PhiPhiLux caspase-3 assay. Cells were stained with the cell permeable fluorogenic caspase 3 substrate PhiPhiLux (G1-D2; Oncolmmunin, Inc.) as per the manufacturer’s instructions followed by flow cytometry (Komoriya et al., 2000).

FAM-DEVD-FMK caspase-3 assay. Cells were stained with the cell permeable fluorogenic caspase-3 inhibitor FAM-DEVD-FMK (APOLOGIX, Inc.) as per the manufacturer’s instructions followed by flow cytometry.

Mitochondrial potential loss ($\Delta\Psi\text{m}$)

After treatment, cells were stained with MitoTracker red CMXRos (Molecular Probes) as described (Metkar et al., 2000).

Imaging, computers, and software

Images of immunoblots were captured either with a Eastman Kodak digital camera or Saphir Ultra 2 flatbed scanner, exported to Adobe Photoshop®

7.0, after which the Tiff images were placed for final presentation in Adobe Illustrator® 10.0 using a Macintosh Power G4 computer (OS X).

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