Mitochondria-dependent and -independent Regulation of Granzyme B–induced Apoptosis

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Summary

Granzyme B (GraB) is required for the efficient activation of apoptosis by cytotoxic T lymphocytes and natural killer cells. We find that GraB and perforin induce severe mitochondrial perturbation as evidenced by the release of cytochrome *c* into the cytosol and suppression of transmembrane potential ($\Delta\psi$). The earliest mitochondrial event was the release of cytochrome *c*, which occurred at the same time as caspase 3 processing and consistently before the activation of apoptosis. Granzyme K/perforin or perforin treatment, both of which kill target cells efficiently but are poor activators of apoptosis in short-term assays, did not induce rapid cytochrome *c* release. However, they suppressed $\Delta\psi$ and increased reactive oxygen species generation, indicating that mitochondrial dysfunction is also associated with this nonapoptotic cell death.

Pretreatment with peptide caspase inhibitors zVAD-FMK or YVAD-CHO prevented GraB apoptosis and cytochrome *c* release, whereas DEVD-CHO blocked apoptosis but did not prevent cytochrome *c* release, indicating that caspases act both up- and downstream of mitochondria. Of additional interest, $\Delta\psi$ suppression mediated by GraK or GraB and perforin was not affected by zVAD-FMK and thus was caspase independent. Overexpression of Bcl-2 and Bcl-X_L suppressed caspase activation, mitochondrial cytochrome *c* release, $\Delta\psi$ suppression, and apoptosis and cell death induced by GraB, GraK, or perforin.

In an in vitro cell free system, GraB activates nuclear apoptosis in S-100 cytosol at high doses, however the addition of mitochondria amplified GraB activity over 15-fold. GraB-induced caspase 3 processing to p17 in S-100 cytosol was increased only threefold in the presence of mitochondria, suggesting that another caspase(s) participates in the mitochondrial amplification of GraB apoptosis. We conclude that GraB-induced apoptosis is highly amplified by mitochondria in a caspase-dependent manner but that GraB can also initiate caspase 3 processing and apoptosis in the absence of mitochondria.

Key words: granzyme B • apoptosis • mitochondria • cytochrome *c* • caspase

Cytotoxic lymphocytes can kill their target cells either through activation of the Fas receptor (1) or by the action of granule proteins granzyme B (GraB)[1] and perforin (2, 3). Both mechanisms activate the cysteine aspartyl proteases (caspases) that are the effector proteases of apoptosis, which ultimately lead to the disassembly of the cell through hydrolysis of cytoplasmic and nuclear proteins (4, 5). Fas interacts directly with caspases through a linker molecule at the cell membrane (6, 7), whereas GraB enters the cytoplasm and initiates processing of caspases after perforin signaling (8–10). GraB activates apoptosis through its ability to cleave proteins after an aspartic acid (11, 12) similar to caspases (13), and can directly cleave in vitro translated caspases 3, 7, 8, and 10 between the large and small subunit (4). This initial processing of the caspase protein is followed by autocatalytic removal of the prodomain and tetramer assembly into the mature protein (14, 15).

The proteolytic specificity of caspases can be separated into at least three subgroups (13). The caspases 2, 3, 7 subfamily proteases have a preference for an aspartic acid in the

[1]*Abbreviations used in this paper:* BA, bongkrekic acid; CsA, cyclosporine acid; $\Delta\psi$, mitochondrial transmembrane potential; DEVD-CHO, Ac-Asp-Glu-Val-Asp-acid aldehyde; FA-FMK, Cbz-Phe-Ala-fluoromethyl ketone; GraB, granzyme B; GraK, granzyme K; PT, permeability transition; Rh123, Rhodamine 123; ROS, reactive oxygen species; YVAD-CHO, Ac-Tyr-Val-Ala-Asp-acid aldehyde; zVAD-FMK, Cbz-Val-Ala-Asp-fluoromethyl ketone.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/01/131/13 $2.00
Volume 189, Number 1, January 4, 1999 131–143
http://www.jem.org

P4 position and are considered downstream in the caspase cascade (4, 13). IL-1β–converting enzyme (ICE) subfamily proteases differ from the first group by recognition of a bulky amino acid (Trp, Tyr) in the P4 position. A third group prefers leucine/isoleucine/valine in P4, including caspases 6, 8, 9, and probably 10, all of which are upstream caspases. This latter recognition sequence is shared with GraB. In the Fas activation pathway, caspase 8 interacts with Fas through FADD/Mort (6, 7) and is therefore upstream on the cell death pathway, whereas other caspases including caspase 3 are activated by caspase 8 (16) and are downstream.

Since GraB can process and activate caspase 10, 8, 7, and 3 in vitro and in vivo (17–20), it may be able to initiate apoptosis by activating either upstream or downstream caspases within the cell death pathway. However, recent work suggests that mitochondria play an important if not key role in the regulation of apoptosis (21–26). Mitochondrial function is disturbed early in the apoptotic response and may be important in propagating death signals. Mitochondria release cytochrome *c* (25, 26), which interacts with Apaf-1, the recently cloned Ced-4 homologue in humans (27). In the presence of dATP (24), Apaf-1 and cytochrome *c* can activate caspase 9 and then caspase 3 (28). Another mitochondrial event associated with apoptosis is the loss of transmembrane potential ($\Delta\psi$), which is seen early in response to an apoptotic signal and is thought to occur as the result of the opening of mitochondrial permeability transition (PT) pores (22–24).

Bcl-2 in the outer mitochondrial membrane, and to a lesser extent as a free cytosolic protein, is important for the suppression of apoptosis and mitochondrial manifestations of apoptosis (29–31). The *Caenorhabditis elegans* Bcl-2 homologue Ced-9 forms a complex with Ced-4 and the caspase Ced-3, and this complex can regulate Ced-3 activation (32, 33). Bcl-2 also suppresses cytochrome *c* (27, 28) and apoptosis-inducing factor (34) release from mitochondria, which prevents the activation of caspases. Bcl-2 has been reported to inhibit GraB-induced apoptosis (35) as well as CTL apoptosis mediated by granules (granzymes and perforin) (36, 37).

Because GraB can cleave and activate both up- and downstream caspases, it has been generally assumed that once this is accomplished the caspase cascade will continue to amplify itself and hydrolyze the relevant substrates in the cytoplasm and nucleus to complete apoptosis. Such a scheme would exclude the participation of mitochondria and the mitochondrial amplification mechanisms. We have attempted to determine whether or not GraB is truly as efficient as the in vitro data would suggest and bypass mitochondria. We report here the existence of a mitochondrial-regulated, caspase-dependent GraB apoptotic cell death pathway. In addition, we find that caspase-independent cell death induced by perforin and perforin/granzyme treatment is also associated with mitochondrial dysfunction.

Materials and Methods

Cell Lines and Reagents. The cell lines HeLa, Rat-1, U937, and the Bcl-2 transfectant Rat-1/Bcl-2, as well as U937 transfected with Bcl-2 and Bcl-X_L, were maintained in α-MEM (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL). The peptide aspartyl caspase inhibitor Cbz-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK), and control peptides Cbz-Phe-Ala-fluoromethyl ketone (FA-FMK) (Enzyme System Products), Ac-Asp-Glu-Val-Asp-acid aldehyde (DEVD-CHO), and Ac-Tyr-Val-Ala-Asp-acid aldehyde (YVAD-CHO) (Peptide International, Louisville, KY) were prepared as 10-mM stock solutions in DMSO. Bongkrekic acid (BA) was provided by Dr. J.A. Duine (Delft University, Delft, The Netherlands). Cyclosporine A (CsA) was purchased from Sigma Chemical Co. GraB, GraK, and perforin were purified from the granules of a rat NK large granular lymphocyte leukemia cell line using a previously described procedure (11). Recombinant GraB was prepared from a baculovirus expression system as previously described (38). A mutant murine GraB in which the active Ser^{203} was converted to an alanine was generated by PCR mutagenesis using the following primers: sense primer 5′-TCCTTTCGGGGGGATGCT-GGAGGCCCGCTTGTG-3′ and antisense primer 5′-CACAAG-CGGGCCTCCAGCATCCCCCCGAAAGGA-3′. The mutant protein was expressed and purified by the same method (38).

Perforin and Granzyme-mediated Cell Death. Cell lines used in this study were treated with perforin and either of the purified granzymes as previously described (11). In brief, target cells in HBSS/0.4% BSA were added to a buffer (140 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, and 1 mM EGTA, pH 7.2) containing perforin and one of the granzymes followed by incubation at 37°C for the periods of time indicated in each experiment. In experiments using peptide inhibitors, the cells were preincubated at 37°C for 30 min in the peptide before the addition of the perforin/granzyme mixture. Cells incubated with perforin and/or granzymes were evaluated for apoptotic nuclei by chromatin condensation by Hoechst 33258 or DAPI dye staining (10 nM in PBS). Plasma membrane permeability as a measure of cell death was determined by trypan blue dye exclusion. In both assays, between 150 and 300 cells were counted for each data point in three independent assays, and the number of apoptotic or dead cells was expressed as a percentage of the total cell number.

Flow Cytometry. Changes in the $\Delta\psi$ and the levels of reactive oxygen species (ROS) in cells treated with granzymes and perforin was measured by two-color flow cytometry. The $\Delta\psi$ was measured with the mitochondria-specific fluorescent dye rhodamine 123 (Rh123; 2 μM), and ROS levels were quantified by the conversion of hydroethidine (2 μM) to ethidium. Both reagents were added for the final 30 min of the assay. Analysis was performed using an EPICS 753 cell sorter (Coulter Corp.). No fewer than 5,000 events were counted. Data was analyzed using Coulter Elite workstation software (Coulter Corp.).

Immunofluorescence Microscopy. Cytochrome *c* was detected in HeLa or Rat-1 cells cultured on 12-mm diameter round coverslips overnight and then incubated with freshly titered MitoTracker™ Green FM, usually 80 nM, (Molecular Probes) in prewarmed culture medium for 30 min at 37°C for labeling. The discs were washed three times with HBSS and soaked in 80 μl of HBSS containing 10 mM Hepes, 2 mM $CaCl_2$, and 4 mg/ml BSA. In experiments using peptide inhibitors, the target cells were pretreated for 15 min with zVAD-FMK, DEVD-CHO, YVAD-CHO, or FA-FMK. 80-μl aliquots of GraB or GraK (2 μg/ml) and perforin (125 ng/ml) in 140 mM NaCl, 10 mM Hepes, and 1 mM EGTA, pH 7.2 were added to each coverslip. After 3 h of incubation at 37°C, the cells were fixed in 3.7% formaldehyde-PBS for 10 min. The cells were washed three times with PBS and incubated with mouse monoclonal anti–cyto-

chrome *c* antibody (PharMingen) diluted in PBS containing 10% FCS and 0.1% NP-40 for 1 h at room temperature with agitating. After washing three times with PBS, the cells were incubated with Cy3-conjugated goat anti–mouse antibody (Chemicon International Inc., Don Mills, Ontario, Canada) diluted in the same buffer for 1 h. The cells were washed three times in PBS and mounted with 3 μl of Anti-Fade reagent (Bio-Rad) on a glass slide. Image analysis was performed using a Zeiss Axiophot microscope equipped with a cooled CCD camera and driven by IPLabs Spectrum H-SU2 (version 3.0, Signal Analytics) and Multiprobe 1.1 E (Signal Analytics) software.

Western Blot Analysis. Caspases were identified in postnuclear lysates of perforin and granzyme-treated cells after SDS-PAGE on a 15% agarose gel. Once the proteins were transferred to a nitrocellulose membrane, the blot was reacted with rabbit polyclonal antisera directed against the p17 fragment of CPP32/caspase 3 (provided by D. Nicholson, Merck Laboratories, Montreal, Canada). Pro-caspase 3 and its cleavage fragments were visualized by incubating the blot with horseradish peroxidase–conjugated goat anti–rabbit IgG antibody and developing with an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham).

Cytochrome *c* was examined in lysates of target cells treated with GraB and perforin. HeLa cells were harvested and washed three times with HBSS and resuspended at 10^7 cells/ml in 0.4% BSA, 10 mM Hepes, 2 mM $CaCl_2$, and HBSS, pH 7.2. Aliquots of 5×10^6 target cells were added to 2 μg GraB and 80–120 ng perforin was diluted in 140 mM NaCl, 10 mM Hepes, and 1 mM EGTA, pH 7.2. in a total vol of 1 ml. In some experiments the target cells were preincubated with 40 μM zVAD-FMK, 100 μM YVAD-CHO, or 100 μM DEVD-CHO for 15 min at room temperature. After 90 min of incubation at 37°C in an a CO_2 incubator, the cells were centrifuged to separate mitochondria and mitochondria-free cytosol as described by Liu et al. (24), except that the cells in a 0.3-ml vol were dounced 36 times using a Teflon douncer to release mitochondria. Equal volumes of the mitochondria-free cytosol and pooled pellet from the remaining fractions were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with mouse anti–cytochrome *c* (PharMingen) and goat anti–mouse IgG-horseradish peroxidase conjugate (Bio-Rad), and visualized by an enhanced chemiluminescence method (Amersham).

In Vitro Cell-free System. The preparation of S100 fractions from HeLa or U937 cells was done as described by Liu et al. (24). Nuclei from 2×10^8 YAC-1, U937, or Jurkat cells were washed three times with HBSS and resuspended in 2 ml NB buffer (10 mM Hepes-KOH, pH 7.5, 15 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM spermidine, 0.2 mM spermine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5 mM PMSF). The cells were put on ice for 20 min, which allowed them to swell, and then were homogenized by 15 strokes of a douncing homogenizer. The homogenate was centrifuged through a 4.5-ml cushion of NB with 30% sucrose at 800 *g* for 10 min. The pellets (nuclei) were washed once with NB and resuspended in storage buffer (10 mM Hepes-KOH, pH 7.5, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM spermidine, 0.2 mM spermine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5 mM PMSF) at 10^8 nuclei per milliliter and were stored at −80°C in multiple aliquots until use. Rat liver mitochondria were isolated in 0.25 M sucrose as described by Pedersen et al. (39). 20 μl HeLa S100, 10 μl diluted rat mitochondria (100 μg/ml protein or as indicated) in dilution buffer (DB; 20 mM Hepes-KOH, pH 7.5, 2 mM $MgCl_2$ and 1 mM dithiothreitol), 10 μl of diluted target nuclei in DB, and 10 μl purified GraB diluted in 0.7 M NaCl, 10 mM bis-Tris, pH 6.0, was placed in the cell-free apoptosis reaction mixture. After 1 h of incubation in 37°C, the reaction mixture was fixed in 3.7% buffered formaldehyde, stained with DAPI, and then apoptotic nuclei were scored by microscopy with the identity of the slides concealed to the reader.

Results

Perforin and Granzymes Induce Δψ Suppression and ROS Production. In the presence of the pore-forming protein perforin, GraB, but not GraK, rapidly activates apoptosis and is highly Bcl-2 and Bcl-X_L inhibitable (Fig. 1, A and B). GraB or GraK and sublytic amounts of perforin also induce plasma membrane disruption and cell death (Fig. 1 C). To determine whether or not mitochondrial dysfunction is associated with granzyme- and perforin-dependent cell death, tumor cell lines were treated with GraB or GraK plus perforin and examined for loss of Δψ and the generation of ROS using two-color flow cytometry with the fluorochrome Rh123 or hydroethidine, respectively (Fig. 2, A–F). HeLa cells treated with either GraB or GraK in the absence of perforin did not show any reduction in Δψ or ROS generation and were indistinguishable from untreated controls (Fig. 2, A–C). In contrast, cells exposed to perforin alone exhibited a reduced Δψ that was significantly

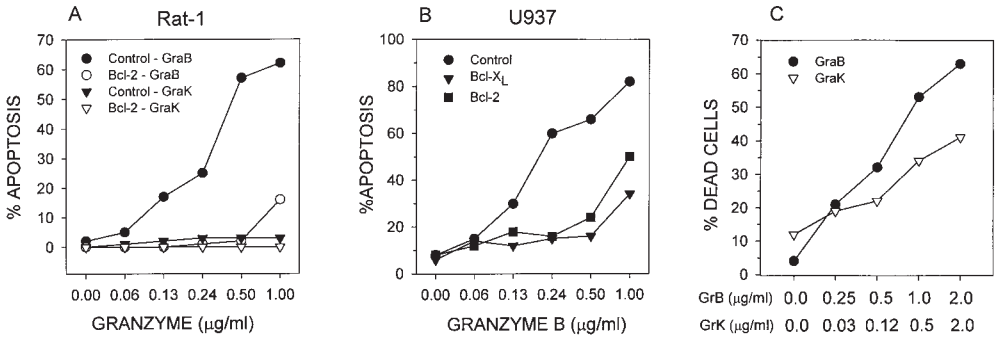

Figure 1. Induction of apoptosis by GraB and perforin. (A) Rat-1 cells or Rat-1 cells overexpressing Bcl-2 were treated with increasing concentrations of GraB or GraK and perforin (80 ng/ml) for 3 h and then the number of apoptotic nuclei were counted after Hoechst staining. (B) U937 cells overexpressing Bcl-2 or Bcl-X_L were treated with GraB and perforin and evaluated for apoptotic nuclei as above. (C) HeLa cells were treated with GraB or GraK at the indicated concentrations and a constant amount of perforin (20 ng/ml) for 1 h and then dead cells were enumerated by trypan blue exclusion. These data are representative of two or three similar experiments.

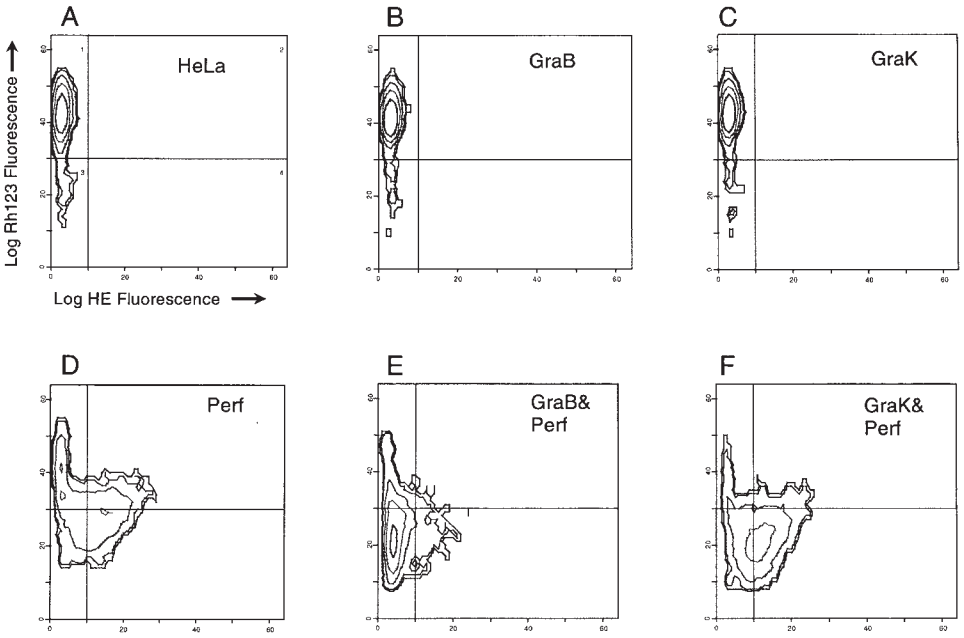

Figure 2. Suppression of $\Delta\psi$ and ROS generation in HeLa cells treated with GraB, GraK, and perforin. HeLa cells were treated with GraB (B), GraK (2 μg/ml) (C), or perforin (Perf) (20 ng/ml) (D) alone, or combinations of GraB and perforin (E) or GraK and perforin (F) for 1 h and then double stained with the membrane potential sensitive dye Rh123 plus hydroethidine (HE) to assess ROS generation as described in Materials and Methods. Cells were then analyzed by flow cytometry and illustrated on a logarithmic scale. These data are representative of three similar experiments.

increased with the addition of either GraB (Fig. 2 E) or GraK (Fig. 2 F). The perforin-mediated reduction in $\Delta\psi$ and ROS production was dose dependent. Addition of a constant amount of either GraB or GraK to increasing amounts of perforin greatly augmented the number of $\Delta\psi^{low}$ cells even at perforin concentrations too low to induce any noticeable mitochondrial dysfunction (Fig. 3, A and B). Treatment with perforin, or perforin and GraK, also increased ROS levels in a dose-dependent manner (Fig. 3 B). Similar results were obtained by repeating these experiments with Jurkat cells. Surprisingly, ROS production was not significantly increased by GraB plus perforin treatment (Fig. 3 B). However, in other experiments cells treated with lower doses of GraB did show increased levels ROS, suggesting that, at the doses of GraB used here, target cells are sufficiently disrupted that their mitochondria are unable to generate ROS.

We next determined the effects of PT inhibitors BA and CsA on GraB-mediated apoptosis. CsA acts on matrix cyclophilin to prevent PT, and BA is a ligand of the adenine nucleotide translocator and can inhibit preapoptotic $\Delta\psi$ disruption and apoptosis in isolated mitochondria (21). CsA was without effect over a broad dose range on several tumor targets. BA produced a six- to eightfold dose-dependent inhibition of GraB-induced apoptosis of HeLa cells, however its inhibitory effect on YAC-1 lymphoma was minimal and never more than twofold (data not shown).

Bcl-2 Inhibits $\Delta\psi$ Suppression and ROS Production Induced by Perforin and Granzymes. Bcl-2 and Bcl-X_L proteins promote cell survival and suppress cell death by many apoptotic stimuli including GraB and perforin, and intact CTL granules (Fig. 1; references 35–37). Bcl-2 expression in the outer mitochondrial membrane prevents mitochondrial PT and $\Delta\psi$ suppression and ROS production to many but not all apoptotic signals (23). We next examined the effects of Bcl-2 on these GraB and GraK-induced mitochondrial changes. Rat-1 cells exposed to either of the granzymes with perforin or just perforin alone exhibited reduced $\Delta\psi$, elevated ROS levels and increased numbers of dead cells (Fig. 4, A–C). In contrast, Rat-1/Bcl-2 treated in exactly the same way showed no $\Delta\psi^{low}$ cells or generation of ROS (Fig. 4, A and B) when compared with nontransfected control Rat-1 targets. The Bcl-2–expressing cells also exhibited a significant reduction in the frequency of dead cells induced by all of the stimuli including perforin alone, perforin and GraB, and perforin and GraK (Fig. 4 C). The suppression of transmembrane potential to GraB was also inhibited by overexpression of Bcl-X_L in U937 cells although only by ~30–35% (data not shown), which is less than that observed with Rat1/Bcl-2.

Release of Mitochondrial Cytochrome c after GraB/Perforin Treatment and its Inhibition by Bcl-2 or Bcl-X_L. Cells undergoing apoptosis release cytochrome *c* from the mitochon-

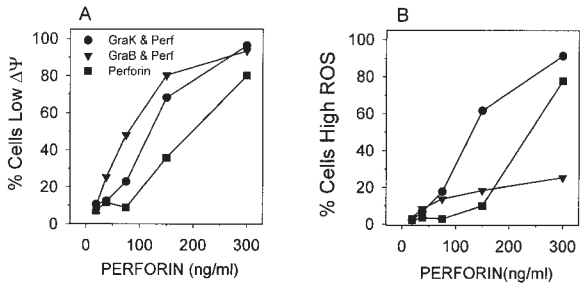

Figure 3. Suppression of $\Delta\psi$ (A) and ROS generation (B) in HeLa cells treated with GraB, GraK, and increasing concentrations of perforin. The experiment was performed as described in Fig. 2 except that GraB and GraK concentration was held constant at 2 μg/ml and the amount of perforin was added as indicated. These data are representative of two similar experiments.

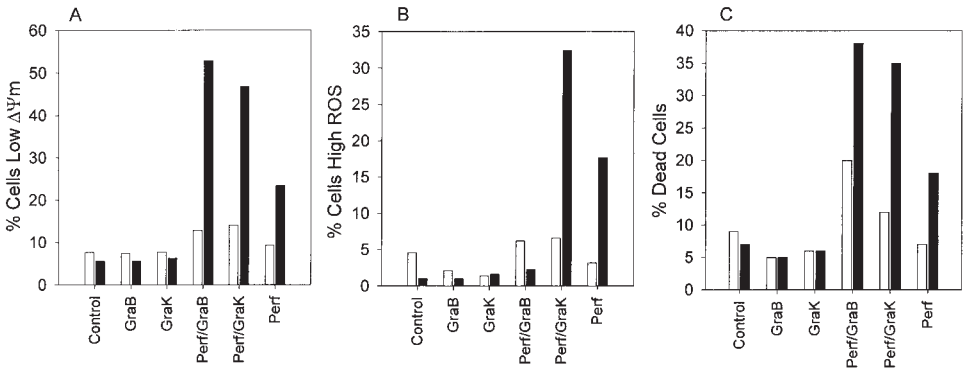

Figure 4. Bcl-2 inhibits the generation of $\Delta\psi^{low}$ cells, ROS, and cell death induced by GraB, GraK, and perforin. Rat-1 cells (black bars) or Rat-1 cells over expressing Bcl-2 (white bars) were treated with GraB (2 μg/ml), GraK (2 μg/ml), or perforin (Perf) (80 ng/ml) as indicated for 1 h and then stained with (A) Rh123 and (B) hydroethidine to measure the percentage of $\Delta\psi^{low}$ or high ROS-expressing cells, respectively, as described in Fig. 2. The percentage of dead cells (C) was quantified after trypan blue staining. The experiment is representative of three similar experiments.

drial intermembranous space into the cytosol (27, 28, 40), which is necessary for Apaf-1 activation of caspase 9 and caspase 3 (28). Bcl-2 blocks cytochrome *c* release and caspase 3 processing in a reconstituted cell free system, thus it has been postulated that cytochrome *c* release is necessary for downstream caspase activation and apoptosis (25–28).

We determined whether cell death signals mediated by either perforin alone, or GraB/GraK plus sublytic amounts of perforin are able to induce mitochondrial cytochrome *c* release in intact cells following a 1-h incubation. We first analyzed the subcellular localization of cytochrome *c* by two-color analysis with anti–cytochrome *c* antibody (Fig. 5, red) and the mitochondrial dye Mitotracker (Fig. 5, green) which produce a yellow punctate pattern when they are colocalized, but leave the mitochondria green when cytochrome *c* is released. Simultaneous nuclear staining with Hoechst dye detects chromatin condensation and apoptosis. The pattern of cytochrome *c* immunofluorescence and nuclear Hoechst staining observed in HeLa cells treated with GraK plus perforin or with perforin alone at sublytic doses resembled untreated controls (Fig. 5 A). That is, cytochrome *c* and the mitochondrial dye Mitotracker showed complete colocalization and Hoechst staining of the nucleus did not detect any apoptotic changes. In contrast, the pattern of staining observed after treatment with GraB and perforin revealed cells undergoing nuclear chromatin condensation, and mitochondria were stained with green Mitotracker but were no longer stained red for cytochrome *c* (Fig. 5 B). Similar observations were made in Rat-1 and MCF-7 cells (data not shown). We detected only faint levels of cytochrome *c* in the cytoplasm, which may have been too dispersed to detect by fluorescent microscopy or may have been released from the cell as a result of plasma membrane damage. In contrast to the above, Rat-1 cells constitutively overexpressing Bcl-2 that were treated with GraB and perforin showed no nuclear changes on Hoechst staining and normal mitochondrial cytochrome *c* localization, identical to Fig. 5 A.

A time course, determined by counting cells retaining mitochondrial cytochrome *c* immunofluorescence after treatment with sublytic amounts of perforin plus GraB at various times, demonstrated that cytochrome *c* was released significantly before the appearance of apoptotic nuclei (Fig. 6). In experiments carried out over longer periods, the number of apoptotic nuclei continued to increase and plateau at the same level as cells showing cytochrome *c* redistribution. In contrast, simultaneous assessment of $\Delta\psi$ in the same samples revealed that the appearance of $\Delta\psi^{low}$ cells paralleled but did not precede apoptosis (Fig. 6).

We next confirmed these observations by Western blotting cytochrome *c* released into the S100 cytosol. After treatment with GraB and perforin, cytosolic cytochrome *c* levels increased and this was not seen in cells over expressing Bcl-2 (Fig. 7 A). The release of cytochrome *c* in U937 cells was detected at lower doses of GraB than were able to

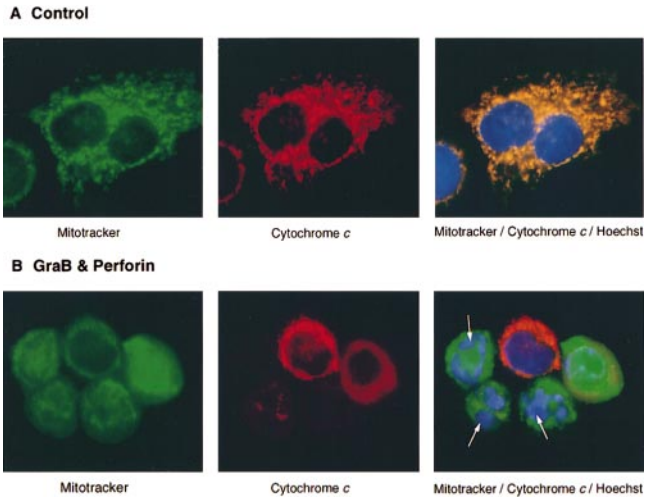

Figure 5. GraB and perforin induce the release of cytochrome *c* from mitochondria detected by immunofluorescent localization. (A) Control HeLa cells were triple stained with Mitotracker (green; left), which localizes in mitochondria and anti-cytochrome *c* antibody detected with Cy3-conjugated second antibody (red; center). In the right panel the colors were overlaid to illustrate colocalization of the Mitotracker and cytochrome *c* in mitochondria (yellow). (B) HeLa cells were treated with GraB (2 μg/ml) and perforin (80 ng/ml) for 1.5 h and then stained as in A. Color overlay of all stains illustrates that cells undergoing apoptosis, even in the earliest stages of chromatin margination (white arrows), have no cytochrome *c* localized to the mitochondria leaving them stained only for Mitotracker (green). Similar responses were observed in more than five separate experiments.

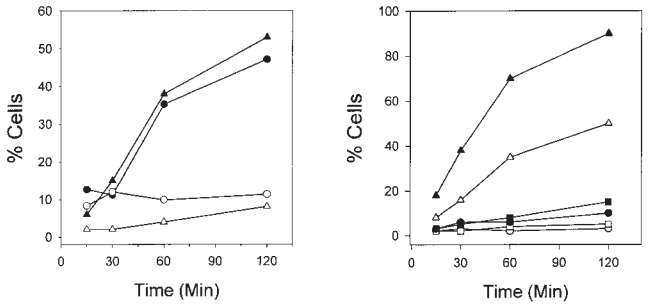

Figure 6. Time course of the release of cytochrome *c*, appearance of $\Delta\psi^{low}$ cells, and apoptosis in cells treated with GraB and perforin. (Left panel) Apoptosis versus $\Delta\psi$ suppression. HeLa cells were treated with GraB (2 μg/ml) and perforin (80 ng/ml) or perforin alone then Rh123 stained and $\Delta\psi^{low}$ cells analyzed by flow cytometry. Cells from the same samples were Hoechst stained for apoptotic cells. ●, GraB/perforin-$\Delta\psi^{low}$; ○, perforin-$\Delta\psi^{low}$; ▲, GraB/perforin-apoptosis; △, perforin-apoptosis. (Right panel) Apoptosis versus cytochrome *c* release. After treatment of HeLa cells with GraB and perforin or perforin alone as described, cells were stained and the percentage releasing cytochrome *c* from mitochondria (as illustrated in Fig. 5 B) was compared with apoptotic nuclei. Virtually all cells undergoing apoptosis had released mitochondrial cytochrome *c* but not all cells releasing cytochrome *c* had apoptotic nuclei. These samples were calculated from at least 200–300 cells and these results are representative of three similar experiments, some of which extended to 4 h of observation. ○, apoptosis-control; □, apoptosis-perforin; △, apoptosis–GraB/perforin; ●, cytochrome *c* release–control; ■, cytochrome *c* release–perforin; ▲, cytochrome *c* release–GraB/perforin.

induce apoptosis (Fig. 7 B), and Bcl-2 and Bcl-X_L over expression were equally effective in suppressing cytochrome *c* release (Fig. 7 B). A time course showed that cytochrome *c* appeared in S100 cytosol as early as 15 min after GraB and perforin treatment but not with perforin treatment alone (Fig. 7 C). The cleavage of caspase 3 was also detected within 15 min before significant numbers of apoptotic cells were detected (Fig. 7 D).

Peptide Caspase Inhibitors Block Apoptosis and Cytochrome c Release But Not Δψ Suppression. Earlier work indicated that inhibition of caspases blocks GraB apoptosis (14, 17). Since GraB activates both mitochondrial cytochrome *c* release and $\Delta\psi$ suppression, we next examined the requirement for caspases in the induction of these mitochondrial changes. Target cells were first incubated with increasing amounts of the tetrapeptide caspase inhibitors that blocked all caspases (zVAD-FMK), or those related to caspase 1 (YVAD-CHO) or caspase 3 (DEVD-CHO), then treated with GraB and perforin to determine the dose of each that would inhibit apoptosis by 70% (Fig. 8 A). Using pretreatment with zVAD-FMK (40 μM) or YVAD-CHO (100 μM) under these conditions completely prevented cytochrome *c* release from mitochondria as detected by immunofluorescent localization (Fig. 8 B). However, cytochrome *c* release was not blocked by the addition of DEVD-CHO (100 μM) despite the nearly complete inhibition of apoptosis, as clearly shown by intact Hoechst-stained nuclei in cells with no mitochondrial cytochrome *c* staining.

We next repeated the peptide inhibition experiments by examining the release of cytochrome *c* into the cytosol from mitochondria detected by Western blot. The appearance of free cytosolic cytochrome *c* and the reduction of

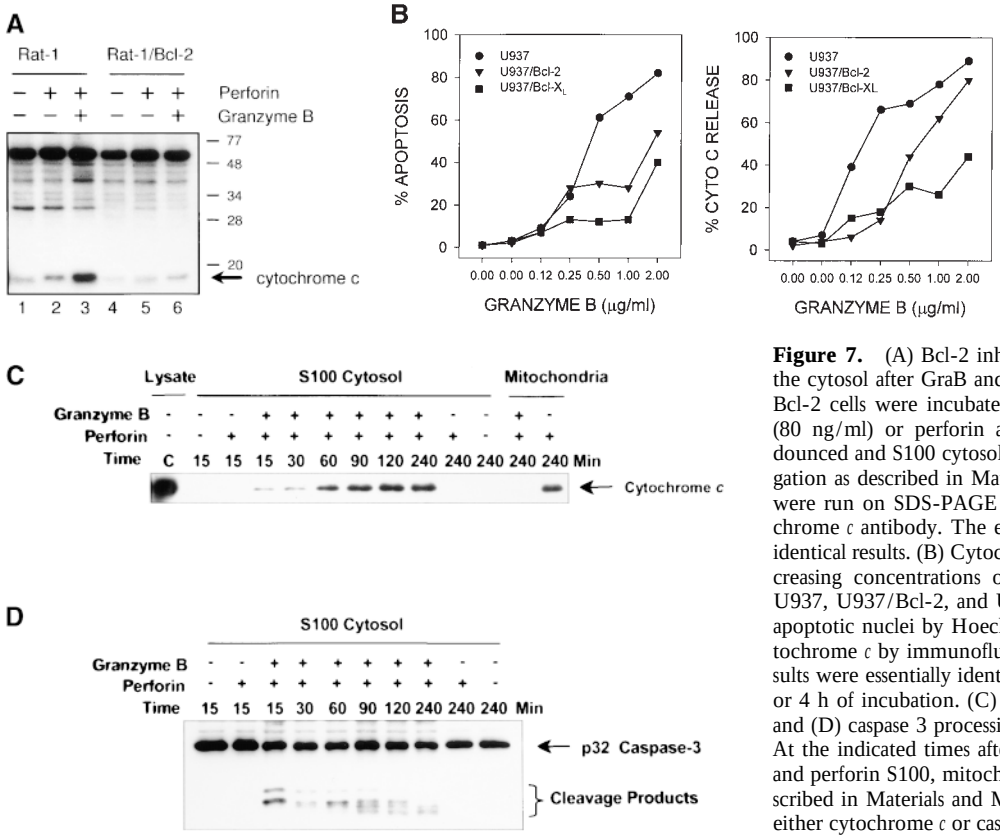

Figure 7. (A) Bcl-2 inhibits the release of cytochrome *c* into the cytosol after GraB and perforin treatment. Rat-1 or Rat-1/Bcl-2 cells were incubated with GraB (2 μg/ml) and perforin (80 ng/ml) or perforin alone for 1.5 h and then cells were dounced and S100 cytosol was prepared by high speed centrifugation as described in Materials and Methods. Cytosol fractions were run on SDS-PAGE and Western blotted with anti–cytochrome *c* antibody. The experiment was performed twice with identical results. (B) Cytochrome *c* release and apoptosis with increasing concentrations of GraB and perforin (80 ng/ml) in U937, U937/Bcl-2, and U937/Bcl-X_L. Cells were counted for apoptotic nuclei by Hoechst staining and for mitochondrial cytochrome *c* by immunofluorescence as described in Fig. 5. Results were essentially identical in three experiments taken after 3 or 4 h of incubation. (C) Western blot of cytochrome *c* release and (D) caspase 3 processing after GraB and perforin treatment. At the indicated times after treatment of HeLa cells with GraB and perforin S100, mitochondrial fractions were prepared as described in Materials and Methods and then Western blotted for either cytochrome *c* or caspase 3.

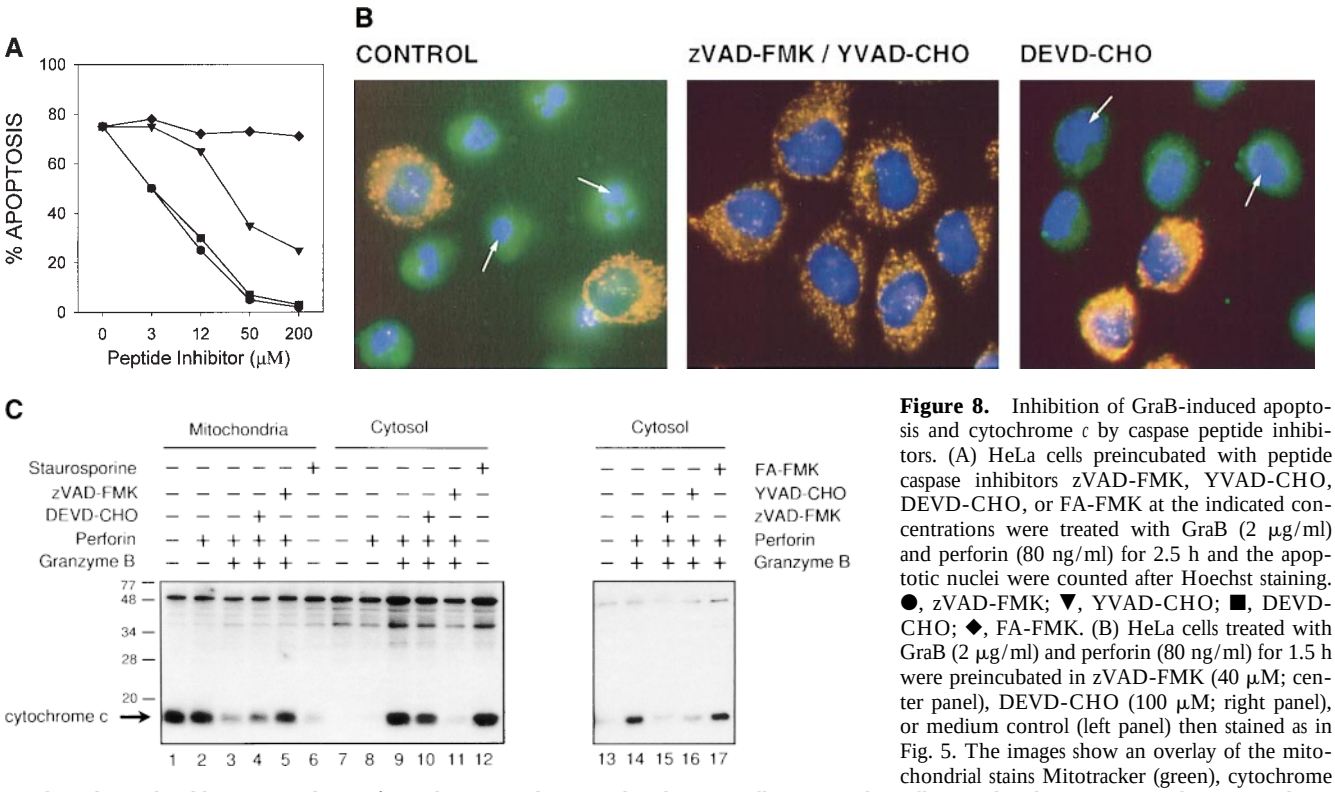

Figure 8. Inhibition of GraB-induced apoptosis and cytochrome *c* by caspase peptide inhibitors. (A) HeLa cells preincubated with peptide caspase inhibitors zVAD-FMK, YVAD-CHO, DEVD-CHO, or FA-FMK at the indicated concentrations were treated with GraB (2 μg/ml) and perforin (80 ng/ml) for 2.5 h and the apoptotic nuclei were counted after Hoechst staining. ●, zVAD-FMK; ▼, YVAD-CHO; ■, DEVD-CHO; ◆, FA-FMK. (B) HeLa cells treated with GraB (2 μg/ml) and perforin (80 ng/ml) for 1.5 h were preincubated in zVAD-FMK (40 μM; center panel), DEVD-CHO (100 μM; right panel), or medium control (left panel) then stained as in Fig. 5. The images show an overlay of the mitochondrial stains Mitotracker (green), cytochrome *c* (red), and Hoechst (blue). Coincidence of cytochrome *c* and Mitotracker shows as yellow. Note that cells treated with DEVD-CHO have no evidence of chromatin condensation (white arrows), yet cytochrome *c* no longer colocalizes with Mitotracker. Cells treated with YVAD-CHO (100 μM) were identical to zVAD-FMK–treated cells (not shown). The concentrations of inhibitory peptide noted above inhibited apoptosis by 70% as determined in A. (C) HeLa cells were treated with perforin alone (lanes 2 and 9) or GraB (2 μg/ml) and perforin (80 ng/ml; lanes 3–5, 9–11, and 14–16), staurosporine (1 μM; lanes 6 and 12), or medium (lanes 1, 7, and 13) for 2.5 h. GraB- and perforin-treated cells were also preincubated in zVAD-FMK (40 μM; lanes 5, 11, and 15), YVAD-CHO (100 μM; lane 16), or DEVD-CHO (100 μM; lanes 4 and 10) peptide inhibitors, control peptide FA-FMK (100 μM; lane 17), or medium (lane 13) and cytochrome *c* in the S100 cytosolic fraction (lanes 7–17) and in the mitochondria-containing fractions assessed by Western blotting (lanes 1–6). The doses of each inhibitory peptide reduced apoptosis by 70% (see A).

mitochondrial cytochrome *c* levels was completely blocked by zVAD-FMK and YVAD-CHO, whereas neither DEVD-CHO nor the control FA-FMK had any effect on cytochrome *c* release (Fig. 8 C). Thus, DEVD-CHO blocks apoptosis independently of cytochrome *c* release, whereas zVAD-FMK and YVAD-CHO appear to inhibit both cytochrome *c* release and apoptosis.

Of additional interest, although zVAD-FMK (20 μM) effectively blocked cytochrome *c* release, it did not suppress the decrease in $\Delta\psi$ or prevent the generation of ROS by GraB and perforin treatment (Fig. 9, A and B). Furthermore, zVAD-FMK only partially inhibited GraB and was unable to affect GraK-mediated cell death as measured by trypan exclusion at a concentration that completely blocked apoptosis as well as the proteolytic conversion of procaspase 3 p32 to p17 (Fig. 9 C). Thus, the decrease in $\Delta\psi$ induced by GraB and perforin is not zVAD-FMK inhibitable and is therefore caspase independent.

Mitochondrial Regulation of GraB Apoptosis, and Caspase 3 Processing and Activation, in an In Vitro Cell-free System. We next directly determined the role of mitochondria in activating GraB apoptosis in an in vitro cell-free system. Previously we had shown that the simple addition of GraB without perforin to cell lysate initiates apoptosis of isolated nuclei and this is associated with the processing and activation of caspase 3 (14). We now reexamined this model using mitochondria-free S100 cytosol. The absence of cytochrome *c* on Western blotting confirmed that the S100 had no contaminating mitochondria. GraB-induced apoptotic activity is detectable without mitochondria (Fig. 10 A). However, the addition of an enriched mitochondrial fraction enhances apoptosis >15-fold with activity detectable at 120 ng/ml of GraB. These experiments were repeated using nuclei and S100 cytosol from several cellular sources, including HeLa, YAC-1 or U937 cells, and mitochondria from HeLa cells or rat liver with similar amplification of apoptosis in the presence of mitochondria. The level of amplification of GraB apoptosis by mitochondria was dependent on the amount of enriched mitochondrial fraction added to the reaction (Fig. 10 B). To confirm that the activity was due to the proteolytic activity of GraB in our purified preparations, we used recombinant GraB bearing an inactivating Ser^{203} to Ala mutation in the in vitro assay and found that it was unable to initiate apoptosis in the presence or absence of mitochondria in contrast to wild-type recombinant GraB (Fig. 10 C).

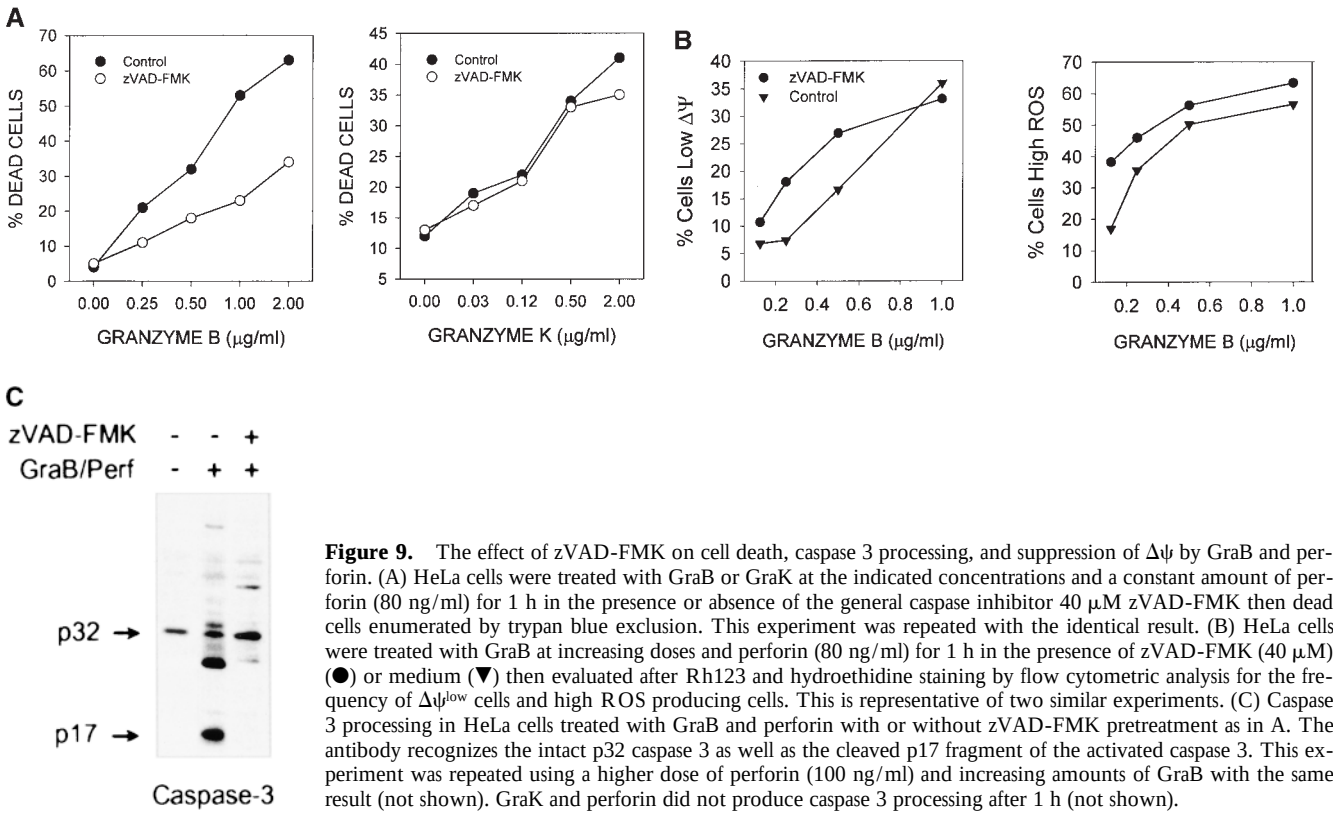

Figure 9. The effect of zVAD-FMK on cell death, caspase 3 processing, and suppression of $\Delta\psi$ by GraB and perforin. (A) HeLa cells were treated with GraB or GraK at the indicated concentrations and a constant amount of perforin (80 ng/ml) for 1 h in the presence or absence of the general caspase inhibitor 40 μM zVAD-FMK then dead cells enumerated by trypan blue exclusion. This experiment was repeated with the identical result. (B) HeLa cells were treated with GraB at increasing doses and perforin (80 ng/ml) for 1 h in the presence of zVAD-FMK (40 μM) (●) or medium (▼) then evaluated after Rh123 and hydroethidine staining by flow cytometric analysis for the frequency of $\Delta\psi^{low}$ cells and high ROS producing cells. This is representative of two similar experiments. (C) Caspase 3 processing in HeLa cells treated with GraB and perforin with or without zVAD-FMK pretreatment as in A. The antibody recognizes the intact p32 caspase 3 as well as the cleaved p17 fragment of the activated caspase 3. This experiment was repeated using a higher dose of perforin (100 ng/ml) and increasing amounts of GraB with the same result (not shown). GraK and perforin did not produce caspase 3 processing after 1 h (not shown).

We next determined whether caspase 3 processing and activation by GraB requires mitochondria. S100 cytosol and nuclei were incubated in increasing amounts of GraB in the presence or absence of mitochondria as described above then Western blotted for caspase 3. We found that in the absence of mitochondria caspase 3 is clearly processed to the p17-p22 products with 2,000 ng/ml GraB (Fig. 10 D). In the presence of mitochondria, GraB-induced cleav-

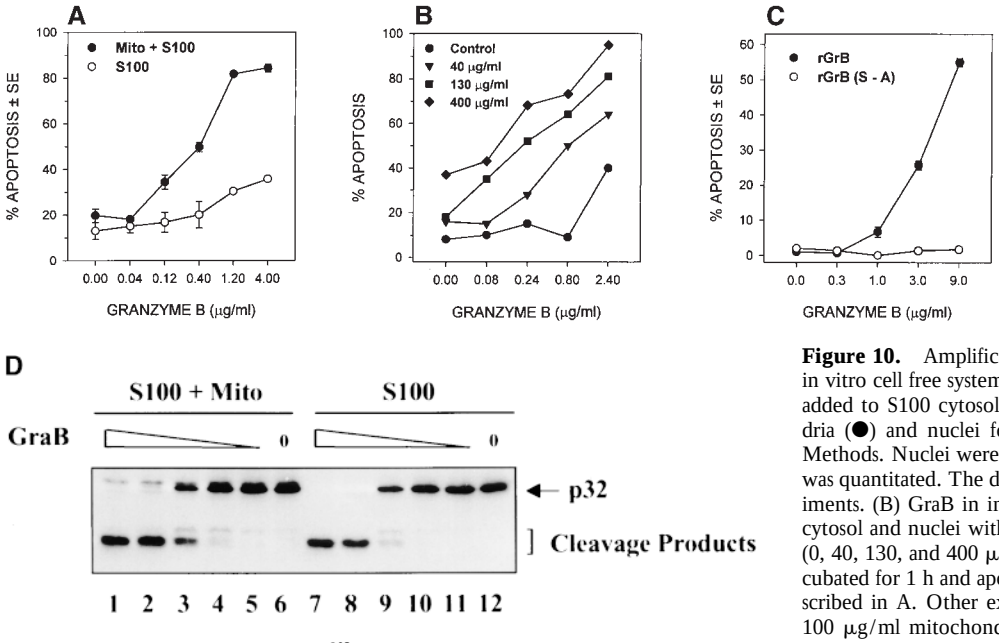

Figure 10. Amplification of GraB-induced apoptosis in an in vitro cell free system. (A) GraB in increasing amounts was added to S100 cytosol (○) or S100 cytosol plus mitochondria (●) and nuclei for 1 h as described in Materials and Methods. Nuclei were stained by Hoechst dye and apoptosis was quantitated. The data are the mean ± SE of three experiments. (B) GraB in increasing amounts was added to S100 cytosol and nuclei with increasing amounts of mitochondria (0, 40, 130, and 400 μg/ml mitochondrial protein), then incubated for 1 h and apoptotic nuclei were enumerated as described in A. Other experiments (A, C, and D) contained 100 μg/ml mitochondrial protein. (C) Recombinant GraB (rGrB; ●) or GraB containing an Ser^{203} to Ala mutation [rGrB(S-A); ○] were incubated with S100 cytosol, mitochondria, and nuclei as described in A. (D) S100 caspase 3 processing after GraB treatment at increasing doses in the presence or absence of mitochondria (Mito). Incubations were carried out as described in A and then cytosol was run on SDS-PAGE followed by Western blotting with anti–caspase 3 antibody. The dose of GraB increased from 0 (lanes 6 and 12) to 70 ng/ml (lanes 5 and 11), 200 ng/ml (lanes 4 and 10), 700 ng/ml (lanes 3 and 9), 2,000 ng/ml (lanes 2 and 8), and 6,000 ng/ml (lanes 1 and 7). The experiment was repeated three times with identical results.

age of caspase 3 was detected at 700 ng/ml, which is a threefold decrease in the dose required to detect processing (Fig. 10 D).

Discussion

This study demonstrates that activation of apoptosis by GraB and perforin is invariably accompanied by mitochondrial perturbation, including the release of cytochrome *c* and loss of mitochondrial transmembrane potential, and that GraB requires the presence of mitochondria to efficiently induce nuclear apoptosis in an in vitro cell-free system.

Disruption of Mitochondrial Transmembrane Potential by Granzymes and Perforin. It has been postulated that $\Delta\psi$ disruption is an early event in apoptosis and that it involves the opening of the PT pores of the inner mitochondrial membrane (21–23). Consistent with these data, we detected $\Delta\psi$ changes measured by the fluorochrome Rh123 or $DiOC_6$ (data not shown) after GraB and perforin treatment. However, we were unable to detect $\Delta\psi^{low}$ cells before the appearance of apoptotic nuclei or caspase 3 processing. Similarly, the lack of effect of CsA and inconsistent inhibition of apoptosis by BA in different target cells does not support a model in which PT initiates GraB apoptosis. However, we cannot exclude the possibility that PT may contribute to the amplification or continuation of apoptosis in some cells once they have been initiated by other signals.

We also find that the suppression of $\Delta\psi$ is insufficient to activate apoptosis. For example, perforin is unable to induce apoptosis and produces a morphology more typical of necrosis (2). The $\Delta\psi$ suppression by perforin may be related to the necrosis-like injury it mediates, which is similar to that associated with reperfusion injury after anoxia or neuro- or hepatotoxins (41). Perforin forms a nonspecific ion pore in the plasma membrane and induces a rapid increase in intracellular Ca^{2+} (42). Increases in cytosolic-free Ca^{2+} can themselves promote PT (43). Thus the $\Delta\psi$ suppression observed in perforin-treated cells may be secondary to this Ca^{2+} influx, which we have found contributes to the ability of perforin to induce cell death (42).

Another point of interest is that $\Delta\psi$ suppression induced by GraB, GraK, and perforin is not inhibited by the general caspase inhibitor zVAD-FMK. Since GraB probably directly activates caspases to initiate the apoptotic pathway, this is a surprising result and suggests that GraB may target another noncaspase protein that controls mitochondrial transmembrane potential. This caspase-independent pathway is also able to induce cell death because the zVAD-FMK inhibitor is only partially effective in blocking membrane damage by GraB and sublytic perforin doses, while completely inhibiting apoptosis at the same doses. Furthermore, zVAD-FMK has no effect on GraK or perforin-induced cell death nor on $\Delta\psi$ suppression (MacDonald, G., and A.H. Greenberg, data not shown). A caspase-independent cell death pathway induced by cytotoxic T lymphocytes and GraB has been described (20, 44). The data presented here suggest that GraB may target noncaspase cytoplasmic or mitochondrial proteins that lead to mitochondrial dysfunction and loss of transmembrane potential and apparent disruption of electron transport with ROS production. This caspase-independent mitochondrial pathway is regulated by Bcl-2, as loss of $\Delta\psi$, ROS production, and membrane damage induced by GraB, GraK, and perforin are inhibited by Bcl-2 overexpression. Thus, we propose that the disruption of mitochondrial function may be a part of a caspase-independent pathway for granzyme- and perforin-mediated cell death.

Control of Mitochondrial Cytochrome c Release in GraB-induced Apoptosis. As noted earlier, the release of cytochrome *c* by mitochondria has been postulated to be an important event in the activation of downstream caspases and apoptosis (24–26, 40). The data presented in this paper are consistent with the hypothesis that mitochondria are an important regulator of GraB-induced apoptosis. GraB induces the release of mitochondrial cytochrome *c* at or before the onset of the nuclear changes of apoptosis. Our experiments show that caspase 3 processing and the release of cytochrome *c* precede apoptosis by GraB and perforin treatment, thus indicating a temporal and perhaps causal relationship. Furthermore, the release of cytochrome *c* into the cytoplasm is blocked by either zVAD-FMK or YVAD-CHO but not DEVD-CHO, suggesting that upstream caspases propagate a signal that determines mitochondrial cytochrome *c* release. Candidates include upstream caspases 6, 8, 9, and 10, and caspase 1–like proteases. Caspases 8 and 10 are upstream caspases that bear the preferred GraB IEXD motif at the interchain processing site (4, 13) and have been shown to be processed during GraB and CTL apoptosis (20, 45), although caspase 10 is more readily processed by GraB in vitro than are other caspases (20). Although some cells derived from mice deficient in caspase 1 are more resistant to GraB (46), caspase 1 cannot be processed by GraB directly in vitro (47). Thus, a YVAD-inhibitable caspase 1–like protease may participate in the pathway but is probably not the primary target for GraB. Alternatively, YVAD-CHO may be nonspecifically inhibiting another upstream caspase. It is also clear from these experiments that a DEVD-CHO–inhibitable caspase controls GraB apoptosis and resides downstream of the mitochondria by virtue of its inability to block cytochrome *c* release. A model for caspases on the apoptotic pathway lying both upstream and downstream of mitochondria has been proposed for Fas (23, 48).

We find that GraB/perforin-induced apoptosis is regulated by Bcl-2 and Bcl-X_L overexpression in agreement with an earlier report (35). Both the release of cytochrome *c* and caspase 3 processing are suppressed in Bcl-2 overexpressing cells. If Bcl-2/Bcl-X_L suppress apoptosis primarily by the inhibition of cytochrome *c* release at the mitochondria then this would still allow GraB to interact with and directly cleave caspase 3 in cells overexpressing Bcl-2/Bcl-X_L. However, Bcl-2 also completely prevents caspase 3 processing by GraB (Shi, L., and A.H. Greenberg, unpublished data). In addition to its effects on cytochrome *c* release, Bcl-X_L acts by complexing with and preventing Apaf-1/Ced-4 from activating caspase 9/Ced-3 (32, 33,

49, 50), and therefore it is possible that caspases in a complex with Bcl-2 and a Ced-4 like mammalian protein are resistant to GraB proteolysis. At this time we cannot distinguish between the two potential mechanisms in which Bcl-2 inhibits GraB activity, either through blocking cytochrome *c* release or by physical interaction with a caspase substrate, thereby preventing direct cleavage. Other inhibitory mechanisms are possible, as Bcl-2/Bcl-X_L can act at sites other than mitochondria, including the endoplasmic reticulum and downstream of mitochondria (51–53).

Although the inhibition of GraB-induced apoptosis is clearly suppressed by Bcl-2/Bcl-X_L, their regulation of CTL is controversial. In some laboratories Bcl-2/Bcl-X_L appears to offer protection against killing mediated by CTL, NK, or LAK cells using a granzyme/perforin- or granule-mediated pathway (54–56). Others find little effect on CTL or NK cell killing in cells overexpressing Bcl-2 (35, 57, 58). Recent experiments by Sutton et al. (35), in which a comparative examination of susceptibility to apoptosis was made using the same cell lines, found a discordant resistance to GraB/perforin-induced apoptosis and susceptibility to CTLs, NK cells, or isolated granules in Bcl-2–expressing cells. These authors proposed that other granule components may provide the effector cells with the means to bypass the Bcl-2 block. These might include other granzymes such as GraA or GraK, which have been shown to induce apoptosis in vitro and contribute to CTL and NK killing alone or in combination with GraB (59–61), or other granzymes in the CTL granules (62). The molecular basis of the ability of CTLs to overcome Bcl-2 remains to be determined but this appears to be independent of the action of GraB and perforin.

Another possibility is suggested by the observation that Bcl-2 inhibition appears to distinguish two pathways of Fas/CD95 killing (58). In this model, Bcl-2 can only inhibit receptor signals generated through mitochondria when there is inefficient caspase 8 recruitment to the death-inducing signaling complex (DISC). With efficient caspase 8 recruitment, mitochondria are bypassed, effector caspases are activated directly, and Bcl-2 does not inhibit the process. Since GraB killing appears to become more mitochondria-independent at higher doses, one could hypothesize that Bcl-2 would be less inhibitory in these conditions because mitochondria are not necessary for apoptosis amplification. Thus CTLs expressing and delivering large amounts of GraB to the target cell would be expected to bypass mitochondria and be unaffected by Bcl-2 overexpression.

Mitochondria Amplify GraB-induced Apoptosis In Vitro. Earlier models suggested that GraB can directly activate caspase 3 to initiate cell death (17). Our data do show that GraB can initiate the processing of caspase 3 in S100 cytosol and that apoptosis is activated in the absence of mitochondria. Similar to earlier experiments in whole cell lysates (14), caspase 3 in S100 cytosol is cleaved by the addition of GraB. This indicates that a mitochondria-independent caspase 3 processing and apoptosis mechanism exists. We cannot distinguish between direct or indirect proteolysis of caspase 3. For example, it has been suggested by other experiments that caspase 10 is a preferred substrate (20), and thus GraB may also cleave caspase 3 indirectly via caspase 10.

Although a mitochondria-independent pathway exists for GraB apoptosis, it is clear that GraB induction of nuclear apoptotic activity is at least 15-fold greater in the presence of mitochondria. The apoptosis is dependent on the amount of mitochondria in the incubation and only proteolytically intact GraB is functional. The replacement of the active Ser^{203} to an Ala completely eliminates GraB activity in this reconstituted cell free system (shown in this study) as well as in intact cells in the presence of perforin (Shi, L., J. Lieberman, and A.H. Greenberg, data not shown). The addition of mitochondria has a two to threefold increase on the efficiency of caspase 3 processing by GraB under the identical conditions that produce a much larger (15-fold) increase in apoptosis. The relatively small enhancement in caspase 3 processing was probably not sufficient to account for the amplification of apoptosis, suggesting that other caspases are recruited by mitochondria, which then amplify apoptosis. That GraB may act through caspases other than caspase 3 is suggested from the finding that GraB/perforin CTL effectors kill target cells from caspase $3^{-/-}$ mice and cleave poly (ADP-ribose) polymerase (PARP) with high efficiency (45). In addition, MCF-7, which is caspase 3 deficient (63), is susceptible to GraB- and perforin-induced apoptosis (Shi, L., and A.H. Greenberg, unpublished data). A mitochondria-regulated pathway would probably be directed through caspase 9, which could then activate downstream caspases such as caspases 3 and 7. Alternatively, GraB may induce the release of another mitochondrial protein that

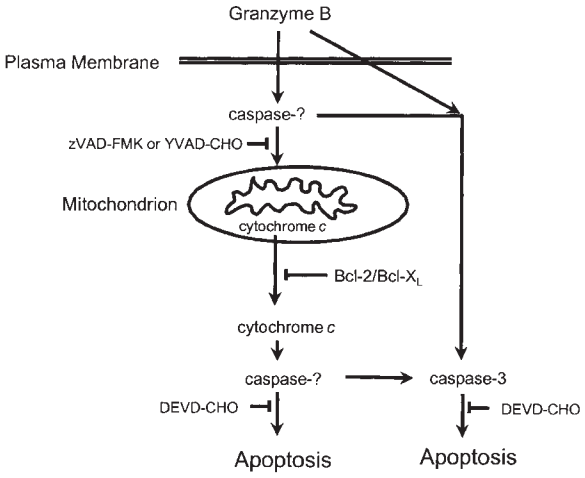

Figure 11. Model of mitochondria-dependent and -independent control of apoptosis induced by GraB. An upstream zVAD-FMK– or YVAD-CHO–inhibitable caspase is targeted by GraB, which then promotes the release of mitochondrial cytochrome *c*. Cytochrome *c* release and apoptosis are Bcl-2/Bcl-X_L regulated. This mitochondrial pathway activates caspase 3 and an unidentified but DEVD-CHO–inhibitable caspase. A second mitochondria-independent pathway regulates caspase 3 processing and apoptosis and is also DEVD-CHO inhibitable. Caspase 3 may be directly activated by GraB or activated indirectly through an upstream caspase that is initially targeted by GraB.

can augment the nuclear apoptotic changes mediated by the downstream effector caspases.

It is not clear from our data whether caspase 3 processing or mitochondrial perturbation is the earlier event, since we see simultaneous release of cytochrome *c* and caspase 3 cleavage after GraB/perforin treatment. For example, GraB may first activate caspase 3 followed by a caspase 3–directed initiation of the mitochondrial amplification mechanism to further expand the processing of downstream caspases. It has been observed recently that caspase 3 cleaves Bcl-2 and Bcl-X_L, which relieves their suppressive effects and allow activation of apoptosis (64, 65). However, if caspase 3 activation preceded mitochondrial perturbation, then DEVD-CHO inactivation of caspase 3 should block cytochrome *c* release by GraB, which it does not under the conditions of our experiments. The alternative interpretation is that GraB targets an upstream caspase(s) that then triggers a mitochondria-directed apoptosis pathway and the activation of downstream caspases. A mitochondria-independent pathway also exists in which GraB either directly cleaves caspase 3, or does so through the processing of an upstream caspase. The much greater activity observed in the presence of mitochondria at lower GraB doses suggests that this is the primary amplification pathway for apoptosis (see model in Fig. 11).

In conclusion, we have identified the mitochondrial regulation of GraB-induced apoptosis. We suggest that GraB-induced apoptosis is highly amplified by mitochondria in a caspase-dependent manner but that GraB can also initiate caspase 3 processing and apoptosis in the absence of mitochondria. We also propose that perforin alone or perforin and granzymes induce caspase-independent mitochondrial disruption and cell death.

We thank Dr. Don Nicholson for the anti-caspase 3 antibody and Dr. Ed Rector for his help with flow cytometry analysis. We also thank Zhinan Xia for his help in developing the mutant recombinant GraB.

This work was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada (NCIC). A.H. Greenberg is a Terry Fox Cancer Research Scientist of the NCIC.

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Received for publication 6 July 1998 and in revised form 11 September 1998.

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