Granule B-mediated Apoptosis Proceeds Predominantly through a Bcl-2-inhibitable Mitochondrial Pathway*

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Cytotoxic T lymphocytes kill virus-infected and tumor cell targets through the concerted action of proteins contained in cytolytic granules, primarily granzyme B and perforin. Granzyme B, a serine protease with substrate specificity similar to the caspase family of apoptosis-cysteine proteinases, is capable of cleaving and activating a number of death proteins in target cells. Despite the ability to engage the death pathway at multiple entry points, the preferred mechanism for rapid induction of apoptosis by granzyme B has yet to be clearly established. Here we use time lapse confocal microscopy to demonstrate that mitochondrial cytochrome c release is the primary mode of granzyme B-induced apoptosis and that Bcl-2 is a potent inhibitor of this pivotal event. Caspase activation is not required for cytochrome c release, an activity that correlates with cleavage and activation of Bid, which we have found to be cleaved more readily by granzyme B than either caspase-3 or caspase-8. Bcl-2 blocks the rapid destruction of targets by granzyme B by blocking mitochondrial involvement in the process.

Cytotoxic T lymphocytes (CTL) and natural killer cells induce apoptosis in their targets through the concerted action of effector molecules contained in cytolytic granules that engage the death pathway (1, 2). Granzyme B enters the target cell and, with another granule protein, perforin, triggers all of the characteristic manifestations of apoptosis, providing the principal mechanism of killing by CD8+ CTL and natural killer cells (3). Granzyme B is a serine protease that shares substrate specificity with many members of the caspase family of cysteine proteases (4). In fact, granzyme B cleaves and activates the apical caspase, caspase-8, as well as caspases-3 (5, 6), -6, and -7 (7, 8). Granzyme B can directly activate caspase-3 and is capable of triggering apoptosis at multiple points of the caspase-dependent pathway (9, 10) and therefore is not absolutely dependent on caspase-8 cleavage. This pathway differs from another common death pathway utilized by CTL, signaling through the Fas surface receptor by Fas ligand expressed on the surface of the CTL. Apoptotic signaling through Fas requires an obligate activation of caspase-8 (11) and can proceed via mitochondria-dependent or -independent pathways (12). The mitochondrial pathway involves the release of cytochrome c for caspase activation and apoptosis (13, 14).

Release of mitochondrial cytochrome c is a pivotal event in the apoptosis of many cell types induced by many stimuli (15, 16). Upon release, cytochrome c binds Apaf-1 and promotes the formation of an oligomeric Apaf-1 apoptosome that recruits and activates the effector caspase, caspase-9 (17–19). In receptor-mediated apoptosis the requirement for cytochrome c release is dependent on the type of cell triggered to die, and the decision to utilize the mitochondrial route appears to rely primarily on the concentration of caspase-8 (20). Although the same pathways are used in granule-mediated apoptosis, the conditions governing the premitochondrial events remain to be clearly established.

Granule-driven CTL killing represents a very effective means to induce apoptosis largely because of the ability of granzyme B to engage the death pathway at multiple entry points. Despite recent advances outlining the mechanism(s) of action of granzyme B within the target cell, there are still numerous conflicting reports regarding the requirements and dependence of granzyme B-mediated apoptosis on caspases and the potential for inhibition by the anti-apoptotic proto-oncogene, Bcl-2. It is clear that granzyme B is capable of cleaving and activating a number of key enzymes in the caspase cascade, notably caspases-8 and -3 (10, 21–23), but it is unclear what preference, if any, exists for each potential event. In this report we address the contribution of some of these aspects of granzyme-mediated apoptosis. Also, because granzyme B can activate the effector caspases directly, we sought to determine the necessity for cytochrome c release and its contribution to the efficient death in target cell apoptosis.

In early studies involving Bcl-2 in CTL-mediated apoptosis, it appeared that target cell expression of the proto-oncogene could confer some protection from CTL (24–26). Cytochrome c release in target cells has been shown to occur, but it is not clear whether it is a requirement for death. In this report we...
follow single cells within populations to ascertain the order and extent of cytochrome c release in the context of its (in)dependence on caspase activation. We have also utilized our experimental system to assess the effects of Bcl-2 on both cytochrome c release and subsequent apoptosis. In this way we determined that: 1) Bcl-2 inhibits cytochrome c release and the ultimate death of the target cell; and 2) granulysin B induces target cell cytochrome c release in a caspase-independent manner via Bid proteolysis. Because apoptotic Bid expressing cells do not release cytochrome c, we also conclude that when necessary, granulysin B bypasses the mitochondria via direct activation of effector caspases rather than overriding Bcl-2 by proteolytic degradation or some other mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Jurkat and Jurkat-Bcl-2 were cultured in RPMI with 10% fetal calf serum, penicillin, and streptomycin. We also employed an HeLa cell line stably transfected with cytochrome c-GFP (denoted 2H18) as described previously (27). Cytochrome c-GFP was shown to behave identically to cytochrome c in 2H18 in apoptosis assays including side by side immunoblots for cytochrome c and cytochrome c-GFP in subcellular fractions as well as immunofluorescence assays on sections of these cells. Transformation of the gene in the plasmid pEFGPKapo. The transfected cells were selected for resistance to puromycin, and surviving clones resistant to UV- and actinomycin D-induced apoptosis were analyzed for Bid expression by Western blot analysis (Lucidis Biotech; Philadelphia, PA). The data from one clone (2H18-Bcl-2) are reported here. Both 2H18 and 2H18-Bcl-2 were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. All cells were grown at 37 °C under 5% CO₂. Granzyme B was purified from the human natural killer cell line YT-Indy as described previously (28). The broad spectrum caspas inhibitor zVAD-fmk was purchased from Enzyme Systems Products (Dublin, CA). Antibodies against cytochrome c (1H5.2C12) were purchased from Pharmingen. Rabbit anti-Bid was raised against the peptide N-REDVFHTVFNINGNLRTYVRSLARNGMD corresponding to a sequence in the C terminus of Bid. Specificity of the Bid antisera was verified by Western blot analysis of purified recombinant Bid and GST-Bid protein. N-terminal protein sequencing was performed by core protein facility at the Scripps Research Institute on an excised fragment transferred onto sequencing polyvinylidene difluoride (Amersham Pharmacia Biotech) stained in Coomassie Blue in the absence of acetic acid. Apoptosis and Cytochrome c Release Assays—CTF-free apoptosis was induced as described previously (28, 29). Briefly, target cells were incubated in medium containing 0.5% serum with either 1 μg/ml granzyme B and 10 plaque-forming units/ml replication-deficient adenovirus type 5 or 100 ng/ml anti-Fas (CH-11) for the times indicated. Phosphatidylserine (PS) externalization was assayed with annexin V-fluorescein isothiocyanate (CLONTECH; Palo Alto, CA) labeling and assessment with a Becton-Dickinson FACScan flow cytometer. Protein lysates from apoptotic cells were produced by direct lysis into gel loading buffer containing SDS and subjected to examination by standard SDS-PAGE techniques. SDS-PAGE techniques are as follows. Measurement of the reduction of full-length Bid protein was performed by densitometric analysis of Western blots. Mitochondria were isolated from Xenopus laevis according to Kluck et al. (30). After incubation at 30 °C under experimental conditions noted in the text, mitochondria were removed from solution by centrifugation at 14,000 rpm for 10 min at 4 °C. Pellets containing mitochondria were resuspended in gel sample buffer containing SDS and 2-mercaptoethanol. Mitochondrial and supernatant protein samples were analyzed for cytochrome c by standard immunoblotting techniques after denaturing PAGE. Granulysin B Substrate Assays—The cDNA encoding full-length human Bid was expressed as a GST fusion protein in the vector pGEX-4T-1 in BL21(DE3) cells as described earlier (31). Bid was used as a GST fusion protein or on its own after GST was removed by thrombin digestion followed by purification over GSH-Sepharose (Amersham Pharmacia Biotech). Radiolabeled Bid, caspase-3, and caspase-8 were produced in vitro via transcription and translation (Promega, Madison, WI) from full-length clones in the vector pCDNA-3. Specific activity of each translation product was calculated according to the amount of incorporated [35S]Met radiolabel relative to the number of methionine residues in each polypeptide. Granulysin B digests were performed at 30 °C under conditions described previously (32). Samples were analyzed by SDS-PAGE, fixed in gel, dried, and exposed to Kodak X-Omat radiography film. All reactions were carried out with concentrations of substrate (3 μM) well below the Km, and first-order kinetics were assumed. kcat/Km values were calculated from the linear relation between log(S/So) versus granzyme B concentration where S is the concentration of substrate at time t, and So represents the initial substrate concentration. FACS Analyses—2H18 and 2H18-Bcl-2 cells cultured in flat-bottomed 96-well plates were harvested at the times indicated using 0.25% trypsin. Floating cells and trypsin-treated cells were combined and pelleted in a round bottomed 96-well plate. The medium was aspirated, and the cells were resuspended in 100 μl of ice-cold CLAMI buffer (80 mM KC1 and 25 μg/ml digitonin in phosphate-buffered saline). The cells were incubated on ice for 5 min and analyzed directly for GFP content by FACS analysis. Because cytosolic cytochrome c-GFP is released into the CLAMI buffer, in which mitochondria had not released cytochrome c-GFP were ~0.5–1 log brighter in FL-1 than cells in which the mitochondria had released their cytochrome c-GFP. PS exposure was detected by staining the cells with 10 μM viva annexin V-Alexa 568 and exposed to Kodak X-Omat radiography film. All reactions were carried out with concentrations of substrate (3 μM) well below the Km, and first-order kinetics were assumed. kcat/Km values were calculated from the linear relation between log(S/So) versus granzyme B concentration where S is the concentration of substrate at time t, and So represents the initial substrate concentration. RESULTS The Primary Granulysin B-mediated Death Pathway Involves Mitochondrial Cytochrome c Release—To study the molecular events during target cell apoptosis, we utilized an experimental system of granule-mediated killing wherein purified granulysin B is added directly to the medium of target cells in the presence of a replication-deficient adenovirus (28, 33, 34). Although granulysin B is capable of entering the target autonomously, apoptosis does not occur in the absence of virus. The granulysin B adenovirus system has been shown to trigger all biochemical and cellular manifestations of target cell apoptosis observed when target cells are treated with granulysin B and perforin and those treated with whole CTL (9, 28, 29, 33–36). Using 2H18 and 2H18-Bcl-2, novel cell lines stably expressing cytochrome c-GFP ± Bcl-2, we examined molecular events upstream and downstream of mitochondrial cytochrome c release in granulysin-mediated killing. Using this system we sought to establish a preference, if any, given to Bcl-2-inhibitable events by delivering granulysin B at limiting doses. 2H18 and 2H18-Bcl-2 cells were treated with decreasing amounts of granulysin B along with a constant amount of adenovirus. After 2 and 4 h, cells were harvested and quantitatively assayed by flow cytometry for apoptosis by annexin V binding. At concentrations of granulysin B as low as 10 ng/ml we observed rapid release of cytochrome c which corresponded with PS externalization (Fig. 1). Bcl-2 was capable of inhibiting death at doses of granulysin B as high as 1 μg/ml. As observed earlier, the presence of zVAD-fmk, which afforded no protection from cytochrome c release, inhibited PS externalization, which presumably requires caspase activity in short term assays. To determine if granulysin B can overcome the Bcl-2 block at longer time points, we treated 2H18-Bcl-2 cells with granulysin and adenovirus and followed the target cells in culture by time lapse video confocal microscopy. As showed in Fig. 2, when 2H18-Bcl-2 cells were transfected and matured in the presence of granulysin B and adenovirus, there was no release of cytochrome c. After an extensive incubation (24 h), we observed only a small number of apoptotic cells, and we did not observe cytochrome c release in the apoptotic cells. This suggests that granulysin B treatment cannot override the Bcl-2 blockade of cytochrome c release and...
is consistent with the model in which granzyme B is capable of activating effector caspases directly (10, 21, 22). However, it is clear from our observations that the most efficient pathway to death in targets of granzyme B involves release of cytochrome c from the mitochondria.

Cytochrome c Release by Granzyme Is Caspase-independent—To characterize further the events leading to cytochrome c release during target cell apoptosis, we utilized target cells expressing cytochrome c-GFP. HeLa has proven to be an excellent target for killing by granzyme B in combination with adenovirus (28, 29), and therefore we utilized 2H18, the HeLa sublines expressing cytochrome c-GFP described above to study cytochrome c release during granzyme B-mediated apoptosis. The top series of panels in Fig. 3 shows a representative field of 2H18 cells incubated in the presence of granzyme B and adenovirus which shows cytochrome c-GFP release within 60 min as indicated by the diffuse cytoplasmic pattern. The bottom series of panels shows that the broad spectrum caspase inhibitor zVAD-fmk did not diminish the time required to achieve comparable levels of cytochrome c release. Therefore, caspase-8 is not an obligate requirement for cytochrome c release in granzyme B-treated target cells. These data are consistent with the observation that granzyme B/adenovirus induces a drop in the mitochondrial transmembrane potential which does not require active caspases (36).

In a similar set of experiments we treated Jurkat targets with granzyme B and adenovirus in the presence and absence of zVAD-fmk. Fig. 4A shows an immunoblot time course of cytosol isolated from treated Jurkat. In granzyme B/adenovirus-treated cells, cytosolic cytochrome c appeared within 15–30 min, whereas no cytosolic cytochrome c was observed in cells treated with adenovirus alone. In Jurkat incubated in 100 μM zVAD-fmk prior to the addition of granzyme and adenovirus, there was no reduction in the appearance of cytosolic cytochrome c, thus demonstrating further that caspases are not required for this phenomenon to occur. Similarly, experiments in Jurkat-Bcl-2 targets showed that expression of Bcl-2 com-
pletely prevented granzyme B-mediated cytochrome c release as noted by the absence of cytosolic cytochrome c. Analysis of the mitochondrial pellets (Fig. 4A, bottom panel) showed that cytochrome c was maintained in its original cellular fraction.

Cytochrome c Release by Granzyme B-activated Bid—Because granzyme B directly cleaves Bid and cytochrome c release can be achieved without caspase activation, we reasoned that granzyme B might mediate cellular cytochrome c release in a caspase-independent manner through Bid. To test this model, we treated isolated mitochondria with purified Bid processed by granzyme B. Fig. 4B shows an immunoblot of supernatants from mitochondria treated with increasing concentrations of granzyme B-cleaved and uncleaved Bid. At the highest concentrations of untreated Bid tested (100 ng/ml) we observed some cytochrome c release, but Bid that had been activated by prior treatment with granzyme B was significantly more potent with respect to its cytochrome c releasing activity. Full-length GST-Bid, which possesses no cytochrome c releasing activity, became active after treatment with granzyme B. When GST-Bid was processed by granzyme B, the cytochrome c releasing activity was comparable to that observed with processed wild type Bid. It is important to note that mitochondria treated with granzyme B alone did not release cytochrome c, indicating that granzyme B does not directly cleave any mitochondria-associated proteins that result in cytochrome c release. Although granzyme B cleaves Bid at only one of the caspase-8 sites, Bid processed by granzyme B is active in releasing mitochondrial cytochrome c.

Granzyme B cleaves and activates Bid in vitro, so we next set out to ascertain the pattern and requirements for Bid cleavage in target cells treated with granzyme B. Jurkat targets were treated with granzyme B and adenovirus for 3 h at 37 °C.
Whole cell lysates from these cells were analyzed for the presence and cleavage of Bid protein. As shown in Fig. 4C, there was significant cleavage of Bid in granzyme B adenovirus-treated cells, which corresponds with cytosolic cytochrome c release (Fig. 4A). The reduction in full-length Bid protein in granzyme-treated targets was not inhibited in the presence of the broad spectrum caspase inhibitor zVAD-fmk. Whereas granzyme-mediated Bid cleavage was independent of caspase activation, Jurkat treated with anti-Fas required active caspases for Bid cleavage to occur because cleavage of Bid in anti-Fas-treated Jurkat, which proceeds via caspase-8 activation, was inhibited by zVAD-fmk. These data are in agreement with our previous findings that cleavage of Bid in target cells still occurs in the presence of the viral serpin caspase-8 inhibitor, SPI-2 (9). Similar treatment of targets overexpressing Bcl-2 did not block cleavage of Bid by death stimulus, granzyme B adenovirus, or anti-Fas, suggesting that Bid processing occurs upstream of the apoptotic inhibition imparted by Bcl-2.

Kinetics of Bid Cleavage by Granzyme B—Granzyme B cleaves and activates Bid, thus providing a mechanism by which granzyme B can cause caspase-independent cytochrome c release. In light of these observations we analyzed Bid as a substrate for granzyme B. To facilitate purification of the C-terminal cleavage product away from the similar sized N-terminal fragment, we used purified GST-Bid fusion protein. As shown in Fig. 5A, granzyme B cleaves GST-Bid in a dose-dependent manner. After resolution by SDS-PAGE and transfer to polyvinylidene difluoride, the C-terminal 12-kDa cleavage fragment was subjected to N-terminal sequencing, and the sequence that can also be recognized by caspase-8. Even at the highest doses of granzyme B there were no detectable fragments corresponding to cleavage at Asp-75, the preferred caspase-8 cleavage site. This confirms the earlier observation of Li et al. (37) using Bid mutants, suggesting that granzyme B cleaves at Asp-75, whereas caspase-8 prefers Asp-59 and is consistent with the pattern of Bid cleavage products observed during granzyme B-mediated events reported recently (9). It is important to note that although the granzyme B and caspase-8 cleavage sites differ within Bid, both cleavage events are capable of activating Bid.

In Fas-mediated apoptosis the apical death-inducing signaling complex-associated caspase, caspase-8, has been shown to activate Bid to effect cytochrome c release (37, 38). We recently demonstrated that granzyme B can bypass caspase-8 activation (9). We sought to clarify the relevance of granzyme B-dependent Bid cleavage by comparing the preference of granzyme B for Bid and caspase-8 as substrates. We performed in vitro cleavage assays to compare the rates of cleavage of Bid and caspases. Equimolar amounts of radiolabeled in vitro transcribed and translated substrate were digested with increasing concentrations of granzyme B. As shown in Fig. 5, B and C, granzyme B cleaved Bid more efficiently than caspase-8 or caspase-3. Granzyme B at concentrations as low as 3.75 nM cleaved Bid, whereas these concentrations induced little or no caspase-3 or caspase-8 cleavage. Based on the published $k_{cat}/K_m$ values for this cleavage, using similar measurements (22) we calculated relative $k_{cat}/K_m$ values for granzyme B cleavage of Bid (6.0 x $10^5$ M$^{-1}$ s$^{-1}$) and caspase-8 (2.4 x $10^4$ M$^{-1}$ s$^{-1}$). These data suggest that Bid is a better substrate for granzyme B than caspase-8 and caspase-3 by more than 10-fold and support the notion that Bid represents a target protein sensitive to activation by granzyme B. They also support our contention that the Bcl-2-nhibitable mitochondrial pathway is utilized more efficiently by granzyme B than that involving direct activation of caspases.

DISCUSSION

Granzyme B is a critical mediator of target cell death. This is demonstrated by the inability of CTL and natural killer cells from granzyme B$^{-/-}$ mice to induce rapid DNA fragmentation and subsequent apoptosis in allogeneic target cells (39, 40). These knockout mice also have severely depressed ability to overcome infection by cytomegalovirus (39, 41) and ectromelia (42). There is some low efficiency residual killing activity in the granzyme B$^{-/-}$ mice which is attributed to another granule protease, granzyme A, in a mechanism that is thought to provide a backup to the principal mechanism involving granzyme B (43). Double knockout of granzymes B and A results in a phenotype similar to that observed in the perforin knockout mouse (42, 44). The mechanism by which granzyme B activates apoptosis is therefore of significant interest.

Granzyme B cleaves and activates Bid, which provides a caspase-independent means of releasing cytochrome c during CTL granule-mediated apoptosis. In physiological settings it is possible that granzyme B also utilizes caspase-8 activation as a means to bring about the rapid destruction of its targets. However, direct activation of Bid by granzyme B provides a caspase-independent pathway to mitochondria. Thus, caspase-8 may contribute but is not required for cytochrome c release in granule-mediated apoptosis. It is also clear from our data and others (45) that regardless of the availability of caspase-8, the most efficient pathway to target cell apoptosis is via the mitochondria. As one might expect, redundancy is built into the
CTL arsenal, and granzyme B is capable of initiating apoptotic events through a mitochondrial bypass route by direct activation of downstream caspases, but with greatly reduced efficiency. It was shown previously that Bid is cleaved by granzyme B (37), but it remained to be demonstrated that this cleavage event resulted in active Bid with respect to its cytochrome c releasing activity. However, it is abundantly evident from our observations that although the preferred caspase-8 and granzyme B cleavage sites in Bid differ, cleavage of Bid by granzyme B results in productive activation and potent cytochrome c release in granzyme B-treated cells. Panel A, cytochrome c release in Jurkat by granzyme B adenovirus (Ad) is caspase-independent but is inhibited by Bcl-2. Jurkat were treated with granzyme B and adenovirus for the times indicated in the presence or absence of the caspase inhibitor zVAD-fmk or Bcl-2. Cytosol was isolated away from mitochondria and lysates analyzed by immunoblot. Bands corresponding to cytochrome c are marked by asterisks for each treatment. Cytochrome c release was detected in the presence of zVAD-fmk but was inhibited by Bcl-2. Controls in which cells incubated for 120 min with granzyme B or adenovirus alone were also included. Panel B, granzyme B “activated” Bid causes cytochrome c release in isolated mitochondria. Isolated Xenopus mitochondria were treated with full-length Bid or Bid that had been digested with 17.5 ng/ml granzyme B prior to the addition to mitochondria. After incubation of mitochondria with 1, 10, or 100 ng/ml purified Bid or equimolar amounts of GST-Bid at 30 °C for 1 h, mitochondrial pellets were removed by centrifugation, and the supernatants were analyzed for the presence of cytochrome c by immunoblot analysis. Untreated mitochondria incubated at 30 °C and 4 °C were included as negative controls, and the supernatant from a Nonidet P-40-treated mitochondria sample was included as a positive control for cytochrome c release. Panel C, cleavage of Bid is caspase-independent and upstream of Bcl-2. Jurkat and Jurkat stably expressing Bcl-2 were treated with 1 μg/ml granzyme and adenovirus for 3 h in the absence or presence of 100 μM zVAD-fmk. Cell lysates were collected, analyzed by immunoblot for disappearance of the full-length Bid, and quantitated with NIH-Image software. The percentage of Bid cleavage was normalized according to the amount of full-length Bid in untreated cells.

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Fig. 4. Bid cleavage and cytochrome c release are caspase-independent in granzyme B-treated cells. Panel A, cytochrome c release in Jurkat by granzyme B adenovirus (Ad) is caspase-independent but is inhibited by Bcl-2. Jurkat were treated with granzyme B and adenovirus for the times indicated in the presence or absence of the caspase inhibitor zVAD-fmk or Bcl-2. Cytosol was isolated away from mitochondria and lysates analyzed by immunoblot. Bands corresponding to cytochrome c are marked by asterisks for each treatment. Cytochrome c release was detected in the presence of zVAD-fmk but was inhibited by Bcl-2. Controls in which cells incubated for 120 min with granzyme B or adenovirus alone were also included. Panel B, granzyme B “activated” Bid causes cytochrome c release in isolated mitochondria. Isolated Xenopus mitochondria were treated with full-length Bid or Bid that had been digested with 17.5 ng/ml granzyme B prior to the addition to mitochondria. After incubation of mitochondria with 1, 10, or 100 ng/ml purified Bid or equimolar amounts of GST-Bid at 30 °C for 1 h, mitochondrial pellets were removed by centrifugation, and the supernatants were analyzed for the presence of cytochrome c by immunoblot analysis. Untreated mitochondria incubated at 30 °C and 4 °C were included as negative controls, and the supernatant from a Nonidet P-40-treated mitochondria sample was included as a positive control for cytochrome c release. Panel C, cleavage of Bid is caspase-independent and upstream of Bcl-2. Jurkat and Jurkat stably expressing Bcl-2 were treated with 1 μg/ml granzyme and adenovirus for 3 h in the absence or presence of 100 μM zVAD-fmk. Cell lysates were collected, analyzed by immunoblot for disappearance of the full-length Bid, and quantitated with NIH-Image software. The percentage of Bid cleavage was normalized according to the amount of full-length Bid in untreated cells.

Fig. 5. Kinetics of Bid cleavage by granzyme B. Purified GST-Bid was incubated in increasing doses of granzyme B (0.1, 0.25, 1, 2.5, 10, and 25 ng/ml; 3.75 nM–0.94 μM), resolved on standard SDS-PAGE, transferred to sequencing polyvinylidene difluoride, and stained in Coomassie Brilliant Blue. To determine the site at which granzyme B cleaves Bid, we subjected GST-Bid to complete digestion by granzyme B, and the band corresponding to the new C-terminal fragment(s) was excised and subjected to N-terminal sequencing. No other fragments were observed, even at high concentrations of granzyme B. For comparison of Bid, caspase-3, and caspase-8 as substrates for granzyme B, equimolar amounts of in vitro transcribed and translated proteins were subjected to digestion by increasing amounts of granzyme B for 30 min at 30 °C. The amount of granzyme B (0.1, 0.25, 1, 2.5, 10, and 25 ng/ml; 3.75 nM–0.94 μM) was kept consistent between substrates. The cleavage products were analyzed by SDS-PAGE and autoradiography. Concentrations of granzyme B required to cleave detectable amounts of caspase-3 and caspase-8 (lower panels) were capable of cleaving Bid to completion, suggesting that Bid is a better substrate than either of these caspases for granzyme B.
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Granzyme B can activate caspases directly, the preferred route to apoptosis under limiting conditions is via Bid cleavage, mitochondrial outer membrane permeabilization, and cytochrome c release. It is also likely that these events are accompanied by the release of Smac/Diablo from the mitochondrial intermembrane space, which functions to interfere with the inhibitory activity of inhibitor of apoptosis proteins. The latter may block caspases activated by granzyme B, and therefore the involvement of mitochondrial outer membrane permeabilization in granzyme B-induced apoptosis might be through this molecule (or in conjunction with cytochrome c). In any case, the involvement of the mitochondria in granzyme B-induced apoptosis provides an explanation for the ability of Bcl-2 to block CTL-induced apoptosis in many cases, depending on the cells being targeted and the amount of granzyme B that enters those cells.

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