Initiation of Apoptosis by Granzyme B Requires Direct Cleavage of Bid, but Not Direct Granzyme B–mediated Caspase Activation

By Vivien R. Sutton,* Joanne E. Davis,* Michael Cancilla,‡ Ricky W. Johnstone,* Astrid A. Ruefli,* Karin Sedelies,* Kylie A. Browne,* and Joseph A. Trapani*

*From the *Cancer Immunology Laboratory, Peter MacCallum Cancer Institute, Melbourne 8006, Australia; and the ‡The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Melbourne 3050, Australia*

Abstract

The essential upstream steps in granzyme B–mediated apoptosis remain undefined. Herein, we show that granzyme B triggers the mitochondrial apoptotic pathway through direct cleavage of Bid; however, cleavage of procaspases was stalled when mitochondrial disruption was blocked by Bcl-2. The sensitivity of granzyme B–resistant Bcl-2–overexpressing FDC-P1 cells was restored by coexpression of wild-type Bid, or Bid with a mutation of its caspase-8 cleavage site, and both types of Bid were cleaved. However, Bid with a mutated granzyme B cleavage site remained intact and did not restore apoptosis. Bid with a mutation preventing its interaction with Bcl-2 was cleaved but also failed to restore apoptosis. Rapid Bid cleavage by granzyme B (<2 min) was not delayed by Bcl-2 overexpression. These results clearly placed Bid cleavage upstream of mitochondrial Bcl-2. In granzyme B–treated Jurkat cells, endogenous Bid cleavage and loss of mitochondrial membrane depolarization occurred despite caspase inactivation with z-Val-Ala-Asp-fluoromethylketone or Asp-Glu-Val-Asp-fluoromethylketone. Initial partial processing of procaspase-3 and -8 was observed irrespective of Bcl-2 overexpression; however, later processing was completely abolished by Bcl-2. Overall, our results indicate that mitochondrial perturbation by Bid is necessary to achieve a lethal threshold of caspase activity and cell death due to granzyme B.

Key words: granzyme B • Bid • apoptosis • Bcl-2 • perforin

Introduction

CTLs and NK cells utilize two mechanisms to kill virus-infected and premalignant cells, both of which require direct effector–target cell contact. In one mechanism, engagement of cell surface Fas (CD95) molecules by Fas ligand (FasL) on the effector cell recruits a signaling complex to the inner leaflet of the target cell membrane, resulting in activation of procaspase-8 (1). This mechanism mediates lymphoid homeostasis in higher animals (2). The second mechanism utilizes granule-bound toxins, most importantly a membranolytic agent, perforin, and the serine protease, granzyme B, which are liberated from secretory vesicles in the killer cell cytoplasm into the target cell (3). This mechanism is essential for host protection against some virus infections and cellular transformation (4). Studies with gene knockout mice indicate that perforin is indispensable for eliciting apoptosis in concert with granzymes (4). Although perforin cannot induce apoptotic death per se, it provides access for proapoptotic granzymes to death substrates in the target cell, thus initiating the death cascade (5–8). Perforin's essential role probably involves disruption of granzyme-containing target cell endosomes, as its function can be accurately mimicked by endosomolytic agents such as adenovirus (8), listeriolysin, and pneumococcal pneumolysin (PLO)[1] without the requirement for large transmembrane pores (3).

Address correspondence to Joseph A. Trapani, Peter MacCallum Cancer Institute, Locked Bag 1, A'Beckett St., Melbourne 8006, Australia. Phone: 61-3-9656-3726; Fax: 61-3-9656-1411; E-mail: j.trapani@pmci.unimelb.edu.au

[1]*Abbreviations used in this paper:* Apaf-1, apoptotic protease-activating factor 1; IAP, inhibitor of apoptosis proteins; PLO, pneumococcal pneumolysin; t, truncated; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2000/11/1403/11 $5.00
Volume 192, Number 10, November 20, 2000 1403–1413
http://www.jem.org/cgi/content/full/192/10/1403

The granzymes are structurally related, but have diverse substrate preferences. Through its unique ability to cleave after aspartate residues, granzyme B can cleave many procaspases in vitro (9–11) and is highly toxic to target cells (5). Not surprisingly, it has been assumed until now that granzyme B kills cells by direct caspase activation, supplemented under certain circumstances by direct damage to downstream caspase substrates (12). Having accessed the cytosol, granzyme B is rapidly translocated to the nucleus (13) and can cleave poly(ADP-ribose) polymerase and nuclear matrix antigen, sometimes using different cleavage sites than those preferred by caspases (12). Although many procaspases are efficiently cleaved in vitro, granzyme B–induced caspase activation occurs in a hierarchical manner in intact cells, commencing at the level of executioner caspases such as caspase-3, followed by caspase-7 (14). This is in contrast to FasL-mediated killing, which relies on a membrane signal generated through apical caspases such as caspase-8 (15, 16). Surprisingly, granzyme B can also induce death through a caspase-independent mechanism that involves direct damage to nonnuclear structures, although the key substrates in this pathway have yet to be elucidated (17–20). These additional pathways probably safeguard against viruses that delay programmed cell death by expressing serpins such as the efficient caspase-8 inhibitor crmA elaborated by cowpoxvirus (21) and baculovirus p35, which inhibits a broader range of caspases (22).

Although several apoptotic pathways have been attributed to granzyme B, the essential upstream steps that impart the death signal have not been defined. It has been considered a fait accompli by many investigators that this step simply involves the direct activation of caspases; however, this proposition has not been rigorously tested, nor is it consistent with all of the published evidence. For example, we (23, 24) and other groups (25) have shown that Bcl-2 and related inhibitors such as Epstein-Barr virus BHRF1 (26) can block both the caspase-dependent and -independent pathways to granzyme B–mediated apoptosis. Importantly, we confirmed that all granzyme B death pathways were blocked in Bcl-2–expressing cells, using long-term assays of cell growth and clonogenic capacity (24). We found that Bcl-2 overexpression had no effect on granzyme B uptake into the cell cytoplasm or sensitivity to perforin lysis, but its redistribution to the nucleus after perforin addition was totally inhibited (27). MacDonald et al. (28) also recently reported that granzyme B–mediated cell death was enhanced by the addition of mitochondria to in vitro apoptotic extracts of cells treated with granzyme B, and postulated that mitochondrial factors greatly amplify extramitochondrial caspase activation. The finding that Bcl-2 could rescue cells from granzyme B–mediated cell death was unexpected, as Bcl-2 and related molecules are thought to specifically block only those pathways that operate directly through mitochondrial perturbation, such as growth factor withdrawal, irradiation, and exposure to certain toxins. Bcl-2 and BHRF1 suppress the mitochondrial pathway by preventing loss of mitochondrial membrane depolarization and inhibiting the release of cytochrome c and apoptosis-inducing factor into the cytosol (29). Collectively, these results strongly suggested that mitochondrial constituents are essential for granzyme B–mediated apoptosis, and that this pathway can be regulated by Bcl-2. However, it was difficult to reconcile these findings with granzyme B's demonstrated ability to directly activate many caspases in vitro.

Granzyme B's "preference" for activating the mitochondrial pathway raised other important issues. In particular, how does Bcl-2 overexpression regulate granzyme B when there is no evidence that the two molecules interact directly (Sutton, V.R., unpublished data)? Instead, we postulated that the proapoptotic signal from granzyme B is probably transmitted to mitochondria through an intermediary molecule. Much recent attention had been focused on the proapoptotic Bcl-2 family member, Bid, a BH3 domain-only protein that has been shown to be cleaved in vitro by both granzyme B and caspases (especially caspase-8) at separate Asp residues. Recently, Bid cleavage was also shown to occur in cells treated with granzyme B, but the significance of this finding to cell death was not extensively canvassed (30). It has also been proposed that recruitment of the mitochondrial pathway may amplify suboptimal stimulation of the caspase cascade after Fas ligation in some cell types (31). This pathway involves mitochondrial disruption after insertion of truncated (t)Bid into the mitochondrial membrane.

In this study, we examined the possibility that direct Bid cleavage by granzyme B is necessary to transmit the death signal through the mitochondrion. Consistent with our previous data (23, 24), the proapoptotic activity of tBID can be negatively regulated by Bcl-2 overexpression, indicating that this pathway is indispensable for cell death. In the presence of normal Bcl-2 levels, tBid-mediated mitochondrial disruption would be necessary to achieve a lethal threshold of caspase activation, leading to caspase-mediated apoptosis. This mechanism would also account for granzyme B's ability to kill cells independently of caspases (through direct mitochondrial disruption) when caspases are inactivated, for instance by a viral inhibitor.

Materials and Methods

Cell Culture and Gene Transfection. FDC-P1 IL-3–dependent mouse myeloid cells were cultured in DMEM supplemented with 10% FCS and IL-3 in a humidified CO_2 incubator. Jurkat human T leukemia cells were maintained in RPMI supplemented with 10% FCS.

Populations of FDC-P1 cells expressing human Bcl-2 cDNA in the vector pEFpuro, and/or wild-type mouse Bid or one of three Bid mutants in pEF EE1hygro were generated by gene transfection, using electroporation of plasmid DNA, as described (32). One construct encoded Bid cDNA that encoded Glu rather than Asp at position 59 (D59E); another encoded Glu rather than Asp at residue 75 (D75E); and a third encoded Ala instead of Leu at residue 90. Bid molecules were tagged with an "EE" epitope to permit detection with a specific mouse Ab (BAbCO). Wild-type FDC-P1 cells or cells transfected only with the vector backbone expressed low endogenous levels of Bcl-2 (data not shown). The

Bcl-2 construct was used to produce Jurkat cell populations expressing wild-type Bcl-2 in their mitochondria, as described (24).

Characterization of FDC-P1 Cells Expressing Bcl-2 and/or Bid. Intracellular staining of transfected cells was performed as described (33). Primary Abs were mouse mAbs detecting human Bcl-2 or the EE epitope on Bid-expressing constructs. Specific binding of mouse Ig was detected with FITC-conjugated anti–mouse Ig (Silenus). Stained, fixed cells were analyzed on a cytofluorograph (Becton Dickinson). For Western blotting, lysates of washed cells (10^6) were fractionated on SDS-PAGE with reducing agent, transferred to nylon, and probed with the above mAbs. Signals were detected by exposure to x-ray film after chemiluminescent amplification. For growth factor withdrawal assays, cells in exponential growth were washed four times in PBS, then recultured in their usual medium lacking IL-3. Cells were plated in 24-well tissue culture dishes (2×10^5 cells/ml) and cell viability was determined by dye exclusion. Alternatively, cells were cultured in medium containing staurosporine for 24 h and their viability was estimated in the same way.

Apoptosis Assays. Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) assays were performed as described previously (18, 23). Perforin was purified from rat NK cells as described (23). Recombinant PLO was a gift of Dr. J. Paton (Children's and Women's Hospital, Adelaide, Australia). A sublytic dose of perforin or PLO was that producing <10% specific release of ^{51}Cr over 4 h. Purification of human granzyme B from YT cells was as described (34). The granzyme B was free of granzyme A activity and perforin (7, 34). The peptide inhibitors z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), Asp-Glu-Val-Asp-fluoromethylketone (DEVD-fmk), and Phe-Ala-fluoromethylketone (z-FA-fmk) were purchased from Enzyme Systems Products. In some experiments, Jurkat cells were treated with mouse anti-Fas IgM Ab (250 ng/ml CH11; Upstate Biotechnology). Changes in mitochondrial membrane potential were measured by flow cytometry with the mitochondrion-specific fluorescent dye, rhodamine 123, as described (24).

Western Blotting. Protein lysates were transferred from SDS-PAGE to Immobilon membranes using a semidry transfer cell (Bio-Rad Laboratories). For assays examining the release of cytochrome c into the cytosol, cells were exposed to granzyme B and sublytic PLO with or without fmk inhibitors, lysed, and fractionated (35). The membranes were probed with antisera specific for caspase-3 (Transduction Laboratories), caspase-9 (a gift of Dr. X. Wang, University of Texas Southwestern Medical Center at Dallas, Dallas, TX), caspase-8 (a gift of Dr. H. Walczak, German Cancer Research Center, Heidelberg, Germany), Bid (a gift of Dr. X. Wang), or cytochrome c (clone 7H8; BD PharMingen). Bound Ig was detected with rabbit anti–mouse or swine anti–rabbit reagent coupled to horseradish peroxidase after chemiluminescence amplification.

Results

Bid Is Upstream of Bcl-2 in the Granzyme B Death Pathway. Our previous studies showing that Bcl-2 overexpression blocks the apoptotic changes induced by granzyme B (23) and restores clonogenic survival to target cells (24) indicated that Bcl-2 regulates a key upstream event in the granzyme B death pathway. As there is no evidence that Bcl-2 can directly inhibit granzyme B, we hypothesized that Bcl-2 probably acts indirectly on a proapoptotic substrate activated by granzyme B and acting through the mitochondrial pathway. Bid is known to be cleaved in vitro by granzyme B exclusively at the Asp residue at position 75 (D75; and by caspase-8 at an alternative site, D59; reference 36), and therefore seemed a likely candidate to transmit a granzyme B–initiated death signal to the mitochondrion.

We reasoned that if Bid is upstream of Bcl-2 in the granzyme B apoptotic pathway, cooveexpression of Bid in cells overproducing Bcl-2 should relieve the block to cell death. Therefore, we produced uncloned populations of factor-dependent FDC-P1 cells that expressed mouse Bid and/or Bcl-2. Intracellular FACS® staining (Fig. 1) demonstrated cell populations that expressed high, unimodal levels of Bcl-2 alone (FD.bcl2), Bid alone (FD.bid), or both molecules (FD.bcl2/bid). It was demonstrated by immunostaining that overexpressed Bcl-2 was localized to the mitochondrial membrane and endoplasmic reticulum (data not shown). Furthermore, deliberate missorting of the Bcl-2 to the cell membrane abrogated its ability to block granzyme B–mediated cell death (24). This panel of cell lines was exposed to granzyme B (60 nM) and/or a sublytic concentration of perforin. Cells exposed to perforin (Fig. 2) or granzyme B alone (data not shown) remained viable indefinitely, and showed no changes of apoptosis in TUNEL analysis. Consistent with previous findings, FDC-P1 cells exposed to both perforin and granzyme B for 60 min developed apoptotic changes, including DNA fragmentation (TUNEL positive), reduced size (reduced forward scatter), and increased granularity (increased side scatter). These changes were abrogated by Bcl-2 overexpression, but were restored when Bid was cooverexpressed with Bcl-2 or overexpressed alone. Analogous results were obtained when PLO was substituted for perforin (data not shown). These findings clearly indicated that Bid is upstream of Bcl-2 in the granzyme B death pathway.

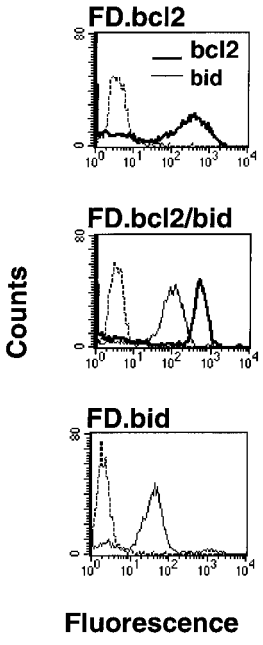

Figure 1. The expression of Bcl-2 and Bid in FDC-P1 transfectant populations. Expression of human Bcl-2 (thick lines) and Bid (thin lines) after gene transfection. FDC-P1 cells expressing either Bcl-2 or Bid alone, or both molecules (middle) were permeabilized, stained, and analyzed by flow cytometry using mouse mAbs specific for human Bcl-2 or EE-tagged Bid. Fluorescence staining in each case is compared with that with the FITC-conjugated anti–mouse reagent alone (dotted lines).

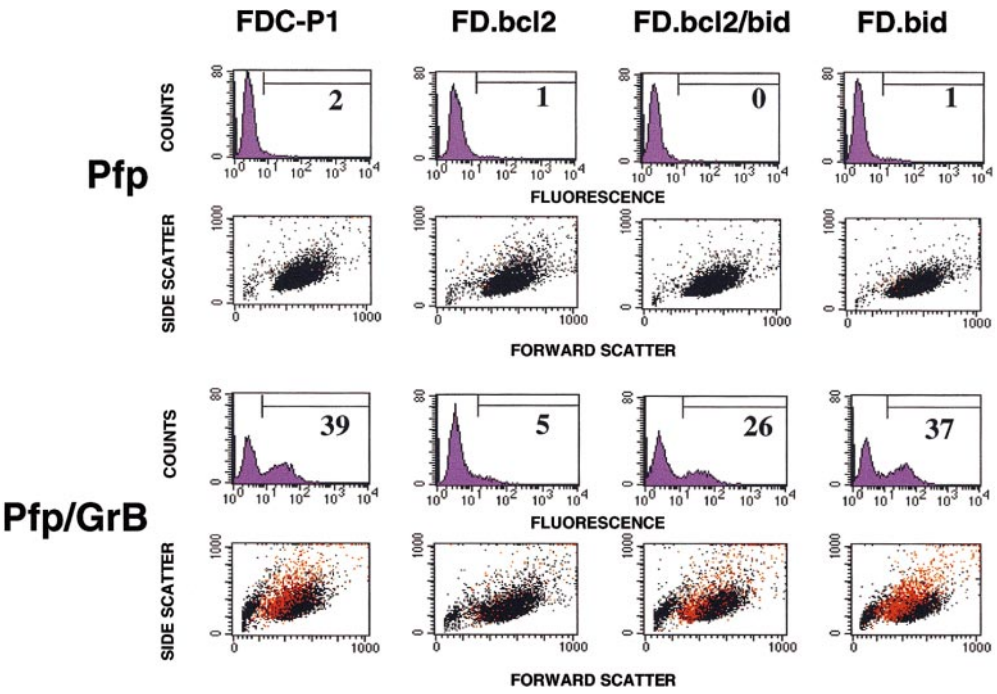

Figure 2. Cooverexpression of Bid overcomes Bcl-2's block of granzyme B–mediated cell death. Cytofluorographic analysis of cells, showing DNA fragmentation (TUNEL assay) and changes in light scatter parameters (forward vs. side scatter) of cells treated with sublytic perforin (Pfp; top panels) or perforin in combination with granzyme B (GrB, 60 nM; bottom panels) for 90 min at 37°C. The cells were wild-type FDC-P1, or FDC-P1 cells stably expressing human Bcl-2, Bid, or both Bcl-2 and Bid (see Fig. 1). The numerals in each panel indicate the percentage of TUNEL-positive cells, to the nearest integer. The experiment shown is representative of four similar experiments.

Bid Does Not Reverse Bcl-2 Inhibition of Apoptosis Due to Growth Factor Withdrawal or Staurosporine. As expected, cell death in response to both IL-3 withdrawal (Fig. 3 A) and staurosporine (Fig. 3 B), stimuli that operate through the mitochondrial pathway (37), was inhibited by Bcl-2. However, in contrast to granzyme B, cells remained completely protected despite overexpression of Bid with Bcl-2 (Fig. 3). Western blots were performed to determine whether Bid is cleaved in response to granzyme B, factor withdrawal, or staurosporine (Fig. 4). Exposure of cells overexpressing only Bid to the combined effects of sublytic perforin and granzyme B resulted in virtually complete loss of intact (~25 kD) Bid within 30 min, and the appearance of truncated forms of ~13, 15, and 16 kD (Fig. 4 A). However, treatment with perforin alone (Fig. 4 A) or granzyme B alone (data not shown) did not result in Bid cleavage. Cooverexpression of Bcl-2 did not prevent Bid cleavage when both granzyme B and perforin were added to the cells, although the rate of Bid depletion was somewhat slowed (see below). These results contrasted starkly when cells were deprived of growth factor or exposed to staurosporine (Fig. 4 B). In cells overexpressing only Bid, incomplete cleavage of 25-kD Bid was observed, but this was totally blocked for >18 h by cooverexpression of Bcl-2. As has previously been noted for staurosporine treatment

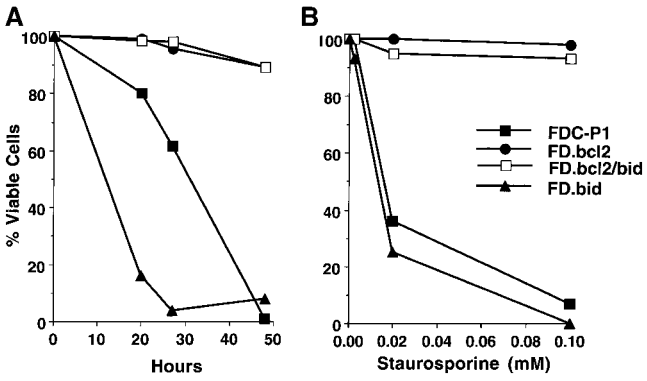

Figure 3. Bid expression does not overcome Bcl-2's block of cell death due to growth factor withdrawal or staurosporine. (A) Wild-type FDC-P1 cells, or FDC-P1 cells stably expressing human Bcl-2, Bid, or both Bcl-2 and Bid (see Fig. 1) were washed extensively and plated in culture medium lacking IL-3. Cell viability was estimated at the times indicated by trypan blue exclusion. (B) The same cell populations were plated in medium containing staurosporine for 18 h, or in normal medium, and cell viability was estimated by trypan blue exclusion. The experiment shown is representative of four similar experiments.

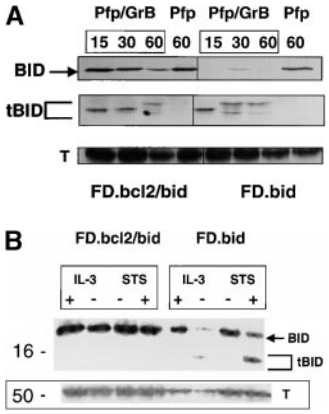

Figure 4. Bid cleavage in Bcl-2–overexpressing FDC-P1 cells after exposure to granzyme B, but not after growth factor withdrawal or exposure to staurosporine (STS). (A) Western blot analysis of whole cell lysates of FDC-P1 cells stably expressing only Bid or both Bid and Bcl-2, after exposure to sublytic perforin alone (Pfp) or a combination of perforin and granzyme B (GrB) for 15–60 min. The signals for uncleaved and tBid are indicated with arrowheads. (B) Western blot of whole cell lysates of the same FDC-P1 cell transfectants as in A after withdrawal of growth factor or exposure to staurosporine for 18 h. Both blots were reprobed with an antiserum specific for tubulin (T; 50 kD) to assess protein loading. The migration of relevant molecular size markers is indicated in kilodaltons at left.

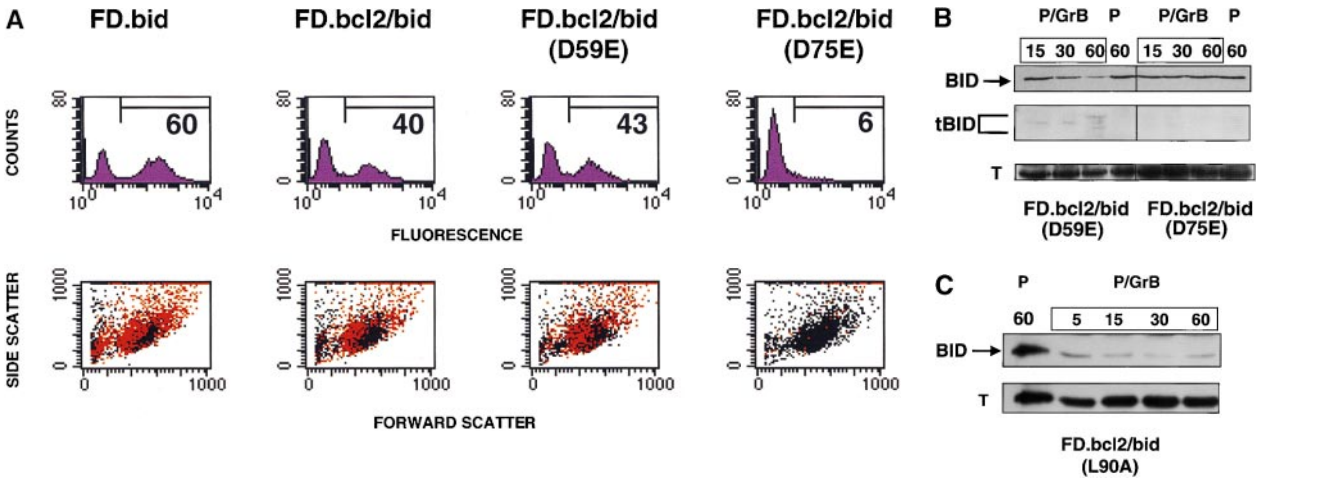

Figure 5. The granzyme B cleavage site of Bid (D75) is essential to overcome Bcl-2's block of granzyme B–mediated cell death, but the caspase-8 cleavage site (D59) is not. (A) Cytofluorographic analysis, showing DNA fragmentation (TUNEL assay) and changes in light scatter parameters (forward vs. side scatter) of cells exposed to sublytic PLO with granzyme B (60 nM) for 60 min at 37°C. The cells were Bcl-2–overexpressing FDC-P1 cells also expressing wild-type Bid or Bid in which the P1 Asp at the granzyme B cleavage site was mutated to Glu (D75E), or in which the P1 Asp at the caspase-8 cleavage site was mutated to Glu (D59E). The numerals in each panel indicate the percentage of TUNEL-positive cells, to the nearest integer. The experiment shown is representative of three similar experiments performed with either PLO or perforin. No significant apoptosis was detected when the cells were exposed to either sublytic quantities of perforin/PLO or granzyme B alone (<2%; data not shown). (B) Western blot using anti-EE antiserum of D59E and D75E cells shown in A. The cells were incubated with sublytic PLO (P) with or without granzyme B (GrB; 30 nM) at 37°C for 15–60 min, as indicated. Tubulin (T) staining has been used to ensure approximately equal protein loading in the samples. (C) Western blot analysis of whole cell lysates of FDC-P1 cells stably expressing L90A-mutated Bid and Bcl-2, after exposure to sublytic PLO or a combination of PLO and granzyme B for 15–60 min. The signal for uncleaved Bid is indicated. Staining of the same samples with antitubulin (T) was used to normalize the amount of protein loaded in each lane.

(38), it is likely that Bid cleavage in response to IL-3 withdrawal is also downstream of Bcl-2.

The Granzyme B Cleavage Site in Bid (Asp 75) Is Essential for Reversing Bcl-2's Block of Cell Death. As independent Bid cleavage sites have been identified for granzyme B and caspases, including caspase-8 (36), it was important to identify which site is necessary for granzyme B–induced cell death. Therefore, we made a further series of FDC-P1 transfectant cell lines. In one, Bid was overexpressed with the granzyme B cleavage site abolished by mutation of D75 to Glu (D75E). As a control, a similar mutation was made in the preferred caspase-8 site (D59E). We compared the ability of both Bid mutants and wild-type Bid to restore cell death inhibited by Bcl-2 overexpression (Fig. 5 A). When the cells were treated with perforin (or PLO) and granzyme B, mutation of the granzyme B cleavage site (D75E) abolished the apoptotic phenotype seen with wild-type Bid. By contrast, loss of the caspase-8 site (D59) did not prevent Bid from overcoming Bcl-2's block of cell death. No Bid cleavage was seen when the granzyme B site was lost (Fig. 5 B), despite the availability of caspase sites, including D59. In contrast, the Bid mutant lacking the caspase-8 site was still cleaved in the typical way. Loss of Bid cleavage with the D75E mutant, but not by the D59E mutant, indicated that the cleavage was mediated directly by granzyme B, and did not result from activation of caspase-8 by granzyme B (see also below). It has previously been shown that mutation of the Bid BH3 domain inactivates Bid by preventing its interaction with Bcl-2 (39). When we coexpressed this mutant (L90A) with Bcl-2, exposure to granzyme B resulted in Bid cleavage, as indicated by loss of the signal for intact Bid (Fig. 5 C). However, this mutant was completely unable to relieve Bcl-2's block of granzyme B, as only background levels of cell death (<5%) were seen after granzyme B and perforin addition (data not shown). This indicated that granzyme B–cleaved wild-type or D59E-mutated Bid overcame the block to cell death by interaction with Bcl-2 at the mitochondria.

Cleavage of Endogenous Bid Is Rapid, Precedes Caspase Activation, and Is Not Delayed by Overexpression of Bcl-2. All of the studies described above used FDC-P1 cells expressing supraphysiological levels of Bid and/or Bcl-2. We next examined the processing of endogenous Bid in Jurkat cells exposed to granzyme B. Bid cleavage was once again observed in response to granzyme B (60 nM) delivered by either sublytic perforin or PLO (Fig. 6). All of the endogenous Bid was cleaved within 2 h, consistent with the characteristically rapid activation of apoptosis by granzyme

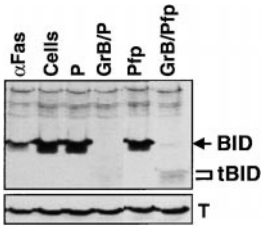

Figure 6. Bid cleavage in Jurkat cells, in response to granzyme B (GrB) and anti-Fas (αFas) IgM. Western blot using anti-Bid antiserum of Jurkat cells either untreated (Cells), incubated with either sublytic Perforin (Pfp) or PLO (P) alone, Pfp/PLO in combination with granzyme B (60 nM) for 2 h, or exposed to anti-Fas IgM (250 ng/ml) for 4 h. Tubulin (T) staining has been used to ensure approximately equal protein loading in the samples.

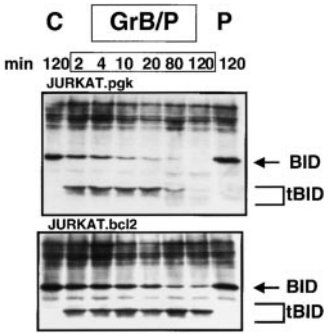

Figure 7. Bid is cleaved early in Jurkat cells exposed to granzyme B (GrB), irrespective of Bcl-2 overexpression. Jurkat cells overexpressing Bcl-2 or transfected with vector backbone DNA only (Jurkat-pgk) were exposed for the times indicated to granzyme B (60 nM) and sublytic PLO. Control cells were exposed to no stimulus (C) or PLO alone (P). At each time point, the cells were flooded with ice-cold medium, pelleted in a microfuge, washed twice in ice-cold medium, then lysed for Western blot analysis with anti-Bid antiserum (see Materials and Methods). The migration of intact and tBid is indicated by the arrowheads.

B (40). Cells exposed to anti-Fas IgM (250 ng/ml) for 4 h showed somewhat reduced Bid processing, consistent with slower death kinetics. We next compared the rate of Bid processing in Jurkat cells expressing Bcl-2 or transfected with empty vector alone (Fig. 7). In Jurkat.pgk cells treated with granzyme B and porin, the signal due to intact Bid was substantially reduced within just 2 min, and we observed the coincident appearance of tBid molecules (~13 and 16 kD). Intact Bid was hardly visible by 80 min, and absent by 120 min. Overexpression of Bcl-2 had only a minor effect on Bid processing. Cleavage of Bid was still seen by 2 min; however, complete cleavage of intact Bid was somewhat slowed in comparison with Bcl-2–nonexpressing cells. It is likely that some of the later Bid cleavage seen in the Jurkat-pgk cells was secondary to caspase activation, as these cells underwent cell death whereas Jurkat-Bcl2 cells remained viable and had little caspase activity (see below).

Kinetics of Bid and Caspase Cleavage in Response to Granzyme B and Fas Ligation. We next compared the time course of Bid cleavage with cleavage of procaspase-3 and -9 in cells treated with granzyme B or Fas ligation (Fig. 8 A). Caspase-9 plays a pivotal role in activating downstream caspases after high order complex formation with cytochrome c liberated from mitochondria and cytosolic apoptotic protease-activating factor 1 (Apaf-1 [41]). Given the apparent dependence of granzyme B–mediated killing on the mitochondrial pathway, it was notable that procaspase-9 was processed later than procaspase-3, with the appearance of the signature 35-kD heavy chain only after the cells had been exposed to granzyme B for 120 min (discussed further below). As observed previously, Bid cleavage in response to granzyme B was rapid (complete by 20 min).

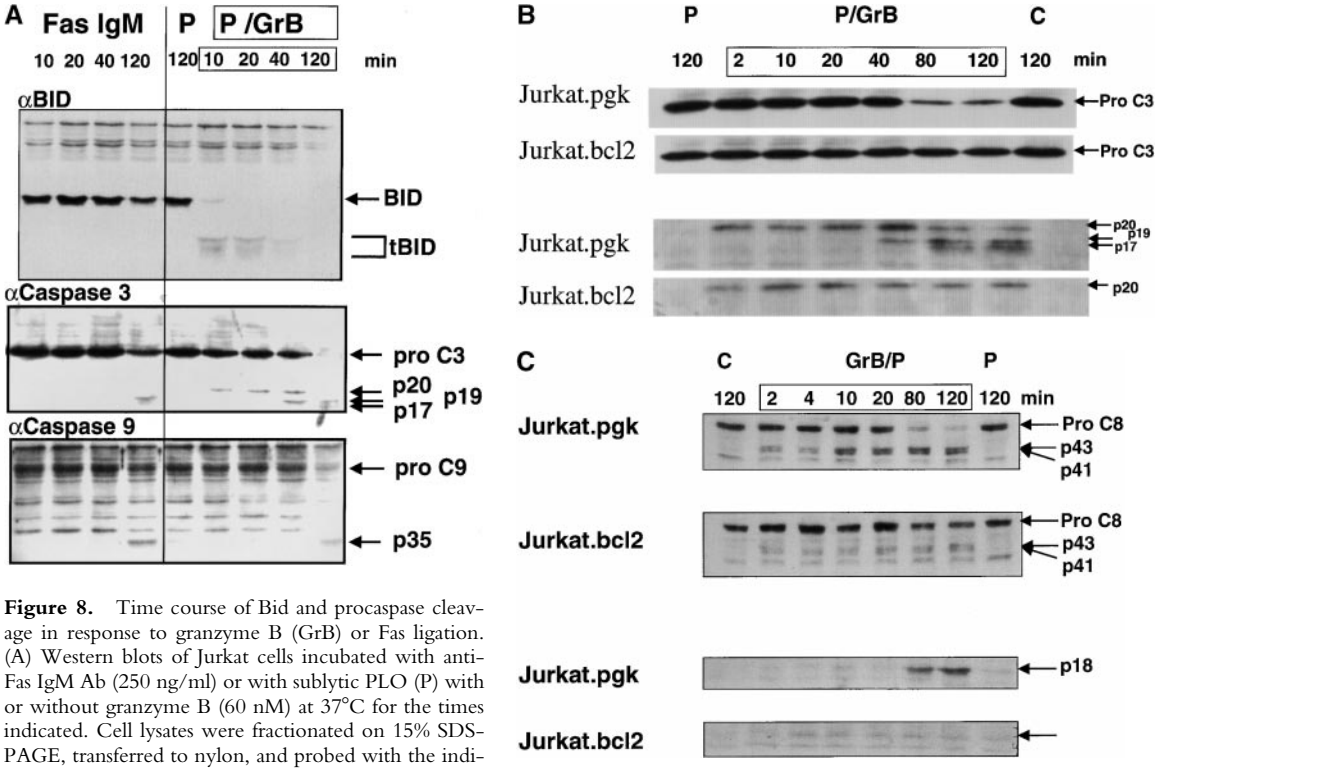

Figure 8. Time course of Bid and procaspase cleavage in response to granzyme B (GrB) or Fas ligation. (A) Western blots of Jurkat cells incubated with anti-Fas IgM Ab (250 ng/ml) or with sublytic PLO (P) with or without granzyme B (60 nM) at 37°C for the times indicated. Cell lysates were fractionated on 15% SDS-PAGE, transferred to nylon, and probed with the indicated antisera (see Materials and Methods). (B) Western blot using anti–caspase-3 antiserum of Jurkat cells overexpressing Bcl-2 or transfected with vector backbone (Jurkat.pgk). The cells were untreated (C) or incubated with sublytic PLO (P) with or without granzyme B (60 nM) at 37°C for the times indicated. To facilitate comparison, signals for 32-kD procaspase-3 (Pro C3, top panels) are shown after a short exposure to x-ray film (15 s), and those for the cleavage products (bottom panels) are from longer exposure (2 min). (C) Western blot using anti–caspase-8 antiserum of Jurkat cells overexpressing Bcl-2 or transfected with vector backbone (Jurkat.pgk). The cells were untreated (C) or incubated with sublytic PLO (P) with or without granzyme B (60 nM) at 37°C for the times indicated. To facilitate comparison, signals for 55-kD procaspase-8 (Pro C8, top panels) are shown after a short exposure to x-ray film (25 s), and those for the cleavage products (bottom panels) are from longer exposure (3 min).

By comparison, treatment with anti-Fas IgM produced only partial loss of the Bid signal by 120 min. Caspase-3 is the earliest caspase activated during granzyme B–mediated apoptosis of Jurkat cells (14). Partial activation of procaspase-3 was seen within 10 min of exposure of cells to granzyme B and either porin, with the appearance of a ~20-kD form, a signature species that results from direct cleavage of procaspase-3 by granzyme B at D175 (3, 10, 18). However, further processing to the mature and fully active p19 and p17 forms (10) was seen only after 40 min. Procaspase-3 cleavage to p19/17 in response to Fas ligation was observed after 120 min without accumulation of the 20-kD species seen with granzyme B–mediated killing (Fig. 8 A). As partial processing of caspase-3 was an early event compared with caspase-9 cleavage (see below), we wished to determine its temporal relationship to Bid processing. Using the same cell lysates as in Fig. 7, we found that the initial processing of procaspase-3 was as rapid as Bid cleavage, and had commenced by the earliest time that could be studied (2 min; Fig. 8 B). Interestingly, initial procaspase-3 cleavage was not delayed by Bcl-2 overexpression. However, further processing to p19 and p17 and loss of 32-kD procaspase-3 seen with Jurkat.pgk cells was not seen over the 2-h experiment. A similar pattern of procaspase-8 activation was also seen (Fig. 8 C). Partial processing to p43 was observed within 2 min irrespective of whether Bcl-2 was overexpressed; however, full processing to the mature p18 chain was seen only in the absence of Bcl-2.

Previously, caspase-3 has been shown to cleave Bid only at D59 (38). However, as both caspase-3 and Bid were processed very rapidly in response to granzyme B, we wished to exclude Bid processing by caspase-3 at the granzyme B–preferred site (D75). Preincubation of Jurkat cells with oligopeptide fmk derivatives that inhibit most caspases had no effect on Bid processing (Fig. 9 A). Neither z-VAD-fmk, a broad spectrum caspase inhibitor, nor DEVD-fmk, which preferentially inhibits caspase-3 and related caspases, had any effect on Bid processing, despite blocking caspase-dependent DNA fragmentation in response to granzyme B (18; see also below; Fig. 9 A). In contrast, Bid processing due to cross-linking of Fas was almost completely blocked by 20 μM z-VAD-fmk (Fig. 9 B). We again concluded that Bid is processed directly by granzyme B at D75, and only secondarily by caspases.

Mitochondrial Perturbation in Response to Granzyme B Is Independent of Caspases. As Bid is processed directly by granzyme B, we predicted that cleaved Bid should be capable of inducing mitochondrial perturbation without a requirement for caspase activation. Therefore, we measured changes in mitochondrial membrane potential under conditions in which caspases were either active or inhibited by fmk compounds (Fig. 10). Consistent with our previous results (18), preincubation of Jurkat cells in z-VAD-fmk or DEVD-fmk reduced DNA fragmentation in response to granzyme B and porin to background levels, confirming that caspases had been blocked. However, neither z-VAD-fmk nor DEVD-fmk had a significant influence on the loss of mitochondrial membrane potential (measured as flux of rhodamine 123 from the cells) in the same cell samples compared with the control inhibitor z-FA-fmk, an inhibitor of chymotrypsin-like proteases. Consistent with our findings that Bcl-2 can abrogate apoptotic changes in response to granzyme B and lead to long-term cell survival, Bcl-2 overexpression in Jurkat cells also inhibited the loss of mitochondrial membrane potential (24). Thus, prior caspase activation is not necessary for mitochondrial perturbation triggered by granzyme B, and this function can be achieved by cleaved Bid, independently of caspases.

The Postmitochondrial Cell Death Signal: The Granzyme B–induced Release of Cytochrome c from Mitochondria Is Largely Dependent on Caspase Activation. Having demonstrated that mitochondrial perturbation is independent of caspase activation, we wished to identify postmitochondrial effector molecules responsible for the execution phase of cell death. In some forms of apoptosis, mitochondrial disruption leads to rapid caspase-9 activation through the liberation of mitochondrial cytochrome c from the intermembrane space into the cytosol. Indeed, Bid cleaved in response to caspase-8

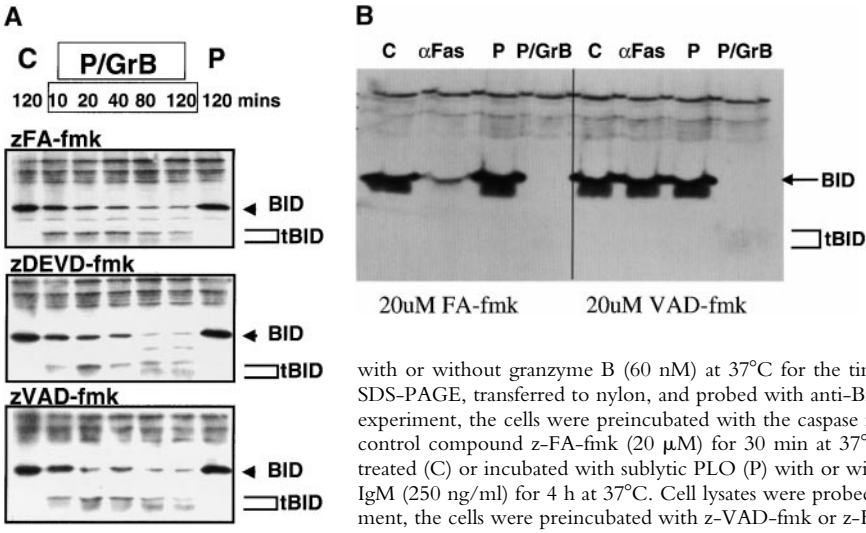

Figure 9. Cleavage of Bid in response to granzyme B (GrB) is independent of caspases, but is caspase dependent after Fas ligation. (A) Western blot of Jurkat cells which were untreated (C) or incubated with sublytic PLO (P) with or without granzyme B (60 nM) at 37°C for the times indicated. Cell lysates were fractionated on 15% SDS-PAGE, transferred to nylon, and probed with anti-Bid antiserum (see Materials and Methods). Before the experiment, the cells were preincubated with the caspase inhibitors z-DEVD-fmk or z-VAD-fmk, or with the control compound z-FA-fmk (20 μM) for 30 min at 37°C. (B) Western blot of Jurkat cells which were untreated (C) or incubated with sublytic PLO (P) with or without granzyme B (60 nM) for 2 h or anti-Fas (αFas) IgM (250 ng/ml) for 4 h at 37°C. Cell lysates were probed with anti-Bid antiserum as in A. Before the experiment, the cells were preincubated with z-VAD-fmk or z-FA-fmk (20 μM) for 30 min at 37°C.

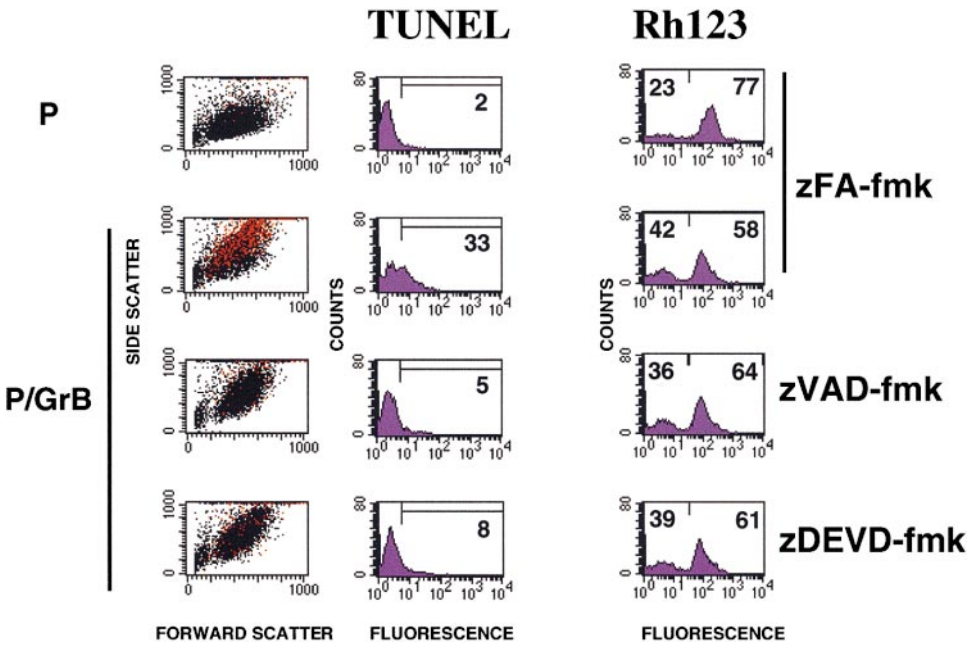

Figure 10. Loss of mitochondrial membrane depolarization in response to granzyme B is independent of caspases. Loss of rhodamine 123 (Rh123) accumulation in Jurkat cells after exposure to granzyme B (GrB, 60 nM) and sublytic PLO (P) for 2 h. The same cell samples were also analyzed for DNA fragmentation by TUNEL and for changes in forward and side scatter characteristics by flow cytometry at 2 h. Before commencement of the experiment, the cells were preincubated with the caspase inhibitors z-DEVD-fmk or z-VAD-fmk, or with the control compound z-FA-fmk (each 100 μM) for 30 min at 37°C. The numerals indicate the percentage of rhodamine-positive or -negative cells, or TUNEL-positive cells, to the nearest integer.

activation is thought to trigger cytochrome c release (36), enabling formation of the apoptosome through recruitment of the adaptor molecule Apaf-1. Given the very early appearance of tBid, we were surprised to find that procaspase-9 cleavage products were not detectable in cells treated with granzyme B and porin until 2 h (Fig. 8 A). Cytochrome c was released from mitochondria into the cytosol in response to granzyme B and PLO; however, little or no release was seen when caspases were inhibited with z-VAD-fmk (Fig. 11). This result both indicated that cytochrome c release is unlikely to contribute to early activation of the caspase-9–dependent pathway in response to granzyme B, and provided a rationale for the observed late cleavage of procaspase-9.

Discussion

This study is the most comprehensive performed to date dissecting the proximal events necessary for granzyme B–mediated cell death. Our results strongly indicate that rapid and direct cleavage of Bid at D75 is essential for transmitting the granzyme B death signal to mitochondria, and that mitochondrial disruption is both indispensable for granzyme B–mediated death and necessary for efficient caspase activation. Direct cleavage of caspases by granzyme B does result in cell death unless caspase activation is also augmented by mitochondrial perturbation. The findings were consistent both in human Jurkat cells expressing endogenous Bid and in mouse FDC-P1 cells in which overexpressed Bid reversed Bcl-2's block of apoptosis. Several lines of evidence suggest that granzyme B cleaves Bid directly, rather than via the caspases. Bid was cleaved as early as 2 min after exposure of cells to granzyme B, and this was not delayed by fmk caspase inhibitors. More importantly, overexpressed Bid lacking the granzyme B cleavage site (D75E) was unable to overcome Bcl-2's block of granzyme B–mediated cell death, whereas Bid, which lacked the caspase-8 cleavage site, still restored apoptosis. Finally, the L90A Bid mutant, which is incapable of interacting with mitochondrial Bcl-2, was cleaved by granzyme B, but failed to restore cell death. This latter finding indicated that wild-type Bid cleaved by granzyme B exerts its effects by interacting with Bcl-2.

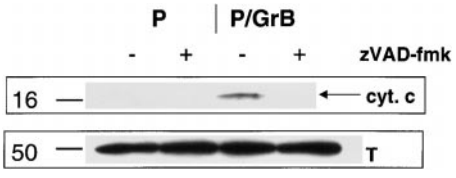

Figure 11. Release of cytochrome c from mitochondria in response to granzyme B is dependent on caspase activation. Jurkat cells were preincubated with 20 μM z-VAD-fmk or without inhibitor for 30 min at 37°C, then exposed to sublytic PLO (P) with or without granzyme B (GrB) for 2 h at 37°C. The cells were then washed, lysed, and cytosolic extracts devoid of mitochondria prepared by ultracentrifugation. The extracts were analyzed in Western blots using antisera for cytochrome c (cyt. c) or tubulin (T) (see Materials and Methods).

Mitochondrial perturbation resulting from direct cleavage of Bid accounts for caspase-dependent and -independent cell death due to granzyme B. Our data allow us to formulate a new model for granzyme B's proapoptotic activity (Fig. 12). Although granzyme B is a potent direct activator of caspases in vitro, it acts predominantly through the mitochondrial pathway in intact cells, and initiates the process by directly cleaving Bid. Although at first perhaps surprising, this mechanism can account for cell death operating either through caspases, or in their absence. In the absence of caspase inhibitors, the release of proapoptotic mitochondrial constituents results in powerful augmentation

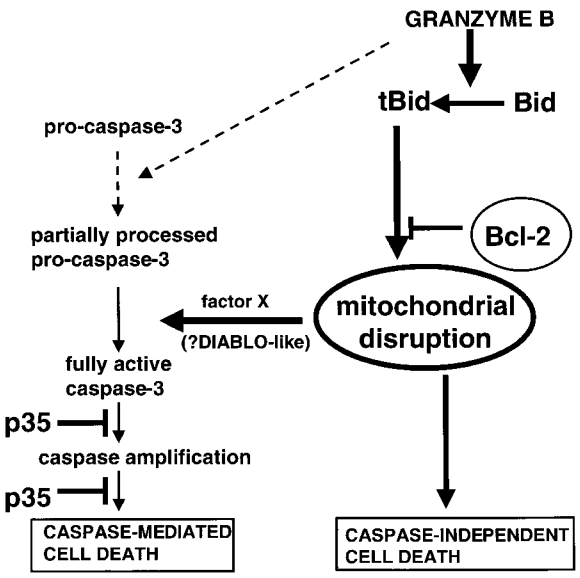

Figure 12. A unifying model of granzyme B–mediated cell death. Immediately upon accessing the cytosol, granzyme B principally cleaves Bid (thick arrow) and partially activates procaspase-3 (dotted arrows). However, caspase-3 is not fully processed unless tBid primarily disrupts the mitochondrion, a step that is regulated by Bcl-2 overexpression. Activation of other caspases is downstream of caspase-3 or related DEVD-fmk–inhibitable caspases. Mitochondrial perturbation by Bid results in cell death that is independent of caspases, and therefore is not blocked by p35. The mitochondrial factor responsible for full caspase activation is unknown (factor X), but is not cytochrome c, the release of which also requires further (secondary) mitochondrial damage resulting from full caspase activation (see text). We speculate, without any evidence, that this factor may be similar to DIABLO/Smac, a protein that, when released from mitochondria, displaces IAP inhibitors from some caspases to allow full activation.

of caspases (the caspase-dependent pathway, discussed further below). Inhibition of caspase activity, for instance by p35, results in loss of DNA fragmentation (17, 18) and poly(ADP-ribose) polymerase cleavage in response to granzyme B (3); however, cell growth and clonogenic assays clearly indicate that caspase-independent death pathways remain intact and cell death still occurs (18, 24). Our model predicts that tBid-induced mitochondrial disruption by itself is sufficient to kill a cell when caspases are not active, as supported by our finding that loss of mitochondrial membrane depolarization occurs despite caspase inactivation with z-VAD-fmk.

The fact that Bcl-2 overexpression fully restores clonogenic capacity to granzyme B–treated cells (24) shows that Bcl-2 governs an essential proximal step that regulates both the caspase-dependent and -independent pathways. We also showed that missorting of Bcl-2 to the inner leaflet of the plasma membrane abolished its ability to block granzyme B (24). In light of this study, it is evident that missorting of Bcl-2 would prevent it from negating the mitochondrial disruption induced after Bid cleavage. Our observation that Bid is the proximal mediator of mitochondrial disruption due to granzyme B is thus consistent both with the centrality of the mitochondrial pathway and the existence of death pathways that operate independently of caspases.

How does granzyme B bring about full caspase activation in intact cells? We observed that although partial processing of caspase-3 and -8 is an early event after exposure to granzyme B, further processing is lost when Bcl-2 is overexpressed. A major question that remains is why caspase activation stalls in Bcl-2–overexpressing cells. It has been proposed that initial cleavage of procaspase-3 by granzyme B is sufficient for further autocatalysis to fully activate caspase-3 (14, 18). Our data suggest that initial granzyme B cleavage of procaspase-3 does not predispose the caspase-3 precursor to full processing, unless mitochondrial perturbation also occurs through tBid (Fig. 12). Mitochondrial disruption classically results in caspase recruitment through cytochrome c–dependent complex formation with Apaf-1 and procaspase-9. However, we found that caspase-9 is activated well after caspase-3, and that cytochrome c is not liberated from mitochondria without prior caspase activation. Therefore, we argue that cytochrome c does not play a primary role in granzyme B–mediated caspase activation. Rather, it is likely that the late release of cytochrome c further amplifies the already activated caspase cascade. This view is supported by the recent finding that overexpression of 27-kD heat shock protein blocked caspase-9–dependent apoptosis by sequestering cytochrome c, but did not influence granzyme B–induced caspase activation (42).

As a mitochondrial cofactor contributing to caspase activation is strongly suggested by our data, we believe a factor other than cytochrome c (factor "X"; Fig. 12) may be responsible for efficient processing of caspase-3. This hypothesis is consistent with the finding that adding mitochondria to granzyme B–activated cytosolic lysate greatly amplified caspase-3 activity (28). In light of this, it was interesting that in non–Bcl-2–expressing Jurkat cells, caspase-3 processing seemed to take place in two stages. After rapid processing to the p20 form ($<$2 min), further maturation was delayed until $\sim$40 min (Fig. 8 A). Soon thereafter, the p19 and p17 forms were clearly present, with depletion of the 32-kD form. A candidate for this facilitatory role is the recently described protein direct inhibitor of apoptosis proteins (IAP) binding protein (DIABLO)/second mitochondria-derived activator of caspase (Smac) which, when released from mitochondria, facilitates further processing of caspases by antagonizing the activity of the inhibitory factor mammalian IAP homolog A (MIHA)/X chromosome–linked inhibitor of apoptosis (XIAP) (43, 44). In Bcl-2–overexpressing cells, the first stage of procaspase processing (inefficient, and due to direct procaspase cleavage by granzyme B) can take place, but not the second, which relies on mitochondrial disruption. Thus, caspase-3 activity never reaches a threshold level that can kill the cell. As the form of tBid produced by granzyme B cleavage at D75 did not induce cytochrome c release, we also speculate that this product may preferentially release an alternative cofactor. In this context, it may be relevant that the release of DIABLO/Smac and cytochrome c have been shown to be separately regulated (45).

In conclusion, this study reinforces our proposition (23, 24) that the mitochondrial pathway is essential for

granzyme B–mediated cell death, and strongly implicates Bid as the molecule that rapidly transmits a death signal to mitochondria. Although direct processing of pro-caspases by granzyme B can occur, this process does not achieve a lethal threshold of caspase activity unless mitochondrial perturbation is also induced by granzyme B–cleaved tBid.

We thank Dr. David Huang for providing FDC-P1 transfectant cells and DNA constructs and for helpful advice. We thank Dr. Xiaodong Wang for providing antisera specific for human Bid and caspase-9, and Dr. Henning Walczak for his antiserum detecting caspase-8. We also thank Andreas Strasser, David Vaux, and Sharad Kumar for helpful discussions.

This study was supported by a project grant (V.R. Sutton, K. Sedelies, and K.A. Browne) and a fellowship (J.A. Trapani) from the National Health and Medical Research Council, Australia. R.W. Johnstone receives a Wellcome Trust Senior Research Fellowship, and J.E. Davis receives a postgraduate scholarship of the Anti-Cancer Council of Victoria.

Submitted: 17 July 2000
Revised: 14 September 2000
Accepted: 18 September 2000

References

1. Rouvier, E., M.F. Luciani, and P. Golstein. 1993. Fas involvement in Ca^{2+}-independent T cell–mediated cytotoxicity. *J. Exp. Med.* 177:195–200.
2. Lynch, D.H., M.L. Watson, M.R. Alderson, P.R. Baum, R.E. Miller, T. Tough, M. Gibson, T. Davis-Smith, C.A. Smith, K. Hunter, et al. 1994. The mouse Fas-ligand gene is mutated in gld mice and is part of a TNF family gene cluster. *Immunity.* 1:131–136.
3. Browne, K.A., E. Blink, V.R. Sutton, C.J. Froelich, D.A. Jans, and J.A. Trapani. 1999. Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. *Mol. Cell. Biol.* 19:8604–8615.
4. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature.* 369:31–37.
5. Shi, L., C.-M. Kam, J.C. Powers, R. Aebersold, and A.H. Greenberg. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J. Exp. Med.* 176:1521–1529.
6. Shi, L., R.P. Kraut, R. Aebersold, and A.H. Greenberg. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med.* 175:553–566.
7. Trapani, J.A., K.A. Browne, M.J. Smyth, and D.A. Jans. 1996. Localization of granzyme B in the nucleus: a putative role in the mechanism of cytotoxic lymphocyte-mediated apoptosis. *J. Biol. Chem.* 271:4127–4133.
8. Froelich, C.J., R. Orth, J. Turbov, P. Seth, R. Gottlieb, B. Babior, G. Shah, R.C. Bleackley, V.M. Dixit, and W. Hanna. 1996. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *J. Biol. Chem.* 271:29073–29079.
9. Srinivasula, S.M., T. Fernandes-Alnemri, J. Zangrilli, N. Robertson, R.C. Armstrong, L. Wang, J.A. Trapani, J. Tomaselli, G. Litwack, and E.S. Alnemri. 1996. The Ced-3/interleukin 1beta converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2alpha are substrates for the apoptotic mediator CPP32. *J. Biol. Chem.* 271:27099–27106.
10. Fernandes-Alnemri, T., R.C. Armstrong, J. Krebs, S.M. Srinivasula, L. Wang, F. Bullrich, L.C. Fritz, J.A. Trapani, K.J. Tomaselli, G. Litwack, and E.S. Alnemri. 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA.* 93:7464–7469.
11. Harvey, N.L., J.A. Trapani, T. Fernandes-Alnemri, G. Litwack, E.S. Alnemri, and S. Kumar. 1996. Processing of the Nedd2 precursor by ICE-like proteases and granzyme B. *Genes Cells.* 1:673–685.
12. Andrade, F., S. Roy, N. Thornberry, A. Rosen, and L. Casciola-Rosen. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity.* 8:451–460.
13. Jans, D.A., P. Jans, L.J. Briggs, V.R. Sutton, and J.A. Trapani. 1996. Reconstitution of nuclear and nucleolar transport of the natural killer cell serine protease granzyme B-dependence on perforin *in vivo* and cytosolic factors *in vitro*. *J. Biol. Chem.* 271:30781–30789.
14. Yang, X., H.R. Stennicke, B. Wang, D.R. Green, R.U. Janicke, A. Srinivasan, P. Seth, G. Salvesen, and C.J. Froelich. 1998. Granzyme B mimics apical caspases. Description of a unified pathway for trans-activation of executioner caspase-3 and -7. *J. Biol. Chem.* 273:34278–34283.
15. Muzio, M., A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, et al. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell.* 85:817–827.
16. Boldin, M.P., T.M. Goncharov, Y.V. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell.* 85:803–815.
17. Sarin, A., M.S. Williams, M.A. Alexander-Miller, J.A. Berzofsky, C.M. Zacharchuk, and P.A. Henkart. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. *Immunity.* 6:209–215.
18. Trapani, J.A., D.A. Jans, K.A. Browne, M.J. Smyth, P. Jans, and V.R. Sutton. 1998. Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. *J. Biol. Chem.* 273:27934–27938.
19. Heibein, J.A., M. Barry, B. Motyka, and R.C. Bleakley. 1999. Granzyme B-induced loss of mitochondrial inner membrane potential and cytochrome c release are caspase independent. *J. Immunol.* 163:4683–4693.
20. Beresford, P.J., Z. Xia, A.H. Greenberg, and J. Lieberman. 1999. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity.* 10:585–594.
21. Tewari, M., and D.M. Dixit. 1995. Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. *J. Biol. Chem.* 270:3255–3260.
22. Clem, R.J., M. Fechheimer, and L.K. Miller. 1991. Prevention of apoptosis by a baculovirus gene during infection of

insect cells. *Science*. 254:1388–1390.
23. Sutton, V.R., D.L. Vaux, and J.A. Trapani. 1997. Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. *J. Immunol.* 158:5783–5790.
24. Davis, J.E., V.R. Sutton, M.J. Smyth, and J.A. Trapani. 2000. Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1. *Cell Death Differ.* 7:973–983.
25. Vaux, D.L., H.L. Aguila, and I.L. Weissman. 1992. Bcl-2 prevents death of factor deprived cells but fails to prevent apoptosis in targets of cell mediated killing. *Int. Immunol.* 4:821–826.
26. Neilan, J.G., Z. Lu, C.L. Afonso, G.F. Kutish, M.D. Sussman, and D.L. Rock. 1993. An African swine fever virus gene with similarity to the proto-oncogene bcl-2 and the Epstein-Barr virus gene BHRF1. *J. Virol.* 67:4391–4394.
27. Jans, D.A., V.R. Sutton, P. Jans, C.J. Froelich, and J.A. Trapani. 1999. BCL-2 blocks perforin-induced nuclear translocation of granzymes, concomitant with protection against the nuclear events of apoptosis. *J. Biol. Chem.* 274:3953–3961.
28. MacDonald, G., L. Shi, C. Vande Velde, J. Lieberman, and A. Greenberg. 1999. Mitochondria-dependent and -independent regulation of granzyme B–induced apoptosis. *J. Exp. Med.* 189:131–143.
29. Susin, S.A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer. 1996. Bcl-2 prevents the release from mitochondria of an apoptotogenic protease. *J. Exp. Med.* 184:1331–1341.
30. Barry, M., J.A. Heibein, M.J. Pinkoski, S.F. Lee, R.W. Moyer, D.R. Green, and R.C. Bleackley. 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol. Cell. Biol.* 20:3781–3794.
31. Schmitz, I., H. Walczak, P.H. Krammer, and M.E. Peter. 1999. Differences between CD95 type I and II cells detected with the CD95 ligand. *Cell Death Differ.* 6:821–822.
32. Huang, D.C.S., L.A. O'Reilly, A. Strasser, and S. Cory. 1997. The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:4628–4638.
33. Bird, C.H., V.R. Sutton, J. Sun, C.E. Hirst, A. Novak, S. Kumar, J.A. Trapani, and P.I. Bird. 1998. Selective regulation of apoptosis: the cellular serpin proteinase inhibitor-9 prevents granzyme B-mediated but not Fas-mediated cell death. *Mol. Cell. Biol.* 18:6287–6398.
34. Trapani, J.A., K.A. Browne, M. Dawson, and M.J. Smyth. 1993. Immunopurification of functional Asp-ase (natural killer cell granzyme B) using a monoclonal antibody. *Biochem. Biophys. Res. Commun.* 195:910–920.
35. Bossy-Wetzel, E., D.D. Newmeyer, and D.R. Green. 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:37–43.
36. Vaux, D.L., G. Haecker, and A. Strasser. 1994. An evolutionary perspective on apoptosis. *Cell.* 76:777–779.
37. Slee, E.A., S.A. Keogh, and S.J. Martin. 2000. Cleavage of BID during cytotoxic drug and UV radiation induced apoptosis occurs downstream of Bcl-2 and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated cytochrome c release. *Cell Death Differ.* 7:556–565.
38. Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell.* 94:481–490.
39. Wang, K., X. Yin, D.T. Chao, C.L. Milliman, and S.J. Korsmeyer. 1996. BID: a novel BH3 domain only death agonist. *Genes Dev.* 10:2859–2869.
40. Trapani, J.A., P. Jans, M.J. Smyth, C.J. Froelich, E.A. Williams, V.R. Sutton, and D.A. Jans. 1998. Perforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction. *Cell Death Differ.* 5:488–496.
41. Li, P., D. Nijhawan, I. Budiharjo, S.M. Srinvasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 14:479–489.
42. Bruey, J., C. Ducasse, P. Bonniaud, L. Ravagnan, S. Susin, C. Diaz-Latoud, S. Gurbuxani, A. Arrigo, G. Kroemer, E. Solary, and C. Garrido. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat. Cell Biol.* 2:645–652.
43. Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.A. Connolly, G.E. Reid, R.L. Motitz, R.J. Simpson, and D.L. Vaux. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell.* 102:43–53.
44. Du, C., M. Fang, Y. Li, and X. Wang. 2000. Smac, a mitochondrial protein that promotes cytochrome-c-dependent caspase activation by eliminating IAP function. *Cell.* 102:33–42.
45. Green, D.R. 2000. Apoptotic pathways: paper wraps stone blunts scissors. *Cell.* 102:1–4.