

# Resistance to Granzyme B-mediated Cytochrome c Release in Bak-deficient Cells

Gui-Qiang Wang,<sup>1</sup> Eva Wieckowski,<sup>1</sup> Leslie A. Goldstein,<sup>1</sup>  
Brian R. Gastman,<sup>2</sup> Asaf Rabinovitz,<sup>1</sup> Andrea Gambotto,<sup>3</sup>  
Shuchen Li,<sup>1</sup> Bingliang Fang,<sup>5</sup> Xiao-Ming Yin,<sup>1</sup>  
and Hannah Rabinowich<sup>1,4</sup>

<sup>1</sup>Department of Pathology, the <sup>2</sup>Department of Otolaryngology, and the <sup>3</sup>Department of Surgery, The University of Pittsburgh School of Medicine, and <sup>4</sup>The University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213

<sup>5</sup>Department of Thoracic and Cardiovascular Surgery, The University of Texas M.D. Anderson Cancer Center Houston, Texas 77030

## Abstract

Granzyme B (GrB), a serine protease with substrate specificity similar to the caspase family, is a major component of granule-mediated cytotoxicity of T lymphocytes. Although GrB can directly activate caspases, it induces apoptosis predominantly via Bid cleavage, mitochondrial outer membrane permeabilization, and cytochrome c release. To study the molecular regulators for GrB-mediated mitochondrial apoptotic events, we used a CTL-free cytotoxicity system, wherein target cells are treated with purified GrB and replication-deficient adenovirus (Ad). We report here that the Bcl-2 proapoptotic family member, Bak, plays a dominant role in GrB-mediated mitochondrial apoptotic events. A variant of Jurkat cells, deficient in Bak expression, was resistant to GrB/Ad-mediated apoptosis, as determined by lack of membranous phosphatidylserine exposure, lack of DNA breaks, lack of mitochondrial outer membrane permeabilization, and unchanged expression of inner mitochondrial membrane cardiolipin. The resistance of Bak-deficient cells to GrB/Ad cytotoxicity was reversed by transduction of the Bak gene into these cells. The requirement for both Bid and Bak, was further demonstrated in a cell-free system using purified mitochondria and S-100 cytosol. Purified mitochondria from Bid knockout mice, but not from Bax knockout mice, failed to release cytochrome c in response to autologous S-100 and GrB. Also, Bak-deficient mitochondria did not release cytochrome c in response to GrB-treated cytosol unless recombinant Bak protein was added. These results are the first to report a role for Bak in GrB-mediated mitochondrial apoptosis. This study demonstrates that GrB-cleaved Bid, which differs in size and site of cleavage from caspase-8-cleaved Bid, utilizes Bak for cytochrome c release, and therefore, suggests that deficiency in Bak may serve as a mechanism of immune evasion for tumor or viral infected cells.

Key words: apoptosis • Bak • Bid • cytochrome c • granzyme B • mitochondria

## Introduction

CTLs and natural killer (NK)\* cells induce apoptosis in target cells by two major mechanisms, including the engagement of the Fas receptor on target cells or the release of cytolytic granules into target cells. Ligation of the Fas re-

ceptor on target cells by Fas ligand on cytotoxic cells initiates a death cascade, which involves activation of caspase-8 as an initiator protease (1). The second major mechanism for T and NK cell cytotoxicity utilizes cytolytic granules, which contain perforin and granzymes that are secreted

Address correspondence to Hannah Rabinowich, University of Pittsburgh Cancer Institute, W952 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15213. Phone: 412-624-0289; Fax: 412-624-7737; E-mail: rabinow@pitt.edu

\*Abbreviations used in this paper: Ad, adenovirus; CMXRos, chloromethyl X-rosamine; Cox IV, cytochrome c oxidase IV; DFF, DNA frag-

mentation factor; GrB, granzyme B; GST, glutathione S-transferase; ICAD, inhibitor of caspase-activate DNase; MIB, mitochondria buffer; NAO, nonyl acrydine orange; NK, natural killer; PARP, poly-(ADP-ribose) polymerase; tBid, truncated Bid; TRAIL, TNF-related apoptosis-inducing ligand; Z-VAD-FMK, Cbz-Val-Ala-Asp-fluoromethyl ketone.

into target cells (2, 3). Studies in gene knockout mice have indicated that perforin is essential for the granule exocytosis mechanism of cell death (4). Although, perforin by itself cannot induce apoptosis, it allows granzymes, cosecreted serine proteases, to access their substrates. Perforin is required for the disruption of granzyme-containing target cell endosomes, a function that can be substituted for by endosomolytic agents, such as adenovirus (Ad, reference 5), listeriolysin, and pneumococcal pneumolysin (6).

Granzyme B (GrB), the prototypic member of the granzyme family of serine proteases, shares substrate specificity with caspases for cleavage after aspartate residues (7). GrB has been reported to cleave caspases, including -3, -6, -7, -8, and -10 *in vitro* (2, 8–10). It has been assumed that it can initiate apoptosis at multiple points along the caspase-dependent apoptotic cascade (11, 12). However, a hierarchical manner has been observed for the *in vivo* activity of this enzyme, with preference for specific substrates (13). GrB can also induce death through a caspase-independent mechanism, that is inhibitable by Bcl-2 (14). Consistent with these observations, recent reports have suggested that the preferred mechanism for rapid induction of apoptosis by GrB is mediated by cleavage of Bid, which initiates a mitochondrial apoptotic cascade (13, 15–17). Bid, a BH3-only proapoptotic Bcl-2 family member, is cleaved by caspase-8 (18, 19), lysosomal proteases (20), or GrB (13, 15–17). Although cleaved at different sites (20), each of the resultant truncated Bids (tBids) translocates to the mitochondrial outer membrane, where it triggers the release of mitochondrial intermembrane cytochrome *c*. Release of cytochrome *c* is essential for the mitochondrial apoptotic cascade (21). Upon its release, cytochrome *c*, in concert with Apaf-1 and dATP, activates caspase-9, leading to the subsequent activation of caspase-3 (22–24). As initiation of apoptosis by GrB requires direct cleavage of Bid, but not direct granzyme-B-mediated caspase activation (15), it thus appears that the cytotoxicity mediated by GrB is regulated by internal mitochondrial mechanisms for releasing cytochrome *c*.

The mitochondrial mechanisms responsible for cytochrome *c* release have not yet been fully elucidated. However, using Bak-knockout mice it has been observed that mitochondrial Bak is required for caspase 8-cleaved Bid to induce release of cytochrome *c* (25). Once inserted into the mitochondrial outer membrane, this fragment of Bid does not function as a poreforming protein, but as a membrane targeted death ligand capable of inducing oligomerization of Bak. It has been proposed that oligomerized Bak serves as a mitochondrial outer membrane pore for cytochrome *c* efflux (26).

It has also been suggested that tBid acts partly by inducing conformational changes in Bax and its translocation to the mitochondria (27, 28). Therefore, it is possible that both Bid and Bax are required for mitochondrial response to GrB. Cytosolic Bax in Jurkat cells has been reported to translocate to the mitochondria in response to GrB (16). However, its requirement for GrB-mediated cytochrome *c* release has not been addressed. Also, GrB cleaves Bid at a

different site than caspase-8, and therefore, these two forms of cleaved Bid might have different binding partners upon translocation to the mitochondria. The requirement for Bak by BrB-cleaved Bid in mitochondrial cytochrome *c* release has also not been addressed.

In the current study, we have used a variant Jurkat cell line deficient in Bak to elucidate the role of Bak in the GrB-mediated mitochondrial apoptotic cascade. We have also used three loss-of-function mouse models, Bid deficiency, Bax deficiency, and combined Bid and Bax deficiency, to determine the relevance of Bid and Bax for the mitochondrial response to GrB. This study has established a regulatory role for Bak downstream of Bid in the mitochondrial mechanism for releasing cytochrome *c* in response to GrB, a prominent component of CTL and NK cytolytic granules.

## Materials and Methods

**Reagents.** Anti-human Bak Abs were from Oncogene (Ab-1, mouse clone AMO3, generated against recombinant Bak $\Delta$ C), BD PharMingen (Ab-2, polyclonal Ab generated against recombinant Bak $\Delta$ C), Biosource International (Ab-3, polyclonal Ab generated against synthetic peptide corresponding to amino acids 23–37 of human Bak) and Accurate Chemical (Ab-4, polyclonal Ab generated against recombinant Bak $\Delta$ C). Anti- $\beta$ -actin mAb (clone AC-15) was purchased from Sigma-Aldrich. Anticytochrome *c* oxidase IV (Cox IV) Ab was from Molecular Probes; rabbit anti-Bid Ab was a gift from Xiaodong Wang (Southwestern Medical Center, Dallas, TX). We also used anti-Bid Ab from BioVision and from Santa Cruz Biotechnology, Inc.; anticaspase-3 was from BD PharMingen; anti-poly-(ADP-ribose) polymerase (PARP) mAb (C2.10) and Cbz-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were from Enzyme System; rabbit anti-DNA fragmentation factor (DFF)45/inhibitor of caspase-activate DNase (ICAD) Ab was from ProSci. TNF-related apoptosis-inducing ligand (TRAIL) protein was from Alexis; cisplatin and taxol were from Bristol-Myers Squibb; FITC-annexin V and propidium iodide were from CLONTECH.

**Preparation of His-tagged tBid and Glutathione S-Transferase-Bak.** Mouse NH<sub>2</sub>-terminal histidine tagged tBid (Ser<sup>76</sup>-Asp<sup>195</sup>) was expressed by the recombinant plasmid, p84-1, that was generated by subcloning a PCR amplicon produced from mouse Bid cDNA. The PCR amplicon generated with the forward primer 5'-GGAATCCATATGTCTGAAAGTCAGGAAGAAATC-3' and the reverse primer 5'-CCGCTCGAGTCAGTC-CATCTCGTTTCTAAC-3' was subcloned into Nde 1 and Xho 1 sites of the bacterial expression vector pET-14b (Novagen). *Escherichia coli* strain BL21(DE3) cells were transformed and cultured at 37°C in Terrific Broth. The induction of expression was started at an OD<sub>600</sub> of 0.8–1.0 by the addition of 0.4 mM, isopropyl  $\beta$ -D-thiogalactoside with continued incubation of the culture at 37°C for 2–3 h. The bacterial pellets were resuspended and sonicated in a buffer containing 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. After centrifugation, the cleared supernatants were passed through a His-Bind nickel agarose affinity chromatographic column precharged with 50 mM NiSO<sub>4</sub> (Novagen). The columns were washed with wash buffer containing 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. His-tagged tBid was eluted with elution buffer containing 400 mM imidazole, 500 mM NaCl, and 20 mM Tris-

Cl, pH 7.9, and was further purified using a Sephadex G-50 column equilibrated with PBS.

The plasmid pGEX2TKBak $\Delta$ C encoding COOH-terminal truncated Bak (missing the last COOH-terminal 21 residues) as a glutathione S-transferase (GST) fusion protein was a gift from Thomas Chittenden (Apoptosis Technology Inc., Cambridge, MA). *Escherichia coli* strain DH5 $\alpha$  (GIBCO BRL) cells were transformed and cultured in LB medium. When the OD<sub>600</sub> reached 0.7–1.0, isopropyl  $\beta$ -D-thiogalactoside was added at a concentration of 0.1 mM to induce the expression of the fusion protein. Bacteria were harvested after a 2–3 h incubation at 37°C and lysed by sonication in lysis buffer (1% Triton X-100, 1 mM EDTA in PBS). After centrifugation, the supernatant was incubated with preswollen glutathion beads for 30 min at 4°C. The beads were spun down and washed. GST-Bak $\Delta$ C was eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0. As reported previously (29), the deletion of the COOH-terminal 21 residues, which encompass the transmembrane domain, did not interfere with the insertion of Bak into the mitochondrial outer membrane when directly applied to purified mitochondria.

**Cell Lines and Clones.** Jurkat T leukemic cell line was obtained from American Type Culture Collection. Jurkat cells were grown in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin (complete medium). The Bak-deficient Jurkat clonal cell line was isolated from wild-type Jurkat cells. Neo and Bcl-X<sub>L</sub>-transduced Jurkat cells were a gift from Craig B. Thompson, Abramson Family Cancer Research, Philadelphia, PA.

**Transduction of Jurkat cells by Ad/GT-Bak and Ad/GV16.** Construction of Ad/GT-LacZ, Ad/GT-Bak, and Ad/GV16 was reported previously (30, 31). This binary adenoviral vector system was used to overcome Bak-mediated apoptosis in the packaging 293 cell line. In this binary system, the vector Ad/GT-Bak contains a Bak gene under the control of the GAL4/TATA (GT) promoter and the GAL4/VP16 (GV16) fusion protein. Bak gene expression could then be induced in target cells by coadministration of the Ad/GT-Bak vector with the second adenoviral vector Ad/GV16, which produces the GAL4/VP16 fusion protein. The transduction efficiencies of the adenoviral vectors were determined by assessing the titer needed to infect the cells with Ad/GT-LacZ and Ad/GV16.

**GrB-induced Apoptosis.** CTL-free apoptosis was induced by incubation of target cells with GrB (1–5  $\mu$ g/ml) and replication-deficient Ad type V (10–100 pfu/ml) for 15 min to 24 h. Apoptosis was measured by flow cytometric assessment of phosphatidylserine externalization in cells stained with annexin V-FITC in combination with propidium iodide.

**Western Blot Analysis.** To generate whole cell extracts, cells were lysed in 0.5–1% NP-40, 10 mM Hepes, pH 7.4, 142 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM PMSF, 10  $\mu$ g/ml leupeptin, and 10  $\mu$ g/ml aprotinin. Proteins were resolved by SDS/PAGE and transferred to PVDF membranes, as described previously (32). After probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce Chemical Co.).

**Mitochondria Purification.** To obtain purified mitochondria, Jurkat cells were suspended in mitochondria buffer (MIB) composed of 0.3 M sucrose, 10 mM MOPS, 1 mM EDTA, and 4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, and lysed by Dounce homogenization as described previously (33). Briefly, nuclei and debris were removed by 10 min centrifugation at 650 g and a pellet containing mitochondria was obtained by two successive spins at 10,000 g

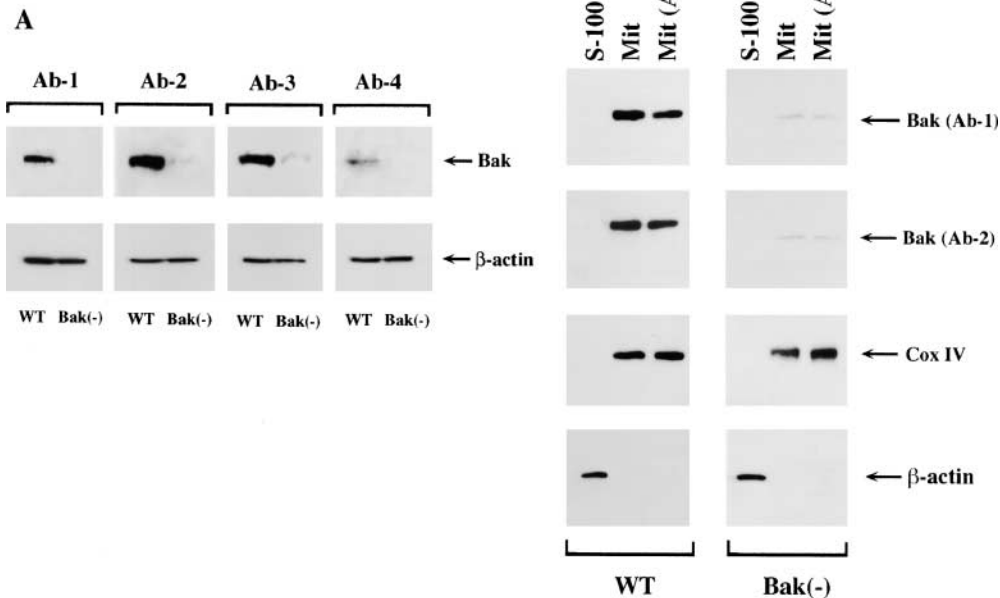
for 12 min. To obtain the S-100 fraction, the postnuclear supernatant was further centrifuged at 100,000 g for 1 h at 4°C. To obtain purified mitochondria, the mitochondrial pellet was re-suspended in MIB and layered on a Percoll gradient consisting of four layers of 10%, 18%, 30%, and 70% Percoll in MIB. After centrifugation for 35 min at 13,500 g, the purified mitochondria were collected at the 30/70 interface. Mitochondria were diluted in MIB containing 1 mg/ml BSA (at least a 10-fold dilution required to remove Percoll). The mitochondrial pellet was obtained by a 30-min spin at 20,000 g and used immediately. When indicated, mitochondria were pelleted, then incubated in 100  $\mu$ l 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, for 20 min on ice to remove loosely attached proteins (34). After alkali treatment, supernatants and mitoplasts were separated by centrifugation and dissolved in 0.5% NP-40 lysis buffer. The fractions were analyzed by immunoblotting.

**Cytochrome c Release Assay.** Purified mitochondria (100  $\mu$ g protein) were incubated with recombinant His-tBid or GST-Bak $\Delta$ C at various doses as indicated, in 100  $\mu$ l MIB at 30°C for 30 min. Mitochondria were pelleted by centrifugation at 10,000 g for 5 min. The resulting supernatants or mitochondria were mixed with lysis buffer and analyzed by SDS/PAGE and immunoblotting for the presence of cytochrome c.

## Results

**Deficient Expression of Bak Protein in Clonal Jurkat Cells.** A clonal cell line isolated from the ATCC wild-type Jurkat cell line was found to be Bak-deficient. The deficiency was determined by immunoblotting of whole cell lysates by four different anti-Bak Abs (Fig. 1 A). To further analyze the expression of Bak, lysates of wild-type or the clonal Jurkat cells were fractionated to yield S-100 cytosol or purified mitochondria. These protein fractions were assessed for the expression of Bak by Western blot analyses. Whereas expression of Bak was detected in mitochondria of wild-type Jurkat cells, only minor expression of Bak was observed in a similar quantity of purified mitochondria from Bak-deficient cells (Fig. 1 B). To assess the mitochondrial localization of Bak, purified mitochondria from either wild-type or Bak-deficient cells were treated with alkali to remove proteins nonspecifically attached to the mitochondria (34). In wild-type Jurkat cells, Bak was found to be a mitochondrial integral membrane protein, as it was detected in the pellet of alkali-treated mitochondria (Fig. 1 B). Levels of protein expression of other Bcl-2 family members in Bak-deficient cells, including Bcl-2, BCL-X<sub>L</sub>, or Bax, were similar to those of the wild-type cell line (data not shown).

**Resistance of Bak-deficient Cells to GrB-mediated Apoptosis.** To study the susceptibility of Bak-deficient cells to GrB-mediated cell death we used a CTL-free system, wherein purified GrB was added in combination with a replication-deficient Ad to the target cell suspension (5, 11, 35). Although GrB enters the cell independently, via its surface receptor (36), efficient apoptosis does not occur in the absence of perforin or a substituting Ad. As assessed by flow cytometric analysis of cells stained with annexin V, treatment of Jurkat cells for 2 h with a combination of GrB (1

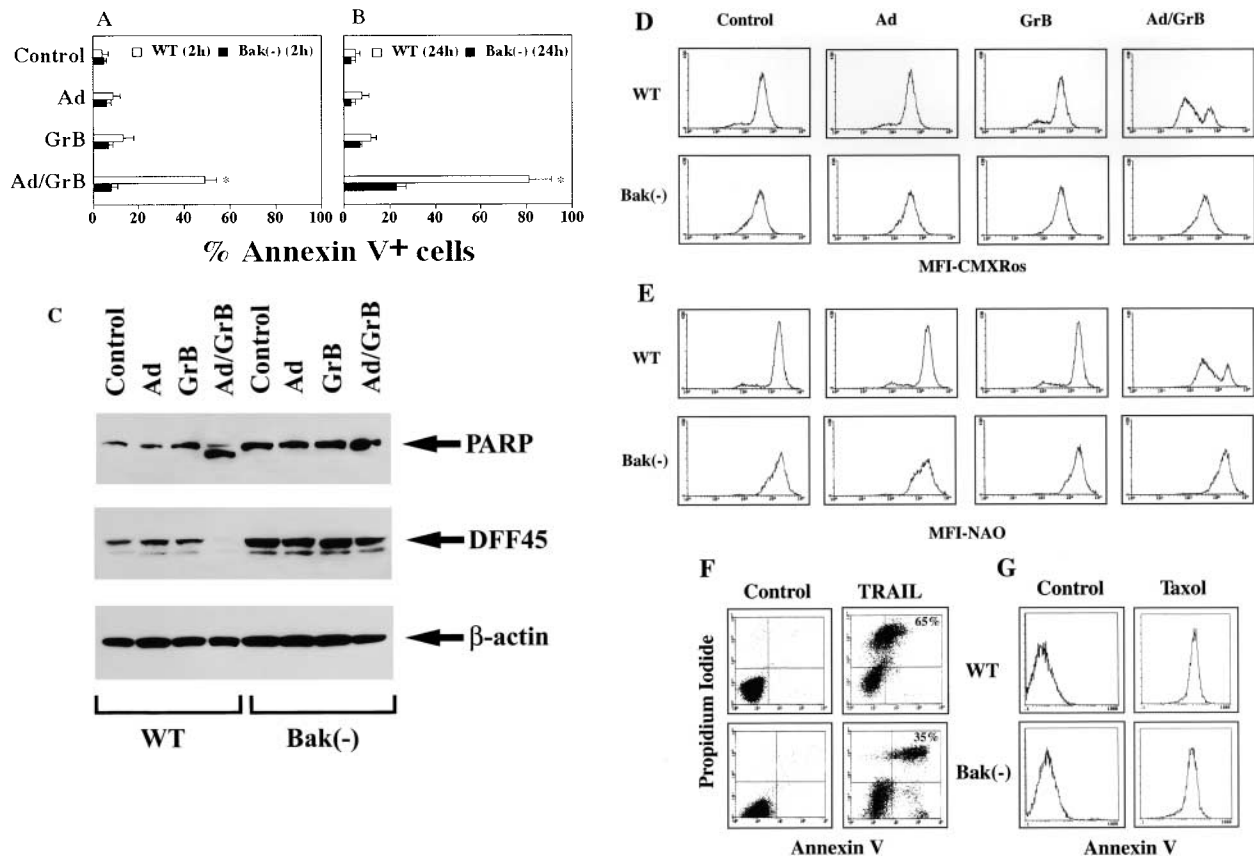


**Figure 1.** Deficient expression of Bak in a clonal Jurkat cell line. (A) Wild-type or the variant Jurkat cell line, Bak<sup>-</sup>, were incubated in 1% NP-40 lysis buffer for 30 min at 4°C. The resultant lysates which contained both cytoplasm and mitochondria, were resolved by SDS/PAGE and assessed by immunoblotting for the presence of Bak. Four different anti-human Bak Ab were used for blotting. The membranes were stripped and reprobed for β-actin to demonstrate equal loading. (B) Expression of Bak in mitochondria of wild-type, but not in mitochondria of Bak-deficient Jurkat cells. Expression of Bak was examined in cytosol (S-100), purified mitochondria, or purified mitochondria treated with alkali to remove nonspecifically attached proteins. These cell fractions were resolved by SDS/PAGE and immunoblotted sequentially by Bak-specific Ab-1 and Ab-2. After additional stripping, the membranes were probed with anti-Cox IV Ab, as a marker for mitochondrial fractions, and with anti-β-actin as a marker for cytosolic proteins.

μg/ml) and Ad (10–100 pfu/ml) induced apoptosis in wild-type, but not in Bak-deficient Jurkat cells (Fig. 2 A). After GrB/Ad treatment for 24 h, ~20% of Bak-deficient cells were annexin V-positive, as compared with ~80% of wild-type cells (Fig. 2 B). Similar results (data not shown) were obtained in cells treated with GrB and LAK extracts that served as a source of perforin (37). These findings suggest that despite the ability of GrB to initiate apoptosis at multiple sites by direct cleavage of caspases or other apoptosis effector proteins, the major apoptotic mechanism of GrB is blocked in our Jurkat Bak-deficient cells. Wild-type cell apoptosis induced by 2-h treatment with GrB/Ad was associated with cleavage of PARP and loss in DFF45/ICAD expression (Fig. 2 C). In similarly treated Bak-deficient cells, no cleavage of PARP or loss in DFF45/ICAD was detected. To assess mitochondrial alterations associated with GrB-mediated apoptosis, we used two fluorescent dyes, which target different mitochondrial components. After 2 h of treatment with GrB/Ad, the cells were stained with the cationic lipophilic dye, chloromethyl X-rosamine (CMXRos), to measure disruption of the mitochondrial transmembrane potential. GrB/Ad treated wild-type, but not Bak-deficient cells, demonstrated reduction in incorporation of this dye (Fig. 2 D). We also assessed mitochondrial changes by nonyl acrydine orange (NAO), which interacts specifically with nonoxidized cardiolipin, a lipid that is exclusively localized in the inner mitochondrial membrane (38). Loss in cardiolipin was detected in wild-type Jurkat cells treated with GrB/Ad, but not in Bak-deficient

cells (Fig. 2 E). These findings suggest that mitochondrial apoptotic events induced in wild-type Jurkat cells by GrB were blocked in our Bak-deficient cells. Despite their differential susceptibility to GrB-mediated apoptosis both wild-type and Bak-deficient Jurkat cells demonstrated sensitivity to apoptosis induced by either TRAIL (Fig. 2 F) or taxol (Fig. 2 G). TRAIL-mediated apoptosis is usually enhanced by a mitochondrial apoptotic loop (1), but may also proceed via a caspase-dependent, mitochondria-independent cascade. In accordance with this model, Bak-deficient cells were TRAIL-sensitive, albeit at a decreased level relative to wild-type Jurkat cells. The reduced susceptibility of Bak-deficient Jurkat cells to TRAIL may relate to the block in the mitochondrial apoptotic loop in these cells (Fig. 2 F). Wild-type and Bak-deficient Jurkat cells were similarly susceptible to taxol (Fig. 2 G), suggesting that Bak was not involved in the mechanism of cytotoxicity mediated by this anticancer drug. These results suggest that Bak-deficiency in Jurkat cells has a significant inhibitory effect on GrB-mediated cytotoxicity, executed mainly by a mitochondrial apoptotic cascade.

*Restored Susceptibility to GrB after Transduction of Bak-deficient Cells with Ad/GT-Bak and Ad/GV16 Vectors.* Using a binary adenoviral vector system to avoid the toxic effects of Ad/Bak on the 293 packaging cells, we successfully produced large amounts of Ad/GT-Bak, whose gene product (Bak) was under the transcriptional control of the GT promoter and GV16 fusion protein (30, 31). The binary adenoviral LacZ vector system (Ad/GT-LacZ plus Ad/GV16)

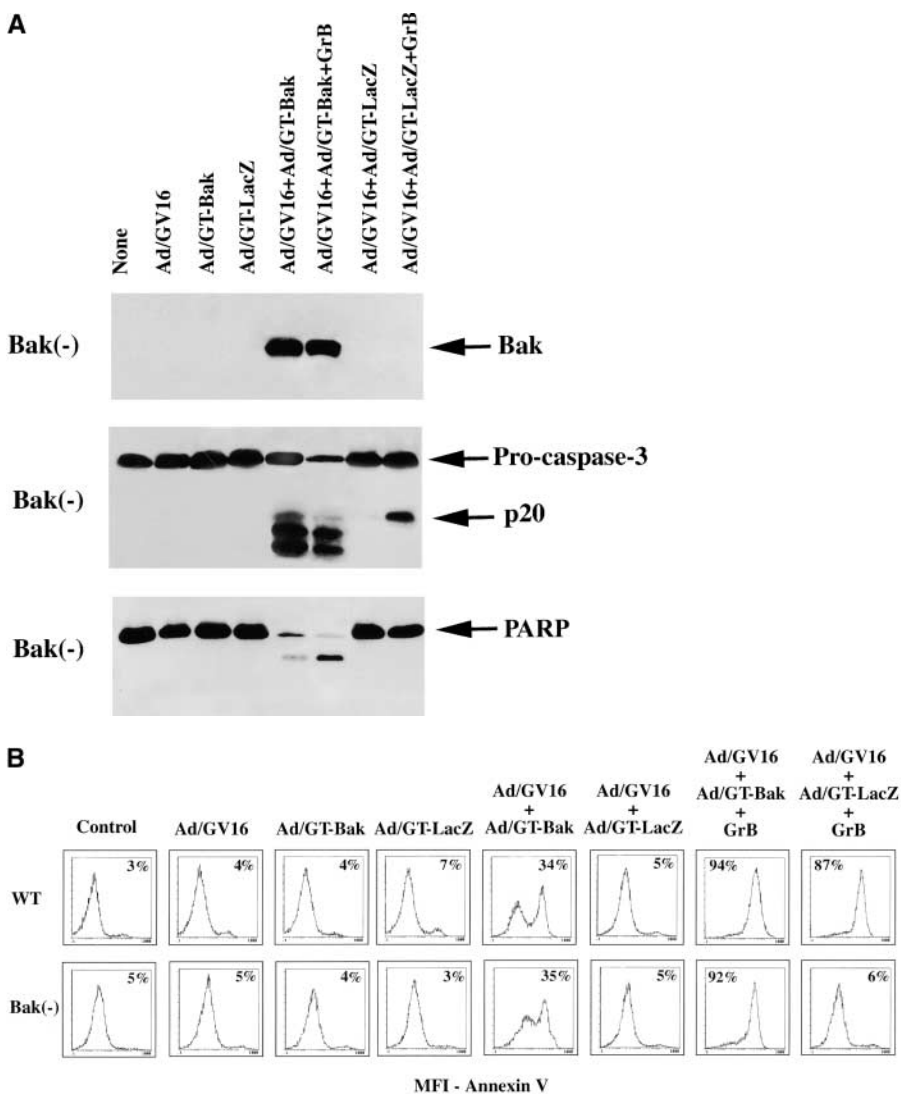


**Figure 2.** Resistance of Bak-deficient Jurkat cells to GrB-mediated apoptosis. (A) and (B) Flow cytometry analysis of staining by annexin V of Jurkat cells treated with Ad (10 PFU/ml), GrB (1  $\mu$ g/ml), and a combination of GrB and Ad for 2 h (A) or 24 h (B) at 30°C. The data are means  $\pm$  SD of results obtained in five independent experiments. The asterisks indicate a statistically significant difference between wild-type and Bak-deficient cells ( $P < 0.05$ , Mann-Whitney  $U$ ). (C) GrB-mediated cleavage of PARP and DFF45/ICAD in wild-type, but not in Bak-deficient cells. Wild-type and Bak-deficient cells were treated with Ad, GrB, or a combination of GrB and Ad, as described previously. The cell extracts were resolved on SDS/PAGE and immunoblotted with anti-PARP mAb or anti-DFF45/ICAD Ab. (D) and (E) Lack of mitochondrial apoptotic events in Bak-deficient Jurkat cells treated with GrB. After 2 h of treatment with GrB and Ad, as described previously, the cells were assessed by flow cytometry for mitochondrial staining with CMXRos or NAO. Staining with CMXRos (100 nM) served to assess changes in mitochondria permeability transition; staining with NAO (100 nM) served to assess loss in mitochondrial cardiolipin. (F) and (G) Susceptibility of Bak-deficient Jurkat cells to TRAIL or taxol. Wild-type or Bak-deficient Jurkat cells were treated with TRAIL (100 ng/ml) or taxol (10  $\mu$ g/ml) for 16 h. The cells were then analyzed by flow cytometry for staining by annexin V or propidium iodide. Percentage of apoptotic cells are indicated for TRAIL.

was used to determine transduction efficiency. As detected by immunoblotting, Bak expression was induced when Ad/GT-Bak plus Ad/GV16 were administered, but not when Ad/GT-LacZ plus Ad/GV16 were used (Fig. 3 A). High level expression of Bak has been reported to induce a rapid cell death (39). As assessed by flow cytometry of cells stained with FITC-conjugated annexin V, we also observed a substantial level of apoptotic cell death ( $\sim$ 35%) in both wild-type and Bak-deficient Jurkat cells 24 h after transduction with Ad/GT-Bak plus Ad/GV16 vectors, but not in mock infected cells (Fig. 3 B). Whereas Bak-deficient cells were resistant to GrB-mediated apoptosis, a significant increase in susceptibility (up to  $\sim$ 90%) was detected in cells transduced with the Bak gene, but not in control cells transduced with LacZ (Fig. 3 B). Apoptosis was also confirmed by detection of active caspase-3 subunits and PARP cleavage in Bak-deficient cells infected with Bak, but not in mock-infected cells similarly treated

(Fig. 3 A). Increased loss in prodomain caspase-3 or PARP was detected in cells infected with Bak and treated with GrB. Interestingly, the p20 subunit of caspase-3, but not the p19 or p17 subunits, was also detected in LacZ transduced cells treated with GrB. This subunit represents the direct cleavage of caspase-3 by GrB (6, 40). However, the partially activated caspase-3 was not sufficient to induce apoptosis, as assessed by annexin V staining (Fig. 3 B) or PARP cleavage (Fig. 3 A). These results confirm the role of Bak deficiency in the observed resistance of these cells to GrB/Ad-mediated apoptosis.

*Abrogation of Cytochrome c release in Bak-deficient Mitochondria.* GrB-mediated cytochrome c release has recently been reported to be regulated by the Bcl-2 family member, Bid (15–17). The initiation of apoptosis by GrB has been reported to require direct cleavage of Bid, and the translocation of the cleaved product to the mitochondria, where it induces release of cytochrome c. Caspase-8–

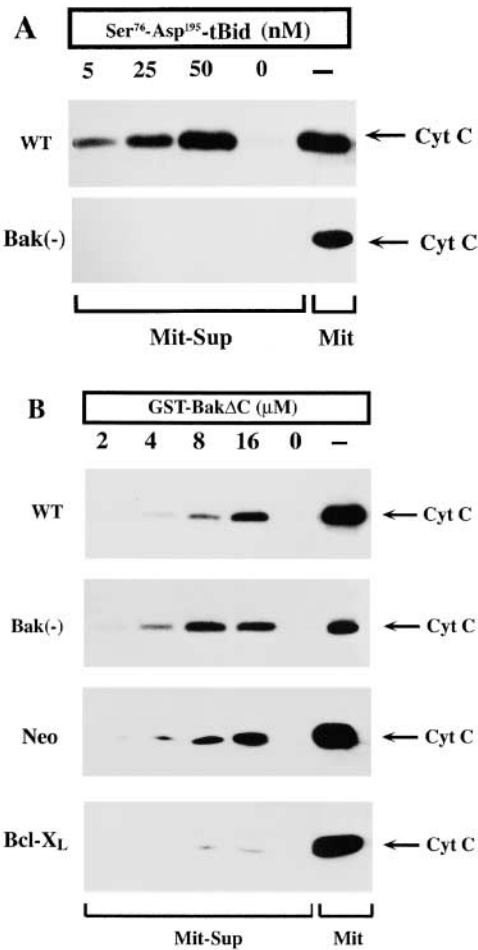


**Figure 3.** Restoration of apoptotic response to GrB after Bak gene transduction. (A) Bak expression, caspase-3 activation and PARP cleavage after adenoviral-mediated transduction of Bak. 24 h after infection of Bak-deficient cells with the indicated combinations of adenoviral vectors, cell extracts were assessed by immunoblotting for Bak expression. The membrane was stripped and reprobed for caspase-3 activation, stripped again, and reprobed with anti-PARP mAb. The p20 caspase-3 subunit in LacZ transduced cells represents the direct cleavage of caspase-3 by GrB. (B) Susceptibility of Bak-deficient cells to GrB 24 h after adenoviral transduction with Bak, but not with LacZ gene. The presence of apoptotic cells was assessed by annexin V staining of wild-type or Bak-deficient cells infected with the indicated combinations of adenoviral vectors and treated with GrB (1  $\mu\text{g}/\text{ml}$ ). Percentage of annexin V-positive cells is indicated.

cleaved Bid has been reported to require mitochondrial Bak for cytochrome c release (25). To further explore the possibility that the block in the response of our Bak-deficient cells to GrB cytotoxicity was related to the requirement of mitochondrial Bak for Bid function, we examined the ability of a recombinant tBid that corresponds in amino acid sequence to GrB-cleaved Bid (Ser<sup>76</sup> to Asp<sup>195</sup>) to induce cytochrome c release from purified mitochondria. In contrast to mitochondria from wild-type Jurkat cells, mitochondria obtained from Bak-deficient cells that were treated with Ser<sup>76</sup> to Asp<sup>195</sup> recombinant tBid did not release cytochrome c (Fig. 4 A). However, release of cytochrome c was observed when purified mitochondria from either wild-type or Bak-deficient cells were treated with recombinant Bak (GST-Bak $\Delta$ C, Fig. 4 B). Cytochrome c release in response to recombinant Bak was inhibited in mitochondria purified from Jurkat cells overexpressing Bcl- X<sub>L</sub> (Fig. 4 B). These results suggest that the resistance of Bak-deficient Jurkat cells to GrB-mediated apoptosis is associated with the abrogation of cytochrome

c release by these mitochondria in response to GrB-cleaved Bid.

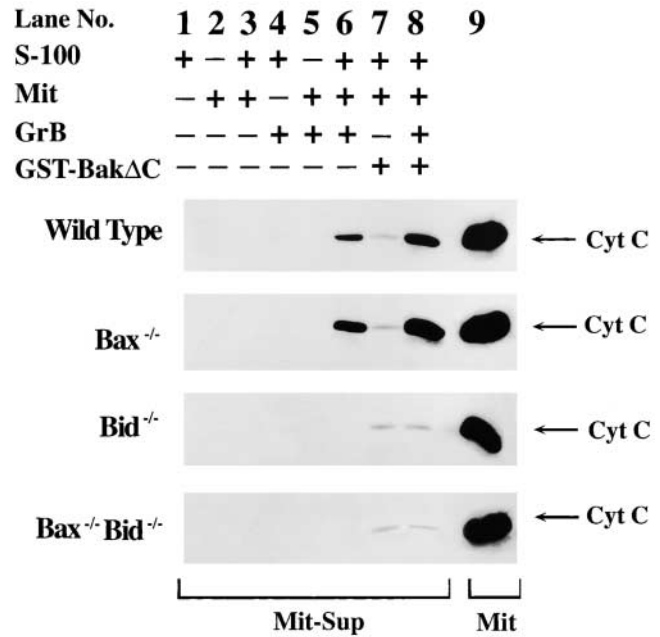
*Bid, but not Bax, Is Required for GrB-mediated Cytochrome c Release.* The cleaved products of Bid generated by either caspase-8 or GrB have been reported to induce the translocation and insertion of cytosolic Bax into the outer mitochondrial membrane (16, 28). To determine the requirement for Bid and/or Bax in GrB-mediated release of mitochondrial cytochrome c, we used liver mitochondria from either Bid<sup>-/-</sup>, Bax<sup>-/-</sup>, or Bid<sup>-/-</sup>Bax<sup>-/-</sup> mice (41, 42). Purified liver mitochondria were incubated with their autologous cytosolic S-100 fractions in the presence or absence of GrB (1  $\mu\text{g}/\text{ml}$ , 1 h, 30°C). GrB induced the release of cytochrome c when incubated with mitochondria and S-100 fractions from either wild-type or Bax<sup>-/-</sup> cells (Fig. 5, lane 6). No release of cytochrome c was detected with mitochondria obtained from Bid<sup>-/-</sup> or Bax<sup>-/-</sup>Bid<sup>-/-</sup> mice (Fig. 5, lane 6). The knockout murine model confirms the requirement for Bid in GrB-mediated cytochrome c release. It appears that although Bax translocates



**Figure 4.** Abrogation of cytochrome c release in Bak-deficient mitochondria in response to recombinant GrB-cleaved Bid. (A) Purified mitochondria (100  $\mu$ g) obtained from wild-type or Bak-deficient Jurkat cells were treated with his-tBid (Ser<sup>76</sup>-Asp<sup>195</sup>) at the indicated concentrations for 30 min at 30°C. The supernatants (Mit-Sup) were boiled in reducing Laemmli buffer, and assessed by immunoblotting for the presence of cytochrome c. Lysed mitochondria from each of the two cell lines tested served as positive controls. (B) Release of cytochrome c by mitochondria from either wild-type or Bak-deficient Jurkat cells is induced by recombinant Bak and inhibited by Bcl-X<sub>L</sub>. Purified mitochondria from wild-type, Bak-deficient, and Neo- or Bcl-X<sub>L</sub>-transduced cells were incubated with GST-Bak $\Delta$ C at the indicated concentrations for 30 min at 30°C. The presence of cytochrome c in the supernatants was assessed as described previously.

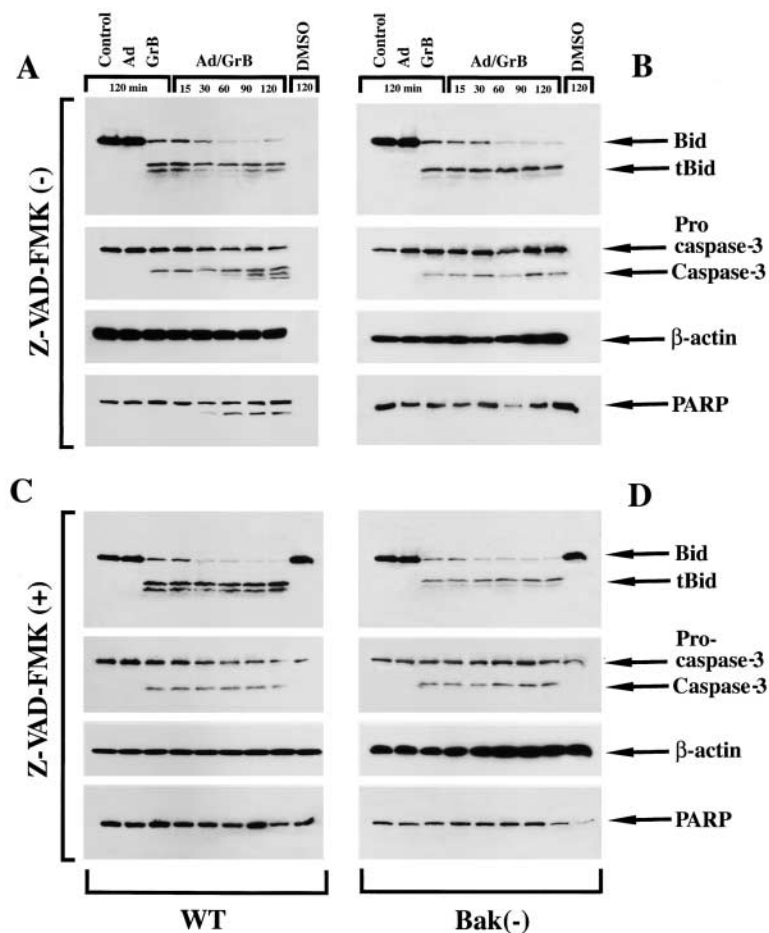
to the mitochondria in response to tBid (16), this process is not essential for GrB-mediated cytochrome c release. As direct application of Bak to purified mitochondria induces release of cytochrome c, we examined whether it would synergize with GrB in the setting of mouse S-100 and mitochondria. Mitochondria from the various mice tested, responded with minor release of cytochrome c when treated with a low dose of recombinant Bak (Fig. 5, lane 7). However, synergy between recombinant Bak and GrB in release of cytochrome c was observed only in wild-type or Bax<sup>-/-</sup> mitochondria (Fig. 5, lane 8).

*A Direct, Caspase-independent Cleavage of Bid Is Unaltered in Bak-deficient Cells.* Our findings suggest that the block in



**Figure 5.** Bid, but not Bax, is essential for GrB-mediated release of mitochondrial cytochrome c. Purified mitochondria and S-100 cytosol fractions were obtained from mouse livers of wild-type, or knockout mice, including Bid<sup>-/-</sup>, Bax<sup>-/-</sup>, and Bid<sup>-/-</sup>Bax<sup>-/-</sup>. Purified mitochondria were coincubated with autologous S-100 fractions in the presence or absence of GrB (5  $\mu$ g/ml) and GST-Bak $\Delta$ C (4  $\mu$ M) for 30 min at 30°C. Supernatants containing mitochondrial proteins were resolved by SDS/PAGE and assessed by immunoblotting for the presence of cytochrome c.

response of Bak-deficient Jurkat cells to GrB localizes to the cytochrome c release mechanism within the mitochondria of these cells. To ensure that the function of Bid upstream of the mitochondria is unaltered, we examined Bid expression and its translocation to the mitochondria of Bak-deficient cells. We first compared the kinetics of Bid cleavage in wild-type and Bak-deficient Jurkat cells treated with a combination of GrB and Ad. Exposure for 15 min of either wild-type or Bak-deficient cells to GrB/Ad was sufficient for processing of significant portion of full-length Bid present in these cells (Fig. 6 A and B). These results demonstrate that Bid cleavage is unaffected by Bak-deficiency. Under the conditions employed, proteolytic processing occurred more readily with Bid than with DFF45/ICAD (Fig. 2 C), reported to be a direct substrate for GrB. To ensure that the observed cleavage of Bid was directly mediated by GrB, rather than via GrB-activated caspases, cleavage of Bid was also examined in cells pretreated with the pan-caspase inhibitor, Z-VAD-FMK. Similar kinetics and levels of processing were observed in wild-type or Bak-deficient Jurkat cells in the absence or presence of Z-VAD-FMK (Fig. 6 C and D). These results suggest that GrB-mediated cleavage of Bid can proceed in a caspase-independent manner. To control for the activity of caspase-3 in GrB-treated cells, the same membranes (Fig. 6) were stripped and reprobed with anti-caspase-3 Ab. In wild-type Jurkat cells, p19 and p17 caspase-3 subunits were detected after 1–2 h of exposure to



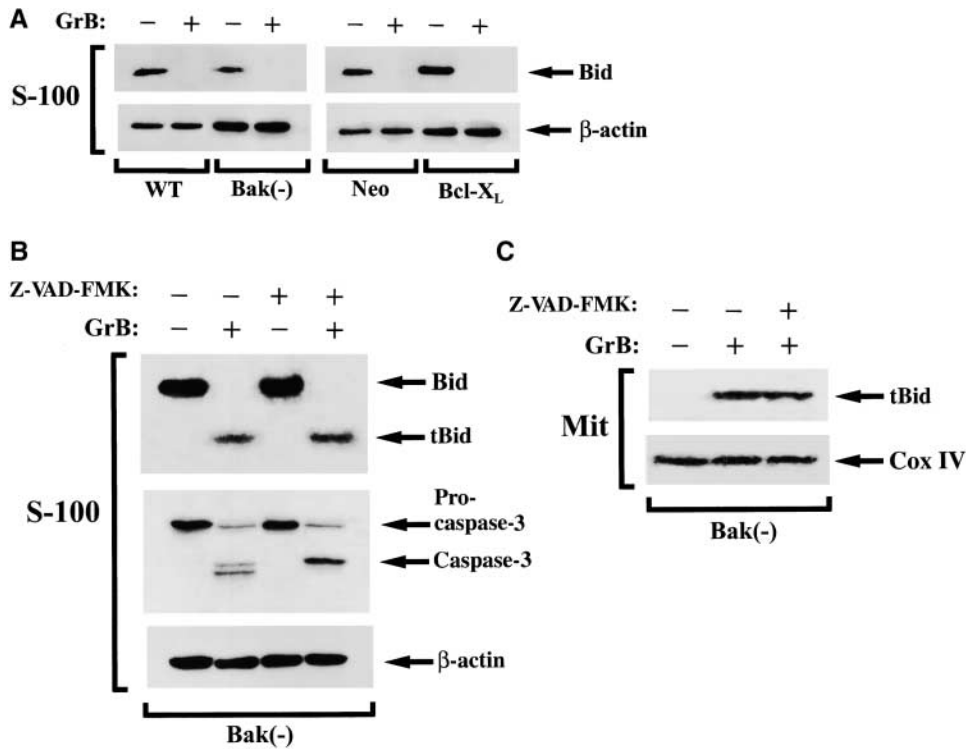
**Figure 6.** GrB-mediated cleavage of Bid is unaltered in Bak-deficient cells and proceeds in the presence of a potent caspase inhibitor. Wild-type and Bak-deficient Jurkat cells were treated with Ad (10 PFU/ml) or GrB (1  $\mu$ g/ml) for 2 h, or with a combination of GrB and Ad for various time periods as indicated. In A and B, the cells were treated in the absence of caspase inhibitors. In C and D, the cells were pretreated with Z-VAD-FMK (100  $\mu$ M) for 2 h before exposure to Ad, GrB, or GrB/Ad. A DMSO control for the solvent of Z-VAD-FMK is included in C and D. At the end of the incubation periods, the cells were lysed, resolved by 15% SDS/PAGE, and immunoblotted for the presence of Bid and its cleavage products. Each of the membranes was sequentially stripped and reprobbed with anticaspase-3 and anti- $\beta$ -actin Ab. For the detection of PARP, the same samples were resolved by 7.5% SDS/PAGE. Please note, the presented  $\beta$ -actin serves as a loading control for Bid and caspase-3 (not for PARP).

GrB/Ad (Fig. 6 A). However, in the presence of Z-VAD-FMK, only the p20 caspase-3 subunit, a direct cleavage product of GrB was detected (Fig. 6 B). The absence of p19 and p17 caspase-3 subunits, suggests that the p20 subunit is unable to undergo further processing in the presence of Z-VAD-FMK. Interestingly, in Bak-deficient cells only the p20 caspase-3 subunit was detected in either the presence or the absence of Z-VAD-FMK. To assess the activity of p20 caspase-3, the same samples were examined for the presence of cleavage products of PARP. Cleavage of PARP was detected only in wild-type Jurkat cells, where full processing of caspase-3 was detected (Fig. 6 A). Caspase-3 p20 detected in wild-type cells in the presence of Z-VAD-FMK, or in Bak-deficient cells appears to be relatively inactive as it failed to cleave PARP (Fig. 6 B–D). In contrast to PARP or DFF45/ICAD whose efficient processing required the presence of both GrB and Ad (Fig. 2 C), Bid cleavage was observed also in cells treated with GrB only. These results further demonstrate that Bid is a preferred substrate for GrB, which upon cell internalization cleaves Bid even in the absence of facilitation by an Ad.

Next, we treated Dounce homogenized extracts of wild-type, Bak-deficient, Neo, and BCL-X<sub>L</sub> transduced Jurkat cells with GrB (1  $\mu$ g/ml, 1 h, 30°C). The extracts

were then separated into cytosol S-100 and mitochondria fractions. GrB treatment resulted in complete processing of Bid, as full-length Bid was not detected in the cytosols obtained from GrB-treated extracts of all the variants of Jurkat cells examined (Fig. 7 A). Thus, Bak-deficiency or overexpression of Bcl-X<sub>L</sub> do not affect GrB-mediated processing of Bid. Interestingly, full processing of caspase-3 was detected in the cytosol fraction of Bak-deficient cell extracts treated with GrB (Fig. 7 B). To ensure that Bid cleavage was not mediated by GrB-activated caspase-3, the extracts were treated with Z-VAD-FMK (100  $\mu$ M, 20 min) before the addition of GrB. Similar processing of Bid was also observed in the presence of the caspase inhibitor (Fig. 7 B). In the presence of Z-VAD-FMK, only p20 caspase-3 was detected in extracts of Bak-deficient cells, whereas both p19 and p17 caspase-3 subunits were detected in the absence of the inhibitor. Thus, in contrast to GrB-treated Bak-deficient cells, in treated extracts full processing of caspase-3 was observed. It appears that the application of GrB directly into the extracts results in a higher dose than would have been internalized via the cell membrane. Such an augmented dose of GrB seems to overcome the lack of mitochondrial contribution to caspase-3 processing in Bak-deficient cells. The cleavage product of Bid was detected in the mitochondria obtained





**Figure 7.** GrB-mediated cleavage of Bid and its translocation to the mitochondria in extracts of Bak-deficient cells. (A) Jurkat cell lines, including wild-type, Bak-deficient, Neo-, or Bcl-X<sub>L</sub>-transduced cells were Dounce homogenized, and then treated with GrB (1 μg/ml) for 1 h at 30°C. The extracts were then separated into S-100 cytosol and mitochondria fractions. Loss of full-length Bid was detected in cytosols of all Jurkat cell variants treated with GrB. The anti-BID Ab used for this blot detects only full length Bid. (B) GrB-mediated cleavage of Bid proceeds in the presence of Z-VAD-FMK. Extracts of Bak-deficient Jurkat cells were incubated with Z-VAD-FMK (100 μM) for 20 min before the addition of GrB (1 μg/ml) for 1 h at 30°C. The extracts were then fractionated into S-100 and mitochondrial fractions, which were assessed by immunoblotting and sequential probing for the presence of Bid, caspase-3, and β-actin. (C) Translocation of GrB-cleaved Bid to the mitochondria of Bak-deficient Jurkat cells. The mitochondrial fraction of extracts of Bak-

deficient cells treated with GrB in the presence or absence of Z-VAD-FMK was assessed by immunoblotting for the presence of Bid, caspase-3, and β-actin. The membrane was stripped and reprobed with anti-Cox IV mAb, as a mitochondrial marker and to demonstrate equal loading.

from extracts of Bak-deficient cells treated in the presence or the absence of Z-VAD-FMK (Fig. 7 C). These results suggest that GrB-cleaved Bid targets the mitochondria within the cell extract. Together, the results shown in Figs. 6 and 7 suggest that the block in GrB-mediated cytochrome c release in Bak-deficient cells is downstream of GrB-cleaved Bid translocation to the mitochondria.

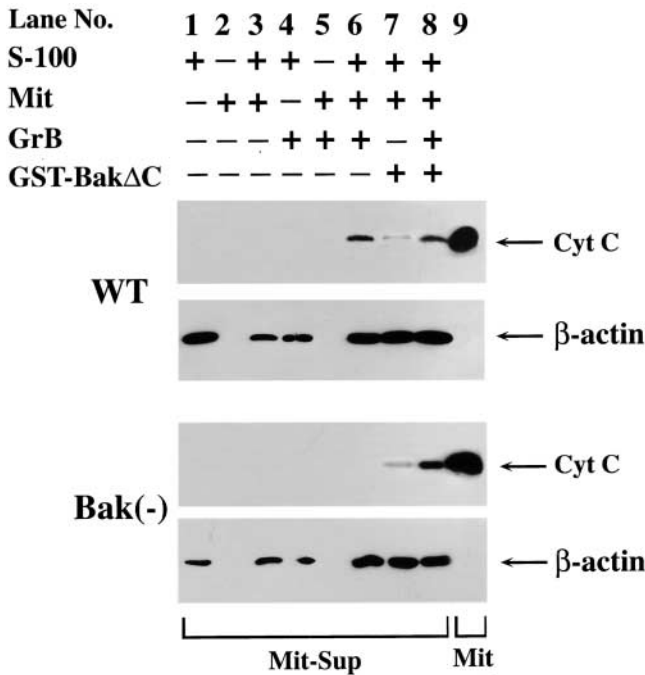
*GrB-mediated Cytochrome c Release Is Abrogated in Bak-deficient Mitochondria, but Restored in the Presence of Recombinant Bak.* To directly investigate the role of Bak in release of mitochondrial cytochrome c in response to GrB, we compared the response of wild-type and Bak-deficient mitochondria to autologous S-100 treated with GrB. Cytochrome c was detected in the supernatant of wild-type mitochondria, but not in supernatants of Bak-deficient mitochondria, treated with S-100 cytosol and GrB (Fig. 8, lane 6). However, in the presence of a low-dose of recombinant Bak, which by itself induced only minor release of cytochrome c (Fig. 8, lane 7), an augmented release in response to GrB was detected (Fig. 8, lane 8). Thus, the presence of recombinant Bak restored the capability of Bak-deficient mitochondria to release cytochrome c in response to GrB and cytosol.

## Discussion

This study is the first to demonstrate that the mitochondrial proapoptotic Bcl-2 member, Bak, plays a role in GrB/Ad-mediated cytochrome c release. Bak deficiency en-

dowed this Jurkat cell variant with a potent mechanism of resistance to GrB/Ad-induced cell death. The significance of Bak was confirmed by transduction of the Bak gene into these cells, which restored susceptibility to GrB-mediated apoptosis. The importance of Bak was further demonstrated in a cell-free system, where GrB-treated S-100 did not induce release of cytochrome c from Bak-deficient mitochondria from the Jurkat cell variant unless recombinant Bak was added.

GrB is a prominent mediator of the apoptotic cascade induced by CTLs or NK in a pathogenic cell. Its significance in the execution of cytotoxic mechanisms against viral-infected cells or allogeneic cells has been established in studies performed in GrB-knockout mice (43–46). As GrB is an aspartase, which can cleave either caspases (47, 48) or death substrates directly (37, 49, 50), it was thought to produce apoptotic events and morphology by short-circuiting the death receptor or mitochondrial pathways of apoptosis. However, in Bak-deficient Jurkat cells treated for up to 24 h with a combination of GrB and Ad, a significant inhibition of apoptosis was observed, whereas wild-type cells were highly susceptible as early as 2 h after similar GrB treatment. These findings suggest that the Bak deficiency serves to block the prominent pathway used by GrB to initiate target cell apoptosis. As Bak represents a mitochondrial protein involved in the mechanism of cytochrome c release, our results imply that a mitochondrial pathway of apoptosis is preferentially activated by GrB to induce apoptosis. Consistent with our findings, it has recently been



**Figure 8.** Restoration of GrB-mediated cytochrome c release from Bak-deficient Jurkat mitochondria in the presence of recombinant Bak. Purified mitochondria and S-100 cytosol fractions obtained from wild-type or Bak-deficient Jurkat cells were treated with GrB (1  $\mu$ g/ml) and/or GST-Bak $\Delta$ C (2  $\mu$ M) for 30 min at 30°C. The supernatants were assessed by immunoblotting for the presence of cytochrome c. The membranes were stripped and reprobed with anti- $\beta$ -actin Ab.  $\beta$ -actin, a cytosolic protein, was detected in S-100, and not mitochondria-only fractions.

concluded that the preferred mechanism for rapid induction of apoptosis by GrB is via initiation of a mitochondrial cascade inhibitable by Bcl-2 (13, 15, 16). Using time lapse confocal microscopy it was demonstrated that mitochondrial cytochrome c release is the primary mode of GrB-induced apoptosis (13). Caspase activation was not required for the observed release of cytochrome c, which was mediated by Bid cleavage and its translocation to the mitochondria. GrB was found to cleave Bid more readily than it cleaves either caspase-3 or -8 (13). We also observed that Bid was the preferred GrB substrate as compared with DFF45/ICAD. Cleavage of DFF45/ICAD by either caspase-3 or GrB, dismantles its inhibitory activity and facilitates its assembly into an active form of DNase (37, 51, 52). Exogenous GrB at a dose which was sufficient to enter the cells and induce Bid processing in 15 min, was too low to directly cleave DFF45/ICAD even after 2-h incubation. This DNase precursor was readily processed in wild-type Jurkat cells, suggesting that DFF45/ICAD processing was enhanced by the mitochondrial release of cytochrome c induced by GrB in wild-type cells, but not in Bak-deficient cells. The cleavage of Bid by GrB represents a caspase-independent event upstream of the mitochondria, as similar kinetics and processing were observed in the presence or the absence of a potent caspase inhibitor in either wild-type or Bak-deficient cells.

The requirement for Bid in GrB-mediated mitochondrial cascade was demonstrated by various experimental approaches, including the immunodepletion of Bid from cytosolic extracts before the addition of mitochondria and GrB (16), or by the inability of Bid with a mutated GrB cleavage site to restore the response to GrB in Bcl-2 overexpressing cells (15). Bid requirement was confirmed in the current study by a Bid knockout murine model. We also used a Bax knockout model to determine its role in the GrB apoptotic cascade. In contrast to Bid, the expression of Bax was not essential for GrB-mediated cytochrome c release. Although Bax does not appear to be required for the GrB mitochondrial cascade, its translocation to the mitochondria as observed in Jurkat cells by Heibien et al. (16), suggests that it plays a facilitating role in the mitochondrial response to GrB.

Bak, a mitochondrial proapoptotic Bcl-2 family member is required for the mechanism of cytochrome c release in response to caspase-8 cleaved Bid (25). The exact nature of the interaction between caspase-8-cleaved Bid and Bak has not yet been fully resolved. Using murine Bak-deficient mitochondria or by treating Bak-containing mitochondria with an anti-Bak blocking Ab, tBid was found to bind in a transient manner to mitochondrial Bak and to release cytochrome c (25). As a result of this interaction, Bak undergoes allosteric activation and an intramembraneous oligomerization into a proposed pore for cytochrome c efflux (25, 26). This study demonstrates that GrB-cleaved Bid can also utilize Bak to induce the release of mitochondrial cytochrome c. Thus, using either human Bak-deficient Jurkat cells or a cell-free system composed of Bak-deficient mitochondria and cytosol derived from these cells, our findings have established an unequivocal role for Bak in the mitochondrial response to GrB. In these two experimental systems, the crucial role of Bak was confirmed by a gain in susceptibility to GrB after restoration of Bak expression. The mechanism of interaction between GrB-cleaved Bid and Bak has not yet been studied.

Partial activation of caspase-3, demonstrated by the appearance of partially processed p20 form, a product of the direct cleavage of procaspase-3 by GrB at D175 (6, 15, 40), was detected over a 15-min exposure to GrB of either wild-type or Bak-deficient Jurkat cells. However, further processing to the p19 and p17 subunits of caspase-3 over a 2-h incubation was only observed in wild-type, but not in Bak-deficient cells. As judged by its inability to cleave PARP, the GrB-generated p20 caspase-3 is relatively inactive, and requires mitochondrial factors to achieve full-processing and activation. Similar observations were made by Sutton et al. in Bcl-2 overexpressing cells (15). These authors speculated that in the absence of mitochondrial perturbation, the XIAP inhibitor, SMAC/DIABLO, was not released into the cytosol, allowing cytosolic XIAP to maintain caspase-3 in an inactive form (53, 54). In accordance with this model, exposure of Bak-deficient cells to exogenous GrB resulted only in the first stage of procaspase-3 processing, suggesting that cytochrome c or other mitochondrial factors, such as SMAC, were required for further

caspase-3 processing. However, we observed full caspase-3 processing in extracts of Bak-deficient cells directly treated with exogenous cytochrome c (data not shown). These findings suggest that an overdose of cytochrome c (which may reflect a nonphysiologic situation) overcomes the lack of SMAC. In a similar manner, we also observed full processing of caspase-3 in extracts of Bak-deficient cells treated directly with GrB in the absence of Z-VAD-FMK, but not in the presence of this pan-caspase inhibitor. These results suggest that the second stage of caspase-3 processing may be achieved in a mitochondria-independent manner in the presence of an augmented dose of GrB, which should be higher than that internalized via its cell surface receptor.

Bid-deficient mice revealed that Bid is a critical caspase substrate for FasL- or TNF- $\alpha$ -induced death of hepatocytes in vivo (41). Thus, in hepatocytes, and probably other types of target cells in which caspase-8 is not efficiently recruited to the DISC (55), a loss in Bid expression may serve to block Fas-dependent cytotoxic mechanisms of CTLs and NK cells. The crucial requirement for Bid in GrB-mediated apoptosis suggests that downregulation of Bid expression will also block a major step in apoptosis triggered by granule exocytosis by CTLs and NK cells. Our findings suggest that deficiency in Bak expression may serve as an efficient mechanism of immune evasion for tumor or viral infected cells, as it blocks GrB-mediated cytotoxicity, and in certain cell types also blocks the Bid-dependent Fas pathway, two major cytotoxic mechanisms used by CTLs and NK cells.

A recent report (20) has identified Bid as a sensor of proteolysis by an endopeptidase, which triggers the mitochondrial mechanism of cytochrome c release. Thus, cleavage of Bid may represent a mechanism by which proteases that have leaked from lysosomes can trigger cell death. Bak-deficiency may serve to protect against such a lysosomal pathology.

It is interesting to note that deficiency in Bak endows Jurkat cells with a potent mechanism of resistance to certain anticancer drugs (29). The significance of Bak for GrB-mediated cytotoxicity suggests that Bak-deficient tumor cells utilize this deficiency as a mechanism of resistance to either cytotoxic drugs or to cytotoxic lymphocytes. These findings may have an important implication for immunotherapy protocols, which target residual disease after treatment with anticancer drugs. Bak deficiency may also interfere with antitumor vaccine therapy, where the cytotoxic activity of specific CTLs could be compromised by the disabling of the GrB-mediated mitochondrial apoptotic cascade in target cells.

We thank Dr. Thomas Chittenden (Apoptosis Technology Inc., Cambridge, MA) for providing the pGEX2KBak $\Delta$ C plasmid, Dr. Xiaodong Wang (Southwestern Medical Center, Dallas, TX) for anti-Bid antibodies, and Dr. Craig B. Thompson (Abramson Family Cancer Research, Philadelphia, PA) for Jurkat cells transduced with Neo or Bcl-X<sub>L</sub>.

This work was supported by The National Institute of Health [NIH] grant RO1CA84134-01 (to H. Rabinowich), The National

Cancer Institute grant PO1DE12321-01 (to H. Rabinowich), American Cancer Society grant RPG-98-288-01-CIM (to H. Rabinowich), The Department of Defense grant BC981056 (to H. Rabinowich), and NIH grant RO1CA83817-01 (to X.-M. Yin).

Submitted: 17 May 2001

Revised: 6 August 2001

Accepted: 18 September 2001

## References

1. Green, D.R. 1998. Apoptotic pathways: the roads to ruin. *Cell*. 94:695-698.
2. Trapani, J.A., J. Davis, V.R. Sutton, and M.J. Smyth. 2000. Proapoptotic functions of cytotoxic lymphocyte granule constituents in vitro and in vivo. *Curr. Opin. Immunol.* 12:323-329.
3. Trapani, J.A., V.R. Sutton, and M.J. Smyth. 1999. CTL granules: evolution of vesicles essential for combating virus infections. *Immunol. Today*. 20:351-356.
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. Froelich, C.J., K. Orth, J. Turbov, P. Seth, R. Gottlieb, B. Babior, G.M. 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.
6. 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.
7. Poe, M., J.T. Blake, D.A. Boulton, M. Gammon, N.H. Sigal, J.K. Wu, and H.J. Zweerink. 1991. Human cytotoxic lymphocyte granzyme B. Its purification from granules and the characterization of substrate and inhibitor specificity. *J. Biol. Chem.* 266:98-103.
8. Darmon, A.J., D.W. Nicholson, and R.C. Bleackley. 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*. 377:446-448.
9. Duan, H., K. Orth, A.M. Chinnaiyan, G.G. Poirier, C.J. Froelich, W.W. He, and V.M. Dixit. 1996. ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J. Biol. Chem.* 271:16720-16724.
10. Chinnaiyan, A.M., W.L. Hanna, K. Orth, H. Duan, G.G. Poirier, C.J. Froelich, and V.M. Dixit. 1996. Cytotoxic T-cell-derived granzyme B activates the apoptotic protease ICE-LAP3. *Curr. Biol.* 6:897-899.
11. Pinkoski, M.J., M. Hobman, J.A. Heibein, K. Tomaselli, F. Li, P. Seth, C.J. Froelich, and R.C. Bleackley. 1998. Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. *Blood*. 92:1044-1054.
12. Pinkoski, M.J., J.A. Heibein, M. Barry, and R.C. Bleackley. 2000. Nuclear translocation of granzyme B in target cell apoptosis. *Cell Death Differ.* 7:17-24.
13. Pinkoski, M.J., N.J. Waterhouse, A. Jeffrey, B. Heibein, B. Wolf, T. Kuwana, J.C. Goldstein, D.D. Newmeyer, R.C.

- Bleackley, and D.R. Green. 2001. Granzyme B-mediated apoptosis proceeds predominantly through a BCL-2-inhibitable mitochondrial pathway. *J. Biol. Chem.* 276:12060–12067.
14. 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.
  15. Sutton, V.R., J.E. Davis, M. Cancilla, R.W. Johnstone, A.A. Ruefli, K. Sedelies, K.A. Browne, and J.A. Trapani. 2000. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J. Exp. Med.* 192:1403–1414.
  16. Heibein, J.A., I.S. Goping, M. Barry, M.J. Pinkoski, G.C. Shore, D.R. Green, and R.C. Bleackley. 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J. Exp. Med.* 192:1391–1402.
  17. Alimonti, J.B., L. Shi, P.K. Bajjal, and A.H. Greenberg. 2001. Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J. Biol. Chem.* 276:6974–6982.
  18. Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang. 1998. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell.* 94:481–490.
  19. Wang, K., X.M. 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.
  20. Stoka, V., B. Turk, S.L. Schendel, T.H. Kim, T. Cirman, S.J. Snipas, L.M. Ellerby, D. Bredesen, H. Freeze, M. Abrahamson, et al. 2000. Lysosomal protease pathways to apoptosis: cleavage of bid, not pro-caspases, is the most likely route. *J. Biol. Chem.* 276:3149–3157.
  21. Li, K., Y. Li, J.M. Shelton, J.A. Richardson, E. Spencer, Z.J. Chen, X. Wang, and R.S. Williams. 2000. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell.* 101:389–399.
  22. Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, 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.* 91:479–489.
  23. Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell.* 90:405–413.
  24. Liu, X., C.N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell.* 86:147–157.
  25. Wei, M.C., T. Lindsten, V.K. Mootha, S. Weiler, A. Gross, M. Ashiya, C.B. Thompson, and S.J. Korsmeyer. 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* 14:2060–2071.
  26. Korsmeyer, S.J., M.C. Wei, M. Saito, S. Weiler, K.J. Oh, and P.H. Schlesinger. 2000. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ.* 7:1166–1173.
  27. Desagher, S., A. Osen-Sand, A. Nichols, R. Eskes, S. Montessuit, S. Lauper, K. Maundrell, B. Antonsson, and J.C. Martinou. 1999. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J. Cell Biol.* 144:891–901.
  28. Eskes, R., S. Desagher, B. Antonsson, and J.C. Martinou. 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol. Cell. Biol.* 20:929–935.
  29. Wang, G.Q., B.R. Gastman, E. Wieckowski, L.A. Goldstein, A. Gambotto, T.H. Kim, B. Fang, and A. Rabinovitz. 2001. A role for mitochondrial Bak in apoptotic response to anticancer drugs. *J. Biol. Chem.* 276:34307–34317.
  30. Fang, B., L. Ji, M. Bouvet, and J.A. Roth. 1998. Evaluation of GAL4/TATA in vivo. Induction of transgene expression by adenovirally mediated gene codelivery. *J. Biol. Chem.* 273:4972–4975.
  31. Pataer, A., B. Fang, R. Yu, S. Kagawa, K.K. Hunt, T.J. McDonnell, J.A. Roth, and S.G. Swisher. 2000. Adenoviral Bak overexpression mediates caspase-dependent tumor killing. *Cancer Res.* 60:788–792.
  32. Johnson, D.E., B.R. Gastman, E. Wieckowski, G.Q. Wang, A. Amoscato, S.M. Delach, and H. Rabinowich. 2000. Inhibitor of apoptosis protein hIAP undergoes caspase-mediated cleavage during T lymphocyte apoptosis. *Cancer Res.* 60:1818–1823.
  33. Petit, P.X., J.E. O'Connor, D. Grunwald, and S.C. Brown. 1990. Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. *Eur. J. Biochem.* 194:389–397.
  34. Goping, I.S., A. Gross, J.N. Lavoie, M. Nguyen, R. Jemmerson, K. Roth, S.J. Korsmeyer, and G.C. Shore. 1998. Regulated targeting of BAX to mitochondria. *J. Cell Biol.* 143:207–215.
  35. Shi, L., S. Mai, S. Israels, K. Browne, J.A. Trapani, and A.H. Greenberg. 1997. Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *J. Exp. Med.* 185:855–866.
  36. Motyka, B., G. Korbitt, M.J. Pinkoski, J.A. Heibein, A. Caputo, M. Hobman, M. Barry, I. Shostak, T. Sawchuk, C.F. Holmes, et al. 2000. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell.* 103:491–500.
  37. Thomas, D.A., C. Du, M. Xu, X. Wang, and T.J. Ley. 2000. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity.* 12:621–632.
  38. Petit, J.M., A. Maftah, M.H. Ratinaud, and R. Julien. 1992. 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. *Eur. J. Biochem.* 209:267–273.
  39. Chittenden, T., E.A. Harrington, R. O'Connor, C. Flemington, R.J. Lutz, G.I. Evan, and B.C. Guild. 1995. Induction of apoptosis by the Bcl-2 homologue Bak. *Nature.* 374:733–736.
  40. Trapani, J.A., D.A. Jans, P.J. Jans, M.J. Smyth, K.A. Browne, 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.
  41. Yin, X.M., K. Wang, A. Gross, Y. Zhao, S. Zinkel, B. Klocke, K.A. Roth, and S.J. Korsmeyer. 1999. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature.* 400:886–891.
  42. Knudson, C.M., K.S. Tung, W.G. Tourtellotte, G.A. Brown, and S.J. Korsmeyer. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science.* 270:96–99.

43. Riera, L., M. Gariglio, G. Valente, A. Mullbacher, C. Museteanu, S. Landolfó, and M.M. Simon. 2000. Murine cytomegalovirus replication in salivary glands is controlled by both perforin and granzymes during acute infection. *Eur. J. Immunol.* 30:1350–1355.
44. Shresta, S., T.A. Graubert, D.A. Thomas, S.Z. Raptis, and T.J. Ley. 1999. Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity.* 10:595–605.
45. Heusel, J.W., R.L. Wesselschmidt, S. Shresta, J.H. Russell, and T.J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell.* 76:977–987.
46. Mullbacher, A., P. Waring, R. Tha Hla, T. Tran, S. Chin, T. Stehle, C. Museteanu, and M.M. Simon. 1999. Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. *Proc. Natl. Acad. Sci. USA.* 96:13950–13955.
47. Andrade, F., S. Roy, D. Nicholson, 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.
48. Medema, J.P., R.E. Toes, C. Scaffidi, T.S. Zheng, R.A. Flavell, C.J. Melief, M.E. Peter, R. Offringa, and P.H. Krammer. 1997. Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. *Eur. J. Immunol.* 27:3492–3498.
49. Browne, K.A., R.W. Johnstone, D.A. Jans, and J.A. Trapani. 2000. Filamin (280-kDa actin-binding protein) is a caspase substrate and is also cleaved directly by the cytotoxic T lymphocyte protease granzyme B during apoptosis. *J. Biol. Chem.* 275:39262–39266.
50. Harris, J.L., E.P. Peterson, D. Hudig, N.A. Thornberry, and C.S. Craik. 1998. Definition and redesign of the extended substrate specificity of granzyme B. *J. Biol. Chem.* 273:27364–27373.
51. Liu, X., H. Zou, C. Slaughter, and X. Wang. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell.* 89:175–184.
52. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature.* 391:43–50.
53. Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L. Moritz, 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.
54. Du, C., M. Fang, Y. Li, L. Li, and X. Wang. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell.* 102:33–42.
55. Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K.J. Tomaselli, K.M. Debatin, P.H. Krammer, and M.E. Peter. 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 17:1675–1687.