Apoptotic Death Concurrent with CD3 Stimulation in Primary Human CD8+ T Lymphocytes: A Role for Endogenous Granzyme B

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A apoptotic death concurrent with CD3 stimulation in primary human CD8+ T lymphocytes: a role for endogenous granzyme B

Mireille Laforge, Nicolas Bidère, Sylvie Carmona, Aurore Devocelle, Bernard Charpentier, and Anna Senik

We exposed primary CD8+ T cells to soluble CD3 mAb plus IL-2 and limited numbers of monocytes (3%). These cells were activated but concurrently subjected to ongoing apoptosis (~25% were apoptotic from day 2 of culture). However, their costimulated CD4+ counterparts were much less prone to apoptosis. The apoptotic signaling pathway bypassed Fas and TNFRs, and required the activity of cathepsin C, a protease which performs the proteolytic maturation of granzyme (Gr) A and GrB proenzymes within the cytolytic granules. Silencing the GrB gene by RNA interference in activated CD8+ T cells prevented the activation of procaspase-3 and Bid, and indicated that GrB was the upstream death mediator. A GrB-specific mAb immunoprecipitated a ~70-kDa molecular complex from cytoplasmatic extracts of activated CD8+ (but not resting) T cells, that was specifically recognized by a nucleocytoplasmic protease inhibitor 9 (PI-9) specific mAb. This complex was also detected after reciprocal immunoprecipitation of PI-9. It coexisted in the cytosol with the 32-kDa form of GrB. As neither were detected in the cytosol of CD4+ bystander T cells (which poorly synthesized GrB), and as silencing the perforin (Pf) gene had no effect in our system, endogenous GrB was likely implicated. Immunoprecipitation experiments failed to reveal Pf in the cytosol of CD8+ T cells, and only a tiny efflux of granular GrA was detected by ELISA. We propose that some GrB is released from cytolytic granules to the cytosol of CD8+ T lymphocytes upon CD3/TCR stimulation and escapes PI-9, thereby mediating apoptotic cell death. The Journal of Immunology, 2006, 176: 3966–3977.

Although the Fas/Fas ligand system is thought to be primarily involved in TCR-induced apoptosis of CD4+ T cells, it is not always essential for TCR-induced apoptosis of CD8+ T cells (1, 2). Alternative TNF-α-based death pathways can operate in activated CD8+ T cells stimulated through the CD3/TCR complex, involving the TNFR2 (1, 3) or the TNFR1 receptors (4). Studies with perforin (Pf)−/− deficient mice have also implicated the pore-forming protein Pf in the in vitro activation-induced cell death elicited in T lymphocytes by CD3 re-cross-linking (5). These various observations show that various pathways are involved in TCR-induced death of activated CD8+ T cells, and the relative importance of each in any particular model needs to be determined.

We previously established a cellular model consisting of primary CD8+ T cells subjected to stimulation through the CD3 co-receptor, using soluble anti-CD3 mAb and exogenous IL-2 (6). In this model, procaspase-3 is first processed at the IETD ↓ S cleavage site, resulting in the production of a p20 fragment (prodomain plus large subunit) and a p12 fragment (small subunit). The full processing of procaspase-3 to the p17 chain (large subunit) is triggered in a second time, simultaneously with outer mitochondrial membrane permeabilization. Given that 1) the partial processing of procaspase-3 was dependent on cathepsin C, a thiol cysteine protease which is concentrated in the cytolytic granules and which is responsible for the proteolytic maturation of the proenzyme forms of granzyme (Gr) A and GrB (7, 8, and 2) that GrB is the only protease to exhibit, together with caspase-8, a specific p20/p12 pattern of aspase activity toward procaspase-3 (9), we suggested that GrB might be involved in our model.

GrB is a serine protease found in the cytolytic granules of CTL and NK cells together with other granzymes and Pf. There is now evidence that GrB plays a key role in granule-mediated apoptosis of target cells (reviewed in Ref. 10). In a cell-free system, GrB cleaves numerous substrates after aspartate residues, and thereby directly process procaspase-3, -7, -8, and -10; it is, however, caspase-3 that ultimately disseminates the caspase cascade and manifests the major executioner activity during GrB-initiated proteolysis of cellular substrates (11). By using procaspase-3-deficient MCF-7 target cells, it was reported that external GrB induces cell death predominantly through caspase-3 activation (12). Results obtained from other cellular models indicate in contrast that the detrimental activity of GrB may be critically dependent on the cleavage and activation of the BH3-only, Bcl-2 family member Bid (13–15).

We investigated whether GrB contributes to the moderate but ongoing apoptotic cell death accompanying CD8+ T cell activation in our cellular model. We used the small interfering RNA
(siRNA)-mediated gene silencing technique (16) to block human GrB translation and determine the consequences of this inhibition on the activation status of caspase-3 and Bid, and on CD8+ T cell survival. It has been shown that endogenous GrB can leak from lytic granules to the cytosol of cytolytic cells, as assessed by complex formation between GrB and the nucleocyttoplasmic proteinase inhibitor (PI)-9 (17). PI-9 is a human serpin produced by both CD4+ and CD8+ T cells. It acts as a "suicide substrate" for GrB and efficiently inactivates its proteolytic activity (18). Overexpression of PI-9 protects cells from GrB-mediated apoptosis (19). Thus, PI-9 might prevent cytolymphocytes from both ectopic and misdirected GrB. PI-6 (the murine equivalent of PI-9) has also been suggested to contribute to the homeostasis control of active memory CD8+ T cells (20). Therefore, we examined whether GrB was associated with PI-9 in the cytosol of CD8+ T cells.

We demonstrate that GrB is directly responsible for the upstream proteolytic/activity events affecting procaspase-3 and Bid in CD3-activated CD8+ T cells, and for the enhanced apoptotic death accompanying the activation of these cells. We show that small amounts of GrB are found in the cytosol of CD3-activated CD8+ (but not CD4+) T cells, not only as PI-9/GrB complexes, but also as free catalytically active molecules. Silencing the Pf gene did not prevent the apoptotic events affecting CD8+ T cells, and no GrB was detectable in the cytosol of bystander CD4+ T cells that had been costimulated with CD8+ T cells. Therefore, it seems likely that mature endogenous GrB leaks from lytic granules to the cytosol upon CD3/TCR stimulation, thus initiating apoptosis.

Materials and Methods

**Human T lymphocyte isolation and culture conditions**

We used peripheral blood leukocytes isolated by using Ficoll-Hypaque from healthy volunteers (Etablissement Français du Sang). Most adherent cells were removed by incubation on plastic dishes and passage over nylon wool columns. Circulating CD4+ and CD8+ T cells were negatively selected using a CD4+ or a CD8+ T cell Isolation kit according to the manufacturer's instructions (Miltenyi Biotec). The purity of each cell population was ≥96% as determined by flow cytometry. Monocytes were added back to the purified cells to a final 3% to allow T cell activation during stimulation. Cat C enzymatic activity was assayed by hydrolysis of the chromogenic Gly-Phe-methylamide (GF-mB) substrate as described by Smyth et al. (23). All these substrates were purchased from Sigma-Aldrich. GrB activity was detected using the acetyl-Ile-Glu-Glu-Pro-Aspartic acid substrate Ac-IEDD-pNA from Calbiochem. Quantification of GrB and GrA were performed using the Pelikine Compact human GrB and GrA ELISA kits from Mab Diagnost.

**Antibodies**

The CH-11 anti-Fas mAb was purchased from Immunotech. The anti-Fas M3 and M33 mAbs were gifts from Dr. D. Lynch (Immunex Research and Development Corporation, Seattle, WA). The neutralizing rabbit polyclonal antiserum produced against rTNF-α was a gift from Dr. J. Wietzerbin (INSERM, Unité 365, Paris, France). The reagents for immunofluorescence studies were as follows: biotin-conjugated anti-GrB mAb (CLB-GB-11) and anti-GrA (GA28) mAb (Mast Diagnostic); anti-human PI mAb (BD Pharmingen) followed by a biotinylated goat anti-mouse IgG2b (Caltag Laboratories, sold by Tebu, Le Puy en Velay, France); anti-PI-9 (7-D8; MBL) followed by an FITC-labeled goat anti-mouse IgG1 (Caltag Laboratories); biotin-conjugated mouse IgG1 and IgG2b controls (Caltag Laboratories); FITC- and tetradohamine isothiocyanate (TRITC)-conjugated streptavidin were from Caltag Laboratories. Primary Abs used for Western blotting were: rabbit antiserum against Smac/Diablo (Oncogene Research Products), human caspase-3 (31A1067; Alexis), Bid (Cell Signaling Technology), and actin (Sigma-Aldrich); goat anti-GrA (Santa Cruz Biotechnology), mouse mAb against human GrB (the 2CS IgG1 or the 2CS/F5 IgG2a from BD Pharmingen), anti-cytochrome c (7H2/12; BD Pharmingen), HtrA2/Omi (196429; Apotech), and anti-cytochrome oxidase subunit II (12C4; Molecular Probes); the IgG2a rat mAb against human PI (Valbiotech).

**Flow cytometry**

Cells were fixed with 3% paraformaldehyde, then incubated with 50 mM NH4Cl, and permeabilized with 0.05% saponin. A saturation avidin/biotin blocking kit (ABCs) was then used according to the manufacturer's instructions, followed by incubation with biotin-conjugated anti-GrB or anti-GrA mAb and FITC-conjugated streptavidin from Caltag Laboratories. Immunostaining was analyzed using a FACScan and CellQuest software (BD Biosciences). For each staining, data from 10,000 events in the lymphocyte gate were collected.

**Confoal laser-scanning microscopy**

Cells were fixed for 30 min at 4°C with 3% paraformaldehyde and spun on glass slides in PBS, washed with PBS, permeabilized with 0.05% Triton X-100, and incubated with the saturation avidin/biotin blocking kit. After three washes, the cells were incubated for 45 min at 4°C with biotinylated anti-GrB (GB11) or anti-GrA (GA28) mAb in PBS supplemented with 0.5% BSA and 2% FCS, and then stained with FITC- or TRITC-conjugated streptavidin from Immunotech (sold by Beckman Coulter) according to the manufacturer's instructions. Nuclei were counterstained for 5 min with 5 μM 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes). For double GrB/PI-9 and GrA/PI-9 immunofluorescence stainings, the cells were sequentially incubated with anti-PI-9, FITC-labeled goat anti-mouse IgG, and normal mouse serum. After saturation with the avidin/biotin blocking kit, they were incubated with GB-11 or GA28, followed by TRITC-conjugated streptavidin. Destabilization of lysosomes/cytolytic granules was estimated by using 10-KDa FITC-dextran molecules (Sigma-Aldrich) as previously described (24). Cells were examined under a CLSM confocal fluorescence microscope (Leica Microsystems).
Subcellular fractionation

Cytosolic extracts were prepared by a selective digitonin-based permeabilization technique according to Foghgaard et al. (25). Briefly, after elimination of dead and apoptotic cells by centrifugation on Ficoll-hypaque, 10^7 lymphocytes were washed twice in PBS and incubated for 5 min on ice with a lysis buffer consisting of digitonin, 250 mM sucrose, 17 mM NaCl, 70 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2 mM EDTA (pH 7.2) supplemented with a protease inhibitor mixture (complete from Roche Applied Science) and 25 μM pepstatin A. Extracts were spun at 300 x g for 5 min and the resulting supernatants were spun again at 10,000 x g for 10 min at 4°C to remove all debris. The final supernatants, called cytosolic fractions, were stored at -80°C. Protein concentrations were determined by the bicinchonic acid method (Pierce). Cytosolic fractions from YT cells were obtained by treating the cells for 5 min on ice with extraction buffer containing 45 μg/ml digitonin.

Immunoblotting

Pellets of 5 x 10^7 lymphocytes were directly resuspended in Laemmli buffer containing 2% SDS and 10% 2-ME, boiled for 5 min and run on 12% SDS-PAGE. After transfer of the proteins onto nitrocellulose membranes, the immunoblots were sequentially incubated with primary Abs and with HRP-coupled secondary reagents from Amersham Biosciences. They were developed by ECL (from Amersham or West Femto from Pierce) using a charge-coupled device camera (Fuji LAS-1000 Plus) and the LumiQuant (Packard). Intensities were quantified using a semi-automated Western blotting system (Tebu-Bio, France). Immunoblotting of cytosolic extracts from CD8^+ T cells and natural killer (NK) cells were performed as described.

Immunoprecipitations

To avoid post-lysis interaction between GrB and PI-9 during the solubilization of GrB^+ cells, immunoprecipitations (IP) were carried as follows: the cells were treated with modified Laemmli buffer (containing 2% SDS, 60 mM Tris-HCL, pH 6.8, and 10% glycerol) and immediately boiled for 10 min. The resulting lysates (200–300 μg) were centrifuged at 15,000 x g and diluted to a final concentration of 0.1% SDS in Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris-HCL (pH 8), 150 mM NaCl) containing protease inhibitors. Cytosolic extracts (at least 250 μg) were obtained by the digitonin-based permeabilization technique above described. Both types of preparations were precleared on 50 μl of protein G-Sepharose (Pharmacia) and 5 μg of control mouse IgG2a Abs, rotating for 2 h at 4°C. After centrifugation at 10,000 x g for 3 min at 4°C, the supernatants were collected and incubated for 1 h with 5 μg of anti-GrB Ab (2CF5/5, an IgG2b from BD Pharmingen) or control isotypic IgG2a Ab. These samples were then mixed with 50 μl of protein G-Sepharose beads and incubated at 4°C with rotation for at least an additional 2 h. The immune complexes obtained were washed four times and resolved by SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes, and the membranes probed with the 7D8 anti-PI-9 mAb (an IgG1) followed by an HRP-conjugated goat IgG specific for mouse IgG1 (Southern Biotechnology Associates, sold by ClinSciences). The blots were then stripped and incubated with the 2CF5/5 anti-GrB mAb (an IgG2a) followed by a HRP-conjugated goat IgG specific for mouse IgG2a (Southern Biotechnology Associate). The reciprocal PL-9 IP was also conducted using the 7D8 mAb, followed by anti-GrB or anti-PI-9 immunoblotting. Immunoprecipitation of PI-9 was conducted in 1% Nonidet P-40 buffer using a mouse IgG2b specific for human PI (BD Pharmingen). Western blot analysis was then performed, using a rat mAb against human PI (Valbiotech) followed by HRP-conjugated rabbit IgG specific for rat IgG (Sigma-Aldrich).

siRNAs and T lymphocyte transfection

GrB and PI-9 gene expression was silenced by the siRNA technique (16, 26). Duplexes of 21-nt siRNA with two 3'-overhanging TT residues were synthesized by Proligo. The sense strand of the siRNA used to silence the GrB gene (GrB-siRNA) was GUCUCUGAAGAGGUGCCGU (position 154–172 relative to the start codon of the GrB mRNA). The sense strand used to silence the PI-9 gene (PI-siRNA) was CCGUGUAAUGCGCCACUATT (position 1533–1551 relative to the start codon of the PI-9 mRNA). A negative control siRNA (GCCAUAUCACUAUGGUGAAGC) was provided by Dr. C. Boucheix (INSERM, Unité 268, Villejuif, France). Purified resting T cells were transfected by electroporation of double-stranded siRNAs (150 pmol/4 x 10^8 T cells in 100 μl) as previously described (24).

Results

A cathepsin-dependent event promotes apoptosis in primary CD3-stimulated CD8^+ T cells

Highly purified CD4^+ and CD8^+ high T cells were stimulated for 4 days with soluble anti-CD3 mAb (250 ng/ml) and IL-2. To allow full activation of the cells, autologous monocytes were added back at the onset of the cultures to make up 3% of the cells. This resulted in similar increases in the forward light scatter for both types of cells (Fig. 1A). Staining with the potential-sensitive dye DiOC_6 revealed that at the end of the stimulation period, more apoptotic cells with dissipated mitochondrial inner membrane potential (Δψm) were generated in CD8^+ T cells than in their CD4^+ counterparts (26 vs 5%). CD8^+ T cell cultures also contained more cell debris resulting from secondary necrosis and subsequent cell demolition. Whole T cell populations were double-labeled with the potential-sensitive dye, CMXROS, and with FITC-anti-CD4 mAb or FITC-anti-CD8 mAb to compare, in kinetics experiments, the fate of cocultivated CD4^+ and CD8^+ high T cells (Fig. 1B). More Δψm^low cells were generated from day 2 of the cultures in activated CD8^+ T cells than in CD4^+ T cells. The enhancement of cell death displayed by activated CD8^+ T cells was CD3/TCR induced. Thus, if the stimulating CD3 mAb was removed after 48 h of stimulation, and purified CD8^+ T cells then grown in IL-2-containing medium, the apoptosis rate of these cells rapidly declined to the level of CD3-stimulated CD4^+ T cells. In contrast, if CD8^+ T cells were replaced in anti-CD3 plus IL-2-containing medium, the apoptosis rate continued increasing (data not shown).

We investigated the upstream molecular actors involved in the apoptotic signaling pathway triggered by CD3 stimulation in CD8^+ T cells. The presence of the blocking Fas-specific M3 mAb (10 μg/ml) from the beginning of culture did not affect the magnitude of apoptotic cell death among activated CD8^+ T cells (Fig. 1C). However, it inhibited the cytotoxic effect exerted by the Fas-specific Ab CH-11 on the same cells (Fig. 1C, insert). The control Fas-specific M33 mAb, which is not able to inhibit CH-11-mediated lysis of Fas^+ cells (27) had no effect. A rabbit antiserum directed at TNF-α (able to neutralize 4 ng/ml TNF-α at the dilution used) also did not prevent the apoptotic death of CD3-stimulated CD8^+ T cells. Thus, it is unlikely that Fas and TNFFR death domain receptors were involved in the initiation of the apoptosis process. In additional experiments (data not shown), we found that continuous exposure of CD8^+ T cells to Z-IETD.fmk, which is a caspase-8 inhibitor, but not a serine protease inhibitor, had no protective effect. This implied that other death receptors capable of recruiting caspase-8 (such as the receptors for TRAIL) were not upstream mediators of apoptosis.

We then used GF.dmk, a synthetic, cell permeable peptide that selectively and irreversibly inhibits the activity of cathepsin C (7). Chronic exposure of CD8^+ T cells during CD3 stimulation to 10 μM GF.dmk (but not GF.dmk used as a control), significantly reduced the occurrence of Δψm^low cells (Fig. 1D). This concentration almost completely inhibited cathepsin C activity within these cells. As a consequence, a moderate but significant 30.3 ± 4.1% increase (p < 0.005, n = 7) in the absolute numbers of viable CD8^+ T cells was observed at the end of the culture period. In contrast, the chronic exposure of CD4^+ T cells to GF.dmk had no influence on their viability. The absolute numbers of viable GF.dmk-treated CD8^+ T cells were increased by GF.dmk treatment to the values for CD4^+ T cells. These results pointed to a granular protease whose proteolytic maturation relies on cathepsin C, as the essential upstream mediator of the enhanced apoptotic death affecting CD3-activated CD8^+ T cells.
FIGURE 1. Primary CD8<sup>+</sup> T lymphocytes stimulated with anti-CD3 and IL-2 display faster apoptotic cell death than activated CD4<sup>+</sup> T lymphocytes. This death is cathepsin C dependent. A, CD4<sup>+</sup> and CD8<sup>+</sup> high human T lymphocytes, highly purified by negative selection over magnetic columns, were stimulated for 4 days with 250 ng/ml OKT3 plus 100 U/ml IL-2 in the presence of 3% autologous monocytes. Lymphocytes were gated by a forward (FSC) vs right angle (90°) light scatter plot (SSC). Numbers in parentheses refer to the mean forward scatter. The cells were incubated with DIOC<sub>6</sub>, and PI, and analyzed by flow cytometry. The positions of apoptotic cells were revealed by incubation with the proto-ionophore, CICCP. One experiment representative of five others is shown. B, Whole T cells were incubated on petri dishes, passed through nylon wool columns, then separated from CD56<sup>+</sup>, CD16<sup>+</sup> NK cells, using the panning technique. At the indicated times, the cells were labeled with FITC-conjugated mAb directed against either CD4 or CD8 and further incubated with red CMXRos to evaluate the percentages of apoptotic cells exhibiting Δψm loss. Δψm<sub>low</sub> cells were identified on gated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. C, Purified CD8<sup>+</sup> T cells were cultivated for 4 days in the presence of the blocking or control anti-Fas mAb (M3 and M33, respectively, at 10 µg/ml), or in the presence of TNF-α antiseraum (normal rabbit serum, NRS, was the negative control). Upper quadrant, The M3 (but not the M33) mAb blocked the cytotoxic effect of the CH11 anti-Fas mAb (2 µg/ml) against activated CD8<sup>+</sup> T cells during a 16 h-assay. D, a, Purified CD8<sup>+</sup> T cells stimulated during 4 days in the chronic presence of the GF.dmk peptide, a cathepsin C inhibitor or in the presence of the control GP.dmk peptide, an inhibitor of dipeptidyl peptidase IV activity (10 µM added each day to the cell cultures). The percentage of Δψm<sub>low</sub> cells was determined by incorporation of the DIOC<sub>6</sub> (3) fluorescent probe. b, Numbers of viable cells, as estimated under the light microscope by morphological criteria and trypan blue exclusion among CD8<sup>+</sup> and CD4<sup>+</sup> T cells stimulated in the presence of GF.dmk or GP.dmk (cumulative doses: 40 µM). c, CD8<sup>+</sup> T cells activated for 4 days in the same conditions, washed three times, lysed, and assayed for cathepsin C activity by hydrolysis of the chromogenic substrates GF-βNA.

* p < 0.005. Results are representative of at least 10 experiments.
As stated above, the early processing of procaspase-3 in CD3-activated CD8<sup>+</sup> T cells (yielding the p20 plus p12 subunits) suggested GrB involvement (6). As CD4<sup>+</sup> and CD8<sup>+</sup> T cells differed in susceptibility to CD3-induced cell death in our cellular model, we investigated whether this difference was correlated with differential GrB expression. A strong GrB signal was detected by flow cytometry in activated CD8<sup>+</sup> T cells whereas activation of purified CD4<sup>+</sup> T cells in our stimulation conditions did not result in any substantial GrB expression as assessed by flow cytometry, immunoblotting, and confocal microscopy (data not shown). However, small amounts of GrA protein were detected in activated CD4<sup>+</sup> T cells by immunoblotting, consistent with the 10–17% GrA<sup>+</sup> cells observed by confocal microscopy and flow cytometry (data not shown). Thus, while plate-bound Abs to CD3/CD46 plus IL-2 stimulate GrB expression in both naive and memory CD4<sup>+</sup> T cells, and while plate-bound Abs to CD3/CD28 plus IL-2 are very effective in driving GrB expression in memory CD4<sup>+</sup> T cells (28), this is not the case when using soluble anti-CD3 plus IL-2 and limiting numbers of monocytes.

**siRNA-induced silencing of GrB gene prevents the proteolytic processing of caspase-3, the activation of Bid to truncated Bid (tBid), and the enhanced apoptotic death of CD3-activated CD8<sup>+</sup> T cells**

We used the siRNA technique to silence the GrB gene and thereby determine whether GrB was involved in the apoptotic death of CD3-stimulated CD8<sup>+</sup> T cells. Resting CD8<sup>+</sup> T lymphocytes were transfected with GrB-siRNA and then stimulated with anti-CD3 plus IL-2. Four days posttransfection, the GrB protein levels in CD3-stimulated cells were 70% lower than in cells transfected with control siRNA, as estimated by immunoblotting and densitometric analysis. The production of neither GrA nor Pf proteins was affected (Fig. 2A). Similarly, the number of GrB-positive cells detected by immunofluorescence fell from 42% in control cultures (mock- and control-siRNA-transfected cells) to 5% in GrB-siRNA-transfected cultures (Fig. 2Ab). We examined the activation status of caspase-3 and Bid in these cells. GrB activates both of them by directly cleaving at specific sites, yielding the p20 subunit of caspase-3 and the p17 subunit (the latter being generated by autocatalysis) (9), and the 14-kDa tBid fragment (13, 14). Under our lysis conditions (cells resuspended in 2% SDS-containing Laemmli buffer and immediately boiled), posttranslational modifications of procaspase-3 and Bid, due to the release of GrB from intracellular granules, was completely abolished as demonstrated by using cell lysates from YT, an NK-like cell line containing abundant cytolytic granules (Fig. 2Ba). However, this processing reaction was observed when radiolabeled-precipitation assay buffer was used, instead of Laemmli buffer, to lyse GrB-positive cells (29). In control CD3-stimulated cultures, both the p20 and the p17 subunits of caspase-3 were detected by Western blotting (Fig. 2Bb), consistent with the substantial proportions of apoptotic (Δψm<sub>low</sub>) cells carried over by the cell cultures. Importantly, procaspase-3 was apparently not processed in GrB-siRNA-transfected CD8<sup>+</sup> T cells. This establishes the causal relationship between GrB activity and procaspase-3 processing in our model. Likewise, the activated tBid fragment, which was detected in control CD8<sup>+</sup> T cell populations, was not detected in GrB-siRNA-transfected CD8<sup>+</sup> T cells, suggesting that it was generated by GrB proteolytic activity. Finally, GrB depletion conferred a significant survival advantage on CD8<sup>+</sup> T cells: the viable cell count was 30 ± 4% (n = 5) higher than in control cell populations (Fig. 2C). Thus, GrB was responsible for procaspase-3 and Bid processing, and ultimately for the enhanced apoptotic death of CD3-activated CD8<sup>+</sup> T cells.

**FIGURE 2.** Depletion of GrB protein by RNA interference results in the suppression of procaspase-3 processing and of tBid production, and enhances the survival of CD3-stimulated CD8<sup>+</sup> T cells. A, Resting purified CD8<sup>+</sup> T lymphocytes were transfected with 1.5 μM GrB-siRNA. Negative controls consisted of non- or mock-transfected cells and of cells transfected with control siRNA. Sixteen hours later, the cells were stimulated with OKT3 and IL-2 for 4 days. a, Western blot analysis of whole cell lysates showing that GrB protein expression was inhibited in cells transfected with GrB siRNA, whereas, for the same amount of protein loaded (see actin), the levels of GrA and Pf protein were unaffected. b, Extinction of GrB- but not Pf-associated immunofluorescence in GrB-siRNA treated cells. White numbers indicate the percentages of GrB-positive cells. B, a, Immunoblots with 2 × 10<sup>5</sup> YT (GrB-positive) cells showing that procaspase-3 and Bid are not inadvertently processed when these cells are immediately lysed and boiled in Laemmli buffer containing 2% SDS. Positive controls consisted of YT cells incubated before lysis with STS (350 nM) for the indicated times. b, The processing of procaspase-3 to p20 and p17 and the generation of tBid were inhibited in whole CD8<sup>+</sup> T cell populations transfected with GrB-siRNA. C, The numbers of viable cells (excluding trypan blue and displaying a normal morphology) were scored in triplicate cultures of CD3-stimulated CD8<sup>+</sup> T cells. The experiment shown is representative of five others.

**GrB is detected by laser scanning confocal microscopy in the cytosol of CD3-stimulated CD8<sup>+</sup> T cells, before mitochondrial destabilization**

The loss of Δψm in activated T lymphocytes exposed to anti-CD3 is a late apoptotic event, accompanying, during apoptosis, the release of proapoptotic proteins from the intermembrane space of mitochondria and cell shrinkage (6). We suspected that some GrB would be detected in the cytosol of some activated CD8<sup>+</sup> T cells,
before mitochondrial permeabilization and ΔΨm loss. To isolate ΔΨm-high cells from whole CD3-activated CD8-high T cell populations, samples were run through a Ficoll-hypaque cushion: the resulting cell preparation contained ≥96% ΔΨm-high cells. Immunoblot analysis failed to detect, in the cytosolic fractions derived from these cells, the presence of several proteins of the intermembrane space of mitochondria, namely Smac/Diablo, Omi/HtrA2, and cytochrome c (Fig. 3B). In shrunken cells (40–60% ΔΨm-low), isolated by fractionation on discontinuous density Percoll gradients, these proteins were in contrast detected, assessing permeabilization of the outer mitochondrial membrane. A punctate immunofluorescence staining of cytochrome c was also seen in confocal microscopy in the vast majority of ΔΨm-high cells, which is compatible with exclusive mitochondrial localization of these proteins (data not shown). We examined the subcellular localization of GrB in CD8-high, ΔΨm-high T cells, using confocal laser scanning microscopy (Fig. 3Ca). At the end of the culture period, 42 ± 0.6% of CD3-activated CD8+ T cells were GrB-positive, and 37 ± 4% of them displayed a diffuse, faint GrB immunostaining surrounding well-delineated red granules (>600 GrB+ cells examined in 12 independent determinations). This suggested that GrB was present in the cytosol of these cells. In contrast, in the 20% resting CD8-high T cells scored as GrB-positive, an entirely punctate GrB immunostaining pattern was detected, compatible with exclusive localization of GrB in cytolytic granules (>150 GrB+ cells examined). Double staining of CD3-activated CD8+ T cells with anti-PI-9 and anti-GrB Abs (Fig. 3Cb) indicated that PI-9, the cognate cytosolic inhibitor of GrB, was distributed throughout the cytosol and to a lesser extent, throughout the nucleus, confirming the results of previous studies (17). In the cells displaying mixed punctate/diffuse GrB immunostaining, the intensity of PI-9 immunostaining seemed to be enhanced close to the cytolytic granules. As PI-9 is not present within the granules (18), the yellow staining in the overlay suggested that some GrB was present in the cytosol and was therefore colocalized with PI-9, close to the cytoplasmic face of the granules. In cells displaying entirely punctate GrB immunofluorescence, there was no yellow overlay, suggesting that GrB was not present in the cytosol.

GrB and PI-9 are coimmunoprecipitated by GrB- and PI-9-specific mAb from the cell lysates and cytosolic extracts of ΔΨm-high CD8+-activated T cells

We sought to verify by immunoblotting whether GrB was present in the cytosol of CD3-activated (ΔΨm-high) CD8+ T cells. Cytosolic fractions were prepared, using a digitonin-based plasma membrane permeabilization technique. The GrB-specific 2C5/F5 mAb delineated by blotting a faint but distinct ~70-kDa migration band, and a band at 32 kDa (corresponding to free GrB), in the cytosol of CD3-stimulated (but not not resting) CD8+ T cells (Fig. 4A). IP experiments were then conducted, using the 2C5/F5 mAb. A ~70-kDa protein was coimmunoprecipitated with GrB from the cytosolic extracts of activated (but not resting) CD8+ T cells (Fig. 4B). This protein was recognized by the PI-9-specific 7D8 mAb, and was therefore a GrB/PI-9 molecular complex. The faint ~38-kDa migration band which was also detected by this Ab, was in all probability a degradation product of this complex. The reciprocal IP of PI-9, followed by anti-GrB or anti-PI-9 immunoblotting, revealed that similar GrB/PI-9 molecular complexes were immunoprecipitated by the 7D8 mAb from the cytosol of activated (but not resting) CD8+ T cells (Fig. 4C). Using cytosolic extracts from healthy (~98% ΔΨm-high) YT cells, which express GrB abundantly, we performed IP of GrB and PI-9, but we failed to detect any GrB/PI-9 complex (Fig. 4D). Lysates of YT made with 1% Triton X-100 contained in contrast the 70-kDa GrB/PI-9 complex and its 38-kDa degradation product, indicating that there was postlysis interaction between GrB and PI-9. These data strongly suggest that digitonin treatment selectively permeabilizes the plasma membrane of the cells, leaving granules intact. However, to exclude the possibility of background permeabilization of granule membranes during the digitonin treatment of activated CD8+ T lymphocytes (which are more fragile than YT cells), thereby confirming that the presence of GrB/PI-9 complexes in the cytosolic preparations of these cells was not artificial, we made use of the fact that preformed GrB/PI-9 complexes are not dissociated by SDS, and resist boiling (18). We directly harvested the cells in lysis buffer containing 2% SDS and immediately boiled them for 5 min to avoid postlysis complex formation between PI-9 and GrB. In these conditions, a ~70-kDa GrB/PI-9 complex was again immunoprecipitated by the 2C5/F5 mAb, together with a major 38-kDa degradation product (Fig. 4Ea). Both were revealed by the 7D8 and

FIGURE 3. Laser scanning confocal microscopy analysis of GrB cellular localization in ΔΨm-high CD8+ high T cells. A, CD3-activated CD8+ high T cells were run through a Ficoll-hypaque cushion, thereby obtaining a preparation consisting of ≥96% ΔΨm-high cells. The protonophore murine CICCP was used for cytofluorometry settings. B, Cytosolic extracts (70 μg) from purified CD8+ T cells; discontinuous Percoll density gradients were used to recover shrunken cells sedimenting at the bottom of these gradients. The cytosolic extracts were analyzed by Western blotting for the presence of indicated mitochondrial proteins. Anti-cytochrome c oxidase II (Cox II) was used to verify the absence of mitochondrial contamination, and anti-actin to assess protein loading. Ca, Resting and CD3-activated CD8+ high T cells were examined by laser scanning confocal microscopy using the GrB-specific GB11 mAb. Nuclei were counterstained with DAPI. Arrows indicate cells with mixed punctate/diffuse GrB immunostaining. Cb, Double immunofluorescence staining of activated CD8+ high T cells using the 7D8 and the GB11 mAb, respectively, specific for PI-9 and GrB.
and from activated CD4 T cells (GrB-positive) of lysates obtained from YT cells (GrB-positive) /H11011 of cytosolic extracts (corresponding to derived from 1 A amount of GrB released (Fig. 5), we found that cytosolic extracts in the corresponding cell lysates, mitochondrial disruption and /H9004 250 GrB-specific mAb. /H9262 to nitrocellulose. The blots were probed with the 2C5/F5 mAb. /H9004 2C5/F5 mAb. /H9274 in the cytosol of CD3-activated CD8 T cells, followed by immunoblotting with the 2C5/F5 mAb. The GrB/PI-9 molecular complexes recognized by both 7D8 and 2C5/F5 Abs. /H11011 The GrB/PI-9 molecular complexes are detected in the cytosolic extracts of YT cells after IP of GrB. The arrow points to the IgG2a Abs (2CF/F5 and control IgG2a) that were used for IP and that were recognized in Western blots by the peroxidase-conjugated secondary Abs. Ten micrograms of proteins obtained from YT lysates indicate postlysis GrB/PI-9 complex formation. /H11001 E, a, Immunoprecipitation of GrB from 300 µg of cell lysates; immunoblotting was first performed with the 7D8 mAb, and after stripping, with the 2C5/F5. The input corresponds to 10 µg of proteins. b, Three hundred micrograms of lysates obtained from YT cells (GrB-positive) and from activated CD4+ T cells (GrB-negative) were subjected to IP and immunoblotting as above.

FIGURE 4. Coinmunoprecipitation of GrB and PI-9 from cellular and cytosolic extracts derived from CD3-activated, ΔψmhighCD8+high T cells. A, Ten micrograms of cytosolic extracts (corresponding to ~2.5 × 10^6 CD8+ T cells) and lysates (from ~5 × 10^5 activated CD8+ T cells) were run on SDS-PAGE and transferred to nitrocellulose. The blots were probed with the 2C5/F5 GrB-specific mAb. B, Immunoprecipitation of GrB from 250 µg of proteins obtained from the cytosolic extracts of resting and activated CD8+ T cells; the immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the 7D8 PI-9-specific mAb. C, Reciprocal IP of PI-9 from 250 µg of proteins obtained from the cytosolic extracts of resting and activated CD8+ T cells, followed by immunoblotting with the 2C5/F5 mAb. * The GrB/PI-9 molecular complexes recognized by both 7D8 and 2C5/F5 Abs. D, No GrB/PI-9 molecular complexes are detected in the cytosolic extracts of YT cells after IP of GrB. The arrow points to the IgG2a Abs (2CF/F5 and control IgG2a) that were used for IP and that were recognized in Western blots by the peroxidase-conjugated secondary Abs. Ten micrograms of proteins obtained from YT lysates indicate postlysis GrB/PI-9 complex formation. E, a, Immunoprecipitation of GrB from 300 µg of cell lysates; immunoblotting was first performed with the 7D8 mAb, and after stripping, with the 2C5/F5. The input corresponds to 10 µg of proteins. b, Three hundred micrograms of lysates obtained from YT cells (GrB-positive) and from activated CD4+ T cells (GrB-negative) were subjected to IP and immunoblotting as above.

2CF/F5 mAb. The 2CF/F5 GrB-specific Ab failed to coprecipitate GrB/PI-9 complexes from the lysates of control CD3-activated CD4+ T cells and from the lysates of GrB-positive YT cells (Fig. 4Eb). Altogether, these results confirmed that some GrB was present in the cytosol of CD3-activated CD8+ T cells in the cytosol of CD3-activated CD8+ T cells, before apparent mitochondrial disruption and Δψm loss. It was present in ~70-kDa GrB/PI-9 molecular complexes and also as 32-kDa mature molecules that were not associated with PI-9 (Fig. 4A), suggesting that these molecules had escaped the neutralizing effect of the serpin.

Low GrB enzymatic activity is found in the cytosol of CD3-activated CD8+ (but not bystander CD4+) Δψmhigh T cells

Using the highly sensitive GrB-ELISA test to measure the total amount of GrB released (Fig. 5A), we found that cytosolic extracts derived from 1 × 10^7 CD3-stimulated (Δψmhigh cells) CD8+ T cells contained 1,905 ± 190 pg/ml GrB (vs 28,295 ± 5,000 pg/ml in the corresponding cell lysates, n = 4). Thus, ~6.6% of total cellular GrB (GrB/PI-9 complexes + catalytically active GrB) was present in the cytosol of activated CD8+ T cells. Only negligible amounts of GrB (~2 pg/ml) were detected in cytosolic extracts derived from 1 × 10^5 CD3-stimulated CD4+ T cells, whether they had been cocultured with CD8+ T cells (vs 40–100 pg/ml in the corresponding cell lysates) or not. Such small quantities could not be detected by Western blotting or laser scanning confocal microscopy (> 200 cells examined, data not shown). As expected, cytosolic extracts from control GrB-positive YT cells were negative for GrB.

We then tested whether the 32-kDa form of GrB, detected in the cytosol of activated CD8+ T cells, expressed enzymatic activity. We used the Ac-IEPD-pNA colorimetric tetrapeptide, a reliable substrate for GrB proteolytic activity (30). Assays were conducted in the presence of the pan-caspase inhibitor, Z-VAD.fmk, to avoid GrB-induced activation of the caspase cascade during cell lysis (31), and prevent the interaction of caspase-8 with its potentially IEPD cognate substrate. This slightly reduced Ac-IEPD-pNA hydrolysis by YT lysates (Fig. 5Bc). Ac-IEPD-pNA hydrolysis by the cytosolic extracts of 1 × 10^5 activated CD8+ T cells could thus be detected: the OD values generated in these conditions were low but significant, consistent with the linear part of the titration curve (OD plotted against GrB units) obtained with serially diluted purified GrB (Fig. 5B, a and b). Cytosolic extracts from CD3-stimulated CD4+ T cells or from bystander CD4+ T cells that had been cocultivated with CD8+ T cells, had no detectable GrB enzymatic activity above that of the substrate control.
Would GrB molecules, potentially released into the culture medium by activated CD8\(^+\) T cells, be selectively reuptaken by CD8\(^+\) T cells? We found, however, that activated CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T cells were equally susceptible to the cytolytic effect of graded doses of granule-purified GrB delivered by a constant sublytic dose of the endosome-disrupting agent, AD (Fig. 5D). This suggested that the two populations did not differ in their capacity to take up external GrB. These data therefore argued for the possibility that GrB, detected in the cytosol of activated CD8\(^+\) T cells, came from endogenous stores.

**Pf is not required for the entry of GrB in the cytosol of activated CD8\(^+\) T cells nor for subsequent apoptotic events**

Substantial amounts of newly synthesized granzymes and Pf are secreted from cytolytic T cells after CD3/TCR cross-linking, concurrent with granule exocytosis (32). Although we did not directly address this possibility, there may be secretion of GrB and Pf, and background levels of granule exocytosis following binding of soluble CD3 mAb to the CD3 coreceptor. If this were the case, released GrB would be internalized within endosomes but the endocytosed GrB would require Pf to be delivered from these vesicles to the cytosol (Refs. 22 and 33), and reviewed in Ref. 34). Therefore, we tested whether Pf was necessary for the entry of GrB in the cytosol of activated CD8\(^+\) T cells and for ensuing GrB-mediated apoptotic events. The Pf gene was silenced by transfecting resting CD8\(^+\)/H11001 T cells with Pf-specific siRNA before CD3 stimulation. Four days later, there was a strong decrease in the amount of Pf protein, but the amount of GrB and GrA proteins were unaffected (immunoblot of Fig. 6Aa). Accordingly, Pf expression was almost totally extinguished in CD3\(^\alpha\)/H11001-activated CD8\(^+\)/H11001 T cells, the numbers of Pf\(^+\)/H11001 cells falling from 42\% in control populations to 4\% in Pf-siRNA-transfected cells, as determined by laser scanning confocal microscopy (Fig. 6Ab). In these conditions, GrB was still diffusing to the cytosol (in 37\% of GrB\(^+\)/H11001 cells), procaspase-3 was still processed, and Bid cleaved to tBid (Fig. 6B). These proteolytic events did not occur in GrB-siRNA-transfected cells. The decrease in Pf protein levels had no effect on the extent of cell death affecting activated CD8\(^+\) T cell cultures (Fig. 6C).

**GrB is preferentially released from the cytolytic granules to the cytosol**

The pictures obtained by confocal laser scanner microscopy suggested that, contrary to GrB, Pf and GrA did not significantly leak from cytolytic granules to the cytosol (Fig. 7A). The cells were

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**FIGURE 5.** Low GrB enzymatic activity is found in the cytosol of CD3-activated CD8\(^+\) (but not bystander CD4\(^+\))/H9004\(^+\)/H9274\(^+\) T cells. A, GrB-ELISA using the cytosolic extracts of 1 \times 10^7 T lymphocytes and 1.5 \times 10^6 YT cells (yielding the same quantities of proteins). CD4\(^+\)/A T cells were purified at the start of cultures and CD4\(^+\)/B T cells were purified after coculture with CD8\(^+\)/H11001 T cells. Values are means \(\pm\) SD of four separate samples. B, a, GrB enzymatic activity of serially diluted purified human GrB as determined by incubation with the Ac-IEPD-pNa colorimetric peptide for 4 h. Hydrolysis of the substrate was determined by absorption at 405 nm and after substraction of the background. b, Linear part of the OD/GrB titration curve. c, GrB enzymatic activity of 20 \(\mu\)g of cell lysates from YT and Jurkat cells determined in the presence or absence of 100 \(\mu\)M Z-VAD.fmk. D, Ac-IEPD-pNa hydrolysis, in the presence of 100 \(\mu\)M Z-VAD.fmk, by cytosolic extracts containing as much quantities of proteins as in A. Values are means \(\pm\) SD of four different samples. D, Susceptibility of CD4\(^+\) and CD8\(^+\)/H9004\(^+\)/H9274\(^+\) T lymphoblasts (obtained after soluble anti-CD3 and IL-2 stimulation) to graded doses of granule-purified GrB added concurrently with a constant sublytic dose of AD (10 PFU/cell). The percentage of \(\Delta\pi_{mem}\) cells were determined by incorporation of the DiOC\(_6\) (3) fluorescent probe.
then doubly stained with anti-GrA and PI-9. As no yellow staining was seen in the overlay, it can be inferred that GrA and PI-9 had distinct subcellular localizations. Fig. 7B shows in addition that small 10-kDa FITC-conjugated dextran molecules that have been endocytosed and transferred to the cytolytic granules (as evidenced by colocalization with GrB) were retained in the vesicles of the lysosomal/granular system, even in GrB- cells displaying a mixed punctate/diffuse immunostaining. This suggests that, contrary to what happens when T cells are exposed to the universal apoptosis inducer, staurosporine (24), the membrane integrity of these vesicles was largely preserved.

Western blot analysis of cytosolic extracts derived from activated (CD8$^{+}$ $\Delta_{\text{mhigh}}$) T cells indicated that Pf and GrA protein levels were below the detection threshold, even when large amounts of protein (up to 75 $\mu$g) were loaded on the gels. By contrast, GrB was readily detected in these conditions (Fig. 7C). We conducted IP experiments to try to detect Pf in cytosolic extracts. We were also unable to detect Pf using this approach (Fig. 7D). We then used the available ELISA technique for the detection of GrA with optimal sensitivity: we found that small amounts of GrA (â¼ 130 pg/ml) were present in the cytosolic extracts of 1 $\times$ 10$^{7}$ activated CD8$^{+}$ T cells (Fig. 7E). However, the GrA detected amounted to <10% of the amounts of GrB detected by ELISA in the same extracts. Together, the results obtained with these approaches indicate that granular GrB was preferentially released into the cytosol of activated CD8$^{+}$ T cells.

**Discussion**

When chronically exposed to soluble anti-CD3 mAb and exogenous IL-2, and in the presence of limiting numbers of monocytes, primary CD8$^{+}$ and CD4$^{+}$ T cells are similarly activated, but only CD8$^{+}$ T cells are driven to synthesize GrB. In this cellular model, moderate but ongoing apoptosis, concurrent with CD3 stimulation, affects CD8$^{+}$ T cells and to a much lesser extent CD4$^{+}$ T cells. Our work shows that small amounts of catalytically active GrB, escaping the neutralizing effect of the cytosolic PI-9 serpin are present in the cytosol of â¼37% GrB$^{+}$CD8$^{+}$ T cells, before $\Delta_{\text{m}}$ loss. By silencing the GrB gene, we show that a GrB-mediated suicide pathway is subsequently triggered, involving activation of...
efflux of GrB to the cytosol in a substantial part of CD3-stimulated to CD2 cross-linking (35). Our results suggest that there is granular inhibitor, PI-9 (17). It was suggested that PI-9 surrounding the can leak from cytolytic granules into the cytosol of cytotoxic cells, as evidenced by complex formation between GrB and its cytosolic as 32-kDa form of GrB, a form which is not stored in the granules but is constitutively secreted from CTLs in the course of degranulation (32). Endogenous GrB can leak from cytolytic granules into the cytosol of cytotoxic cells. In contrast, we hardly detected the 35-kDa form of GrB, which is not stored in the cytosol in a substantial part of CD3-stimulated CD8 T cells, ultimately inducing their apoptotic death.

Silencing the Pf gene by the siRNA methodology strongly suggests that Pf is not involved in the CD3-induced apoptotic pathway. This result implies that background granule exocytosis, possibly occurring in our stimulation conditions, did not cause the death of activated CD8+ T cells by a feedback mechanism, and that GrB did not require Pf to enter the cytosol. Examples of GrB-mediated apoptosis in the absence of Pf have been reported recently. Indeed, murine CD4+CD25+ T regulatory cells can mediate GrB-dependent suppression of T effector proliferation by a contact mechanism (still awaiting elucidation) that does not rely on Pf (36). Exogenous GrB is an interacting partner for the heat shock protein (Hsp) 70 protein expressed at the surface of tumor cells, and the GrB/Hsp70 interaction results in dose-dependent uptake of GrB and induction of apoptosis (37). However, Hsp70 is not expressed on the surface of lymphocytes, so cannot be involved in the delivery of GrB into the cytosol of these cells. Interestingly, rGrB coupled to a Lewis-specific mAb is retargeted to Lewis Y-positive cells, is then internalized in endocytic transport vesicles, and induces cell death without any exogenous endosome-disrupting agent (38). GrB endocytic uptake and release into the cytosol has also been reported in another system of redirected GrB by GrB/Ab conjugates (39). Importantly, free exogenous GrB was not by itself able to induce apoptotic cell death but required the assistance of an endosome-disrupting agent. The mechanism by which the granule membrane was destabilized and GrB liberated to the cytosol in these studies is elusive. Still, these examples suggest that to reach the cytosol, internal GrB may not require the help of agents with endosome-disrupting activity.

However, our results do not exclude the possibility that Pf may participate in CD3-induced cell death. When subjected to re-cross-linking with immobilized anti-CD3, T lymphoblasts from wild-type mice (previously stimulated with soluble anti-CD3 plus IL-2) underwent extensive apoptosis whereas T lymphoblasts from Pf-deficient mice were largely spared (5, 40). As cell death was fratricide independent, it was suggested that internal Pf was involved. Extensive granule exocytosis occurs during this mode of stimulation (41). Somehow solicited, Pf may have induced an enhanced granular efflux of GrB, as hypothesized by the authors (40). In our system (using soluble anti-CD3 plus IL-2), Pf was apparently retained in the cytoplasmic granules of the cells, while GrB was allowed to escape. In this respect, it would be interesting to investigate the subcellular localization of Pf and GrB under strong solicitation of the granular cytolytic system.

The mechanism by which GrB leaks from the granule membrane to enter the cytosol is unknown. A better understanding of the mechanism(s) by which GrB crosses endosomal membranes during target cell lysis could shed light on the way GrB escape from cytolytic granules. Ninety percent of GrB is found in the electronic-dense core of the granules, the rest being over the region containing small internal vesicles (42). Given this compartmentalization, it is possible that only the GrB localized in this region leaks into the cytosol. Most GrB molecules are noncovalently bound to the 250-kDa proteoglycan serglycin in macromolecular complexes (30–50 granzyme B molecules can bind to a single serglycin molecule) (43, 44). These complexes are the physiological form of GrB that is exocytosed into the immunological synapse formed between effector and target cells. They possess the same apoptotic activity against target cells as cationic free GrB. Importantly, GrB can dissociate from the serglycin carrier protein at the target cell membrane, and undergo internalization without the proteoglycan (45). Whether in our model GrB molecules dissociate from the serglycin carrier before being released to the cytosol, or whether they remain in macromolecular complexes, is an unresolved issue. GrB/serglycin complex formation may afford protection to GrB against the PI-9 protease, which could explain why we detected low but significant GrB enzymatic activity in cytosolic extracts from CD8+ T cells. However, the protection afforded was not absolute as GrB molecules were allowed to interact with PI-9.

In this study, we did not address the issue of the relative importance of caspases and Bid in initiating and propagating the GrB-mediated death pathway that takes place in activated CD8+ T lymphocytes. Waterhouse et al. (15) showed that Bid−/− target cells underwent rapid apoptotic death upon exposure to external GrB and Pf, whereas various Bid−/− target cells were resistant to this treatment. These data suggested that Bid was the preferred, critical substrate for GrB in intact cells, and that the translocation of GrB-cleaved tBid to the outer mitochondrial membrane was necessary to induce mitochondrial permeabilization. Other published data have demonstrated that GrB-cleaved tBid induces the release of proapoptotic mediators, including cytochrome c (46, 47), Smac/Diablo and HtrA2/Omi (48, 49), (all of which are involved in activation of the caspase cascade) from the mitochondrial intermembrane space. These findings contrast with those of Metkar et al. (12) who reported that GrB initiated cell death through caspase-3 activation and that a secondary mitochondrial feedback loop was then triggered in MCF-7 cells lacking procaspase-3. As low nanomolar concentrations of external GrB can induce cell death in Bid+/− target cells, whereas much higher concentrations of GrB (>30 times higher) are required to induce cell death in Bid−/− target cells, it has been suggested that the triggering of the Bid or caspase-3 pathway may depend on the concentration of GrB applied to the cells (15). Preliminary experiments, conducted in our experimental setting, with Bid-siRNA, indicated that the inhibition of Bid protein production resulted in enhanced cell survival (data not shown). This suggests that GrB-cleaved tBid contributed to the apoptotic events induced by GrB. However, the specific contribution of caspase-3 in this setting has not yet been assessed and must be analyzed for comparison of the roles of caspase-3 and Bid.

A series of observations, obtained in this study by confocal laser scanning microscopy, IP, and Western blot analysis, strongly suggested that Pf and GrA remained largely sequestered in the cytolytic granules while some GrB was translocated to the cytosol. In line with this possibility, confocal microscopy analysis showed...
that ingested 10-kDa FITC-conjugated dextran molecules were re-
tained in the vesicles of the lysosomal/granular system, whereas
GrB was diffusing outside the granules. However, using the highly
sensitive Gra-ELISA technique, we detected very small amounts
of GrA in the cytosolic extracts of CD8+/T cells. They accounted
for only ~0.03% of total cellular GrA, whereas the GrB
released accounted for ~6% of total cellular GrB in the same cells.
The activity of such small quantities of GrA was below the thresh-
old required to induce apoptosis. Furthermore, as GrA cannot
cleave either caspases or Bid (50, 51), unlike GrB, it cannot make
use of the potent amplificatory mechanisms potentially provided
by these substrates.

We also partly detected by confocal laser scanning microscopy
and by Western blot analysis, the presence of cathepsins B, D, and
L in the cytosol of activated CD8+ T cells (data not shown), which
suggested that these proteases were retained in the lysosomal/gran-
ular vesicles. The fact that silencing the GrB gene was sufficient to
prevent Bid activation, further indicated that pamin-like lysosomal
cathepsins proteases that can activate Bid at neutral pH (52),
only cathepsins B, H, L, S, and K (53), were not involved.
However, one cannot exclude that a tiny flux of cystein cathepsins,
not detectable by the immunological tools we used, were crossing
the granule membrane of GrB+/T cells. If so, they would then be
controlled by endogenous cystatins that bind them tightly and
readily inhibit their activity (54).

The first analyses of GrB knockout mice concluded that they
have no particular phenotype (55). However, recent studies have
indicated that clearance of lymphophoric choriomeningitis virus
infection is delayed in GrB-deficient mice (56). Increased levels
of lymphophoricinhibitoritis virus-specific memory cells (but not ef-
ectors) have been observed after the contraction phase in GrB
knockout mice and in mice transgenic for the serine protease in-
tector cells (57). Moreover, lymphocytic choriomeningitis virus-specific memory cells (but not effector cells) have been observed after the contraction phase in GrB
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