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Granzyme A Loading Induces Rapid Cytolysis and a Novel Form of DNA Damage Independently of Caspase Activation

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Summary

Cytotoxic lymphocytes trigger apoptosis by releasing perforin and granzymes (Grn). GrnB activates the caspase apoptotic pathway, but little is known about GrnA-induced cell death. Perforin was used to load recombinant GrnA and GrnB and enzymatically inactive variants into target cells. GrnA induces single-strand DNA breaks that can be labeled with Klenow polymerase and visualized on alkaline gels. GrnA-induced DNA damage but not cytolysis requires GrnA proteolysis. GrnA-induced membrane perturbation, nuclear condensation, and DNA damage are unimpaired by caspase blockade. GrnA fails to induce cleavage of caspase-3, lamin B, rho-GTPase, or PARP. GrnA-induced cytotoxicity and cleavage of PHAP II, a previously identified GrnA substrate, are unimpaired in Jurkat cells that overexpress *bcl-2*. Therefore, GrnA activates a novel apoptotic pathway.

Introduction

Granule exocytosis is the primary execution pathway for cytotoxic T cells (CTL) to eliminate virally infected cells. CTL granules contain a pore-forming protein, perforin, and a group of serine proteases termed granzymes (Grn) contained in a proteoglycan matrix (Masson and Tschopp, 1985; Pasternack and Eisen, 1985; Podack et al., 1985; Gershenfeld and Weissman, 1986; Bleackley et al., 1988; Jenne and Tschopp, 1988). Additional molecules in the cytotoxic granules, whose function in cytolysis is not completely clear, include the 4-transmembrane protein GMP-17 or p15-TIA-1 (Medley et al., 1996), the RNA helicase leukophysin (Abdelhaleem et al., 1996), the Ca^{2+}-binding protein calreticulin (Dupuis et al., 1993), and β-chemokines (Wagner et al., 1998). Perforin pokes holes in the target cell, allowing the granzymes to enter and initiate apoptosis. However, perforin may not be required for entry into target cells but may be necessary for granzyme entry into the nucleus or exit from an endosomal compartment (Froelich et al., 1996; Shi et al., 1997). GrnB, a protease with Aspase activity, activates the ubiquitous apoptotic caspase pathway by initially cleaving caspases-10, −3, and −7 (Darmon et al., 1995; Duan et al., 1996; Orth et al., 1996; Quan et al., 1996; Talanian et al., 1997; Yang et al., 1998). However, caspase-independent mechanisms of cell death are also initiated by CTL, since cytolysis and loss of mitochondrial transmembrane potential occur in the presence of caspase blockade and are in some cases not inhibited by *bcl-2* overexpression (Chiu et al., 1995; Schroter et al., 1995; Sarin et al., 1997, 1998; Sutton et al., 1997).

GrnA and GrnB dominate granzyme expression after ex vivo CTL activation, although other granzymes are proteolytically active and may be important in specific cell types (i.e., granzyme M in NK cells) or when GrnA and GrnB are genetically disrupted in knockout mice (Garcia-Sanz et al., 1990; Shresta, 1996, Blood, abstract; Simon et al., 1997). Although the gene for *GrnA*, the most abundant of the granzymes, was cloned 13 years ago (Gershenfeld and Weissman, 1986), its role in CTL lysis remains an enigma.

Partially purified GrnA and GrnB, isolated by column chromatography from mouse or rat cytolytic granules, induce apoptosis when introduced into target cells either by perforin or adenovirus exposure or detergent permeabilization (Hayes et al., 1989; Shi et al., 1992a; Froelich et al., 1996). GrnB induces oligosomal DNA fragmentation and apoptotic cell death rapidly by a mechanism that does not require protein synthesis and is inhibited by caspase inhibitors including the poxvirus crmA protein (Darmon et al., 1995; Quan et al., 1995, 1996; Duan et al., 1996; Orth et al., 1996). GrnA induces rapid cell death, but oligosomal DNA degradation occurs via a slow pathway that can be inhibited by cycloheximide (at least in rats [Shi et al., 1992a]) and serine protease inhibitors (Redelman and Hudig, 1983; Poe et al., 1991; Nakajima and Henkart, 1994). The rapid release of degraded soluble DNA 3 hr after target cell exposure to CTL is completely inhibited by caspase-3 inhibition, while the slow release detected after 16 hr is only blocked when both grnA protease and caspase-3 activity are inhibited (Anel et al., 1997). The slow kinetics and low level of ^{125}IUdR release in GrnA-induced lysis has been interpreted by some to mean that GrnA does not have an important role in apoptosis. However, the ^{125}IUdR release assay only measures production of small detergent-soluble DNA fragments.

Noncytotoxic rat basophilic leukemia (RBL) cells, triggered via the IgE receptor and transfected with combinations of murine perforin, GrnA, or GrnB, have been used as a model to identify the key components of granule-mediated lysis. RBL transfected with perforin alone are weakly cytotoxic, as measured by ^{51}Cr release assay, but do not induce target cell DNA degradation. When these cells are additionally transfected with either GrnA or GrnB, they induce higher levels of cytotoxicity and DNA degradation. The transfection of both granzymes A and B into RBL transfected with perforin synergistically enhances both ^{51}Cr release and DNA degradation to levels comparable to that of CTL clones (Shiver et al., 1992; Nakajima and Henkart, 1994; Nakajima et al., 1995).

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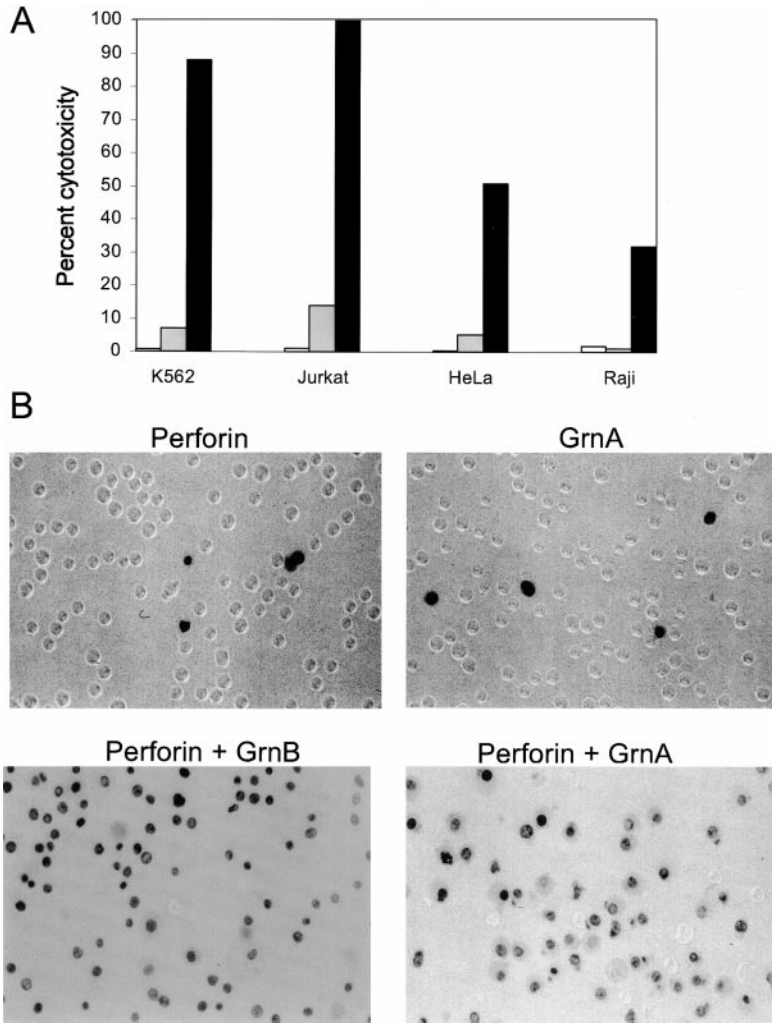

Figure 1. GrnA Loading Induces Cytolysis, PI Inclusion, and Nuclear Condensation

(A) Chromium-labeled target cells were treated with recombinant GrnA (white bars), perforin (shaded bars) or GrnA and perforin (black bars) for 2 hr. The perforin concentration was chosen for each cell line to induce between 2% and 10% specific release.

(B) Perforin loading recombinant GrnB or GrnA into K562 cells, but not GrnA or perforin alone, leads to PI uptake and apoptosis. Two hours after loading, unfixed cells were stained with PI and photographed with backlit fluorescence microscopy (magnification 320×). Although occasional cells treated with perforin or GrnA include PI, nuclear condensation is only seen with the combination of perforin and either Grn.

These results suggest that GrnA and GrnB act independently to induce target cell apoptosis and that these three granule components are the major effectors of granule mediated lysis.

Evidence from knockout (KO) mice rendered deficient in perforin, GrnA, or GrnB support the conclusions of the RBL transfection experiments (Heusel et al., 1994; Kagi et al., 1994; Ebnet et al., 1995; Shresta et al., 1995a, 1995b; Mullbacher et al., 1996). Perforin-deficient mice rapidly succumb to lymphochoriomeningitis (LCMV) infection, have delayed clearance of *Listeria monocytogenes*, and are susceptible to 100-fold lower doses of tumor cells than normal mice. These results support the importance of granule-mediated lysis in the protection against intracellular pathogens and in tumor immunosurveillance and the essential role for perforin. CTL from GrnB-deficient mice have unimpaired cytotoxicity as measured by ^{51}Cr release but a delay in oligosomal DNA fragmentation measured by $^{125}IUdR$ release. No defects in the ability of GrnB-deficient mice to defend against infectious agents have been reported. CTL from GrnA-deficient mice demonstrate unimpaired cytotoxicity and DNA fragmentation. Moreover, GrnA-deficient mice are as capable of withstanding infection with LCMV or *Listeria monocytogenes* or inoculation with syngeneic tumors as wild-type mice. However, GrnA-deficient mice are defective in their ability to protect against hepatic failure and death from ectromelia, a cytopathic mousepox virus (Mullbacher et al., 1996). The susceptibility of the GrnA KO mice to this virus may be due to poxvirus expression of crmA, a GrnB and caspase inhibitor. Therefore, GrnA and GrnB operate independently and synergistically in CTL function.

GrnA, the most abundant of the proteases in human CTL granules, is a tryptic protease that cleaves synthetic substrates with lysine or arginine at the P1 position. Tryptic activity is readily measured by a colorimetric assay based on cleavage of the lysine derivative benzyloxycarbonyl-L-lysine-thiobenzyl ester (BLT) (Pasternack and Eisen, 1985; Odake et al., 1991). GrnA is closely homologous to other serine esterases including the other granzymes, mast cell proteases, and neutrophil cathepsins (Jenne and Tschopp, 1988). It is unique among the granzymes in forming a disulfide-linked homodimer of approximately 50 kDa (Masson et al., 1986).

Because of the high degree of homology between

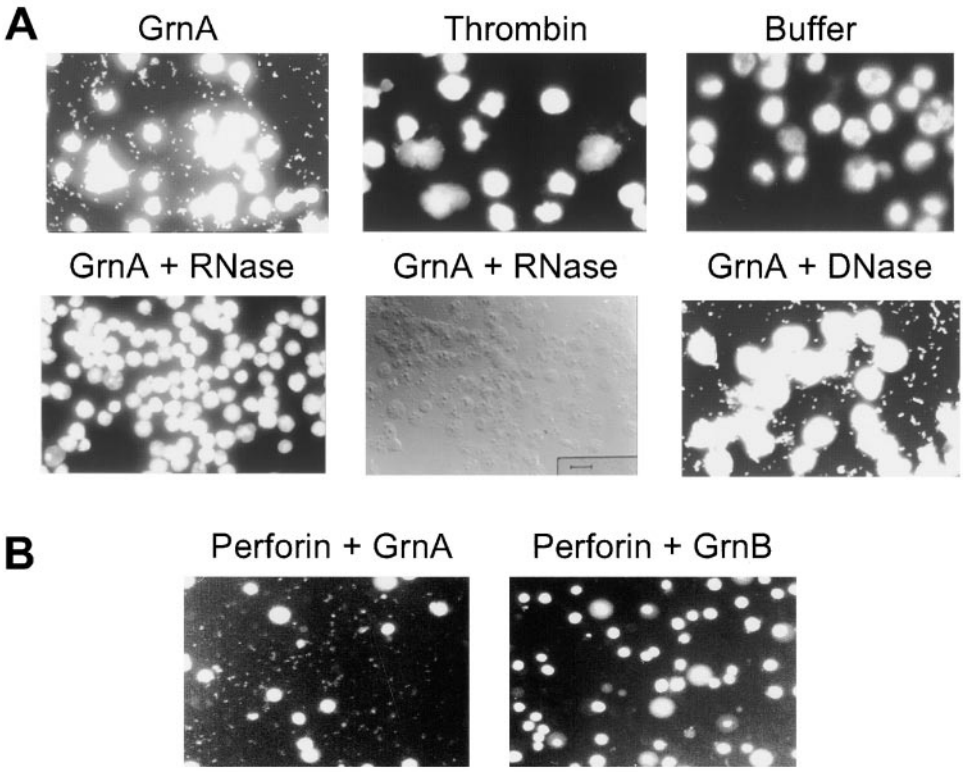

Figure 2. GrnA Loading of Target Cells with Detergent or Perforin Induces Apoptosis and RNA-Containing Membrane Blebs

(A) PI stain of protein-loaded detergent-permeabilized HL60 cells for 2 hr shows membrane blebs containing RNA after GrnA treatment but not after thrombin or buffer. When the slides of GrnA-loaded cells were incubated with DNase or RNase, the PI staining of the blebs disappeared after RNase treatment. However, the blebs were still visible with Nimarski microscopy (magnification 450×, 650×). In this setting PI uptake is not a measure of apoptosis since detergent treatment leads to permeabilization.

(B) Perforin loading of K562 cells for 2 hr with GrnA but not with GrnB induces PI-staining membrane blebs (magnification 320×). With perforin loading alone, only about 10% of the cells stain with PI (data not shown; see Figure 1B).

the granzymes and their similar biochemical properties, purified enzyme preparations may be contaminated with other granzymes. We have been able to produce recombinant active GrnA and GrnB and inactive variants with the active site serine mutated to alanine (Beresford et al., 1997; Xia et al., 1998; MacDonald et al., 1999), which has enabled us to study the characteristics of cell death induced by each of these enzymes. We find that cytolysis but not DNA degradation induced by GrnB is caspase independent. GrnA induces rapid cytolysis and activates a novel form of DNA damage within 2 hr of loading. Both cytolysis and DNA damage by GrnA are independent of caspase activation or caspase substrate cleavage. In addition, overexpression of *bcl-2* does not protect cells from GrnA-mediated death. Although GrnA esterase activity is required for DNA damage, it is not needed for cytolysis.

Results

Granzyme A Loading Induces Cytolysis and Apoptotic Morphological Changes

When purified GrnA is introduced into target cells with perforin, it induces cell death assayed by chromium release. Since purified native GrnA preparations might be contaminated with small amounts of other granzymes with similar physicochemical properties, we verified these results using the recombinant enzyme. Recombinant GrnA was introduced into chromium-labeled target cells using sublytic doses of perforin, determined separately for each cell line to induce 2%–10% specific lysis. GrnA induced between 32% and 100% specific cytotoxicity in Raji, HeLa, Jurkat, and K562 cells compared with 1%–16% with perforin or GrnA alone. Cell lines vary in susceptibility to GrnA loading (Figure 1A). Propidium iodide (PI) staining was used to assay loss of membrane integrity and nuclear condensation after GrnA loading. About 10% of cells stained for PI after loading with perforin or GrnA, comparable to the specific lysis calculated by chromium release. When GrnA or GrnB were loaded with perforin into K562 cells, approximately 80%–90% of cells included PI (Figure 1B). Although nuclear condensation was not seen after loading with only perforin, most PI-staining cells treated with GrnA and GrnB have condensed nuclei. Similar results were found with Jurkat, Cos, Raji, and HeLa cells (data not shown).

With GrnA loading we also observed the formation of membrane blebs. This was initially observed during Triton X-100 loading of GrnA into K562 cells, but it was also present after more physiological perforin loading

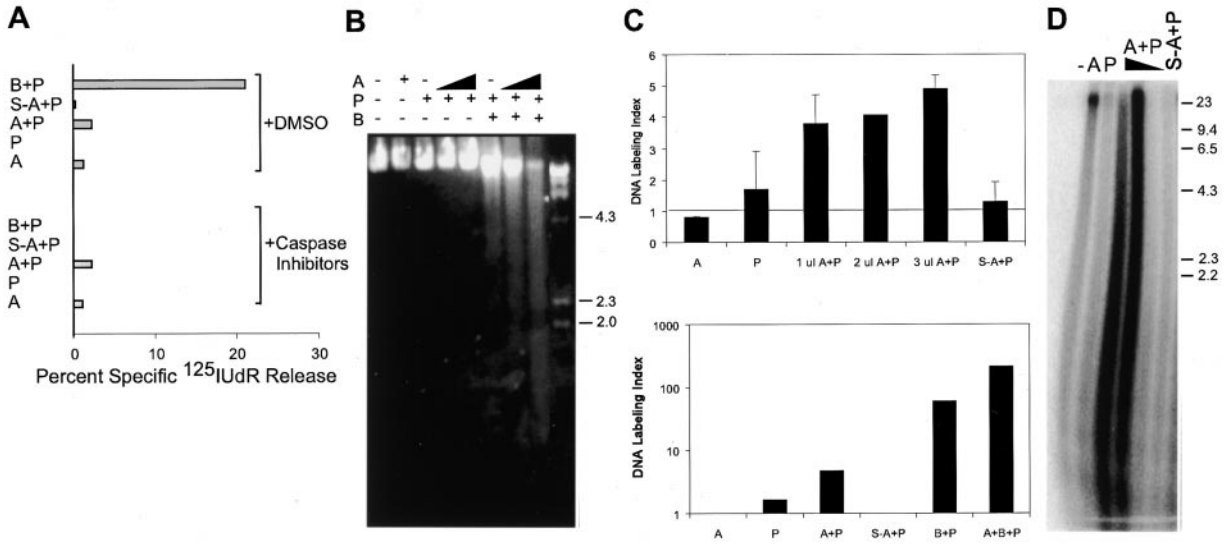

Figure 3. GrnA Loading Induces a Novel Form of DNA Damage

(A) GrnB but not GrnA loading leads to release of detergent soluble DNA fragments. DNA fragment release induced by GrnB is blocked in the presence of the caspase inhibitors z-VAD-FMK and DEVD-FMK. K562 cells were labeled with ^{125}IUdR and incubated for 4 hr following Grn and/or perforin (P) treatment and supernatants were harvested for counting of released DNA fragments (A). DNA fragmentation results were confirmed with agarose gel electrophoresis and autoradiography of extracted DNA (data not shown).

(B) GrnA loading does not induce DNA fragmentation detectable by native agarose gel electrophoresis. However, GrnA enhances GrnB-mediated oligonucleosomal DNA degradation. Cells were loaded with GrnA (2 and 3 μM) and/or GrnB (1 μM) and incubated for 6 hr prior to DNA extraction. DNA was visualized by EtBr staining of an agarose gel.

(C) GrnA but not S-AGrnA induces DNA damage in K562 cells that is detectable by Klenow incorporation of radioactive nucleotides and is dose dependent. Moreover, treatment with both GrnA (1 μM) and GrnB (1 μM) is synergistic (log scale in lower panel). S-AGrnA (3 μM) does not induce radiolabeled breaks.

(D) DNA single-strand breaks, induced after 6 hr loading of GrnA (1, 0.3, and 0.1 μM) but not S-AGrnA (1 μM) into K562 cells, can be visualized after Klenow labeling by denaturing alkaline gel electrophoresis. Radiolabeled fragments were detected beginning within between 2 and 4 hr of adding GrnA (data not shown).

(Figure 2). PI staining blebs were present during GrnA loading but not when cells were loaded with control serine esterases such as thrombin or GrnB. To investigate the nature of the PI-staining material, slides were treated with RNase or DNase before PI staining. RNase but not DNase treatment abrogated the staining, indicating that the blebs contain RNA.

Granzyme A Loading Induces DNA Damage

Whether GrnA has any role in inducing DNA damage has been unclear. Release of ^{125}IUdR-labeled DNA fragments from target cells after native GrnA loading or after targeting by CTL from mice with genetically disrupted GrnB is much delayed compared with that induced by GrnB. Recombinant GrnA loading, unlike GrnB loading, failed to induce oligonucleosomal DNA degradation measured by agarose gel electrophoresis or by release of detergent soluble DNA fragments from ^{125}IUdR labeled cells (Figure 3A). However, GrnA enhanced GrnB-mediated oligonucleosomal DNA degradation (Figure 3B). To detect other forms of DNA damage that might be induced by GrnA, terminal deoxynucleotidyl transferase was introduced into whole cell lysates in the presence of radiolabeled deoxynucleotides after GrnA or GrnB loading. Labeling was detected after GrnB but not after GrnA loading (data not shown). However, the Klenow fragment of DNA polymerase I radiolabeled DNA strand breaks when it was added for 1 hr to whole cell lysates after GrnA loading. Loading of GrnA but not of enzymatically inactive S-AGrnA mutant increased ^{32}P-dCTP incorporation in a dose-dependent manner (Figure 3C). Moreover, treatment with both GrnA and GrnB led to higher incorporation than with either Grn alone. Alkaline agarose gel electrophoresis showed that DNA damage was due to single-stranded breaks, which are first detected between 2 and 4 hr after GrnA loading (Figure 3D; data not shown). These results suggest that GrnA induces DNA damage which is distinct from that induced by GrnB, but may facilitate the apoptotic DNA degradation caused by GrnB.

Granzyme A–Mediated Death Is Independent of Caspase Activation

Because GrnA induces a novel form of DNA degradation, we assessed whether cell death triggered by GrnA loading is dependent on caspase activation. Loss of membrane integrity assessed by ^{51}Cr release after perforin loading of either GrnA or GrnB was not significantly inhibited by caspase inhibition (Figure 4A). Similar results were found for Jurkat cells (data not shown). However, preincubation of cells with both the caspase-3, −7, and −8 inhibitor DEVD-FMK and the broad caspase inhibitor Z-VAD-FMK (Garcia-Calvo et al., 1998) blocked

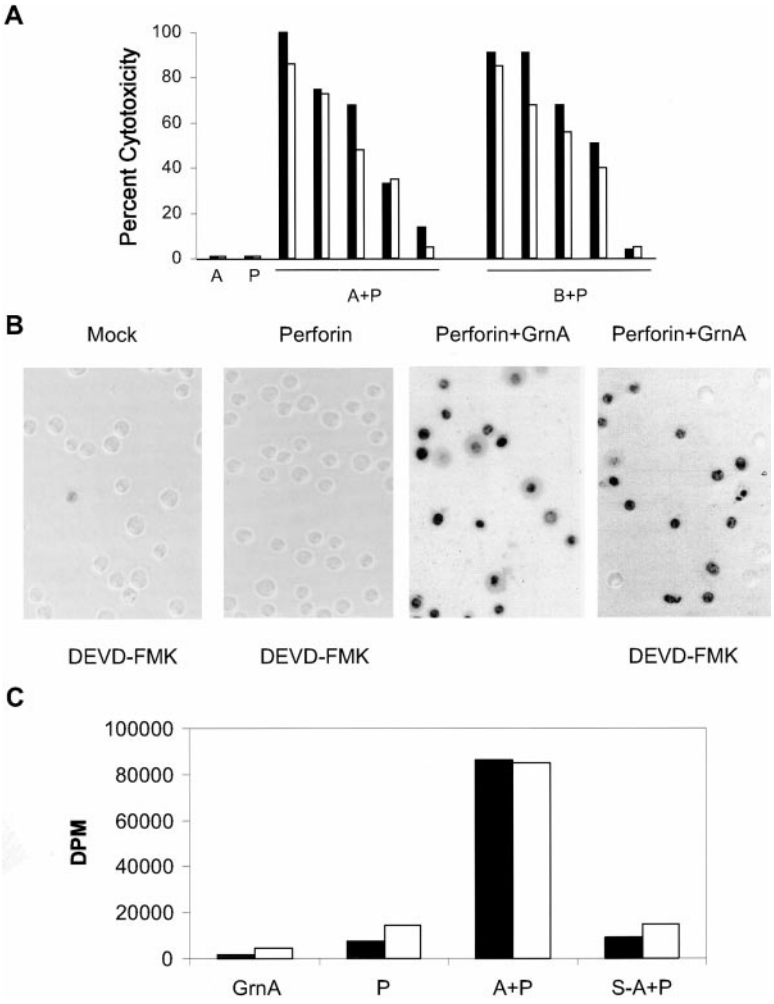

Figure 4. Caspase Inhibition Does Not Block GrnA-Mediated Cytolysis, Nuclear Condensation, or DNA Damage in K562 Cells

(A) ^{51}Cr release was measured after perforin loading GrnA or GrnB for 2 hr in the presence (white bars) or absence (black bars) of z-VAD-FMK and DEVD-FMK. Results with successive 2-fold dilutions of each Grn beginning with 1 μM are shown.

(B) Preincubation with DEVD-FMK does not inhibit apoptosis induced by GrnA loading of K562 cells. Unfixed cells were stained with PI 2 hr after loading and photographed with backlit fluorescence (magnification 320×).

(C) Caspase inhibition with DEVD-FMK and z-VAD-FMK (white bars) does not affect GrnA-mediated DNA damage (measured by Klenow incorporation).

detergent-soluble DNA release produced by GrnB loading (Figure 3A). In contrast, neither DNA damage nor nuclear condensation induced by GrnA loading was inhibited by caspase inhibitors (Figures 4B and 4C).

Cleavage of caspase-3 is a common final pathway in caspase-mediated cell death initiated by diverse agents. Caspase-3 activation by GrnA was assessed by measuring cleavage of the colorimetric substrate DEVD-pNa and by looking for caspase-3 degradation by immunoblot. Caspase-3 was not activated in GrnA-treated Jurkat cell extracts but was activated after GrnB treatment (Figure 5A). In cell lysates, GrnB degraded caspase-3 to p20 but GrnA, S-AGrnA, or the S-AGrnB did not (Figure 5B). Similarly, caspase-3 was degraded only in GrnB-loaded cells (Figure 5C).

To verify further the caspase independence of GrnA-mediated cell death, cleavage of caspase pathway substrates after GrnA loading was evaluated. PARP, lamin B, and Rho-GTPase were cleaved in cells loaded with GrnB (but not with GrnA) within 1 hr of loading (Figures 5C and 5E). However, PHAP II, a previously identified GrnA substrate, was cleaved in GrnA-loaded cells but not in GrnB-loaded cells. The 25 kDa N-terminal fragment of PHAP II seen after perforin loading GrnA has the same size as the fragment visualized in vitro after incubation of purified cellular PHAP II with recombinant GrnA (Beresford et al., 1997).

bcl-2 Overexpression Does Not Inhibit Granzyme A Cytolysis

Overexpression of *bcl-2* inhibits caspase-dependent apoptosis induced by Fas ligation, irradiation, growth factor withdrawal, or chemotherapeutic drugs (Sentman et al., 1991; Itoh et al., 1993; Reed, 1994; Boise et al., 1995). However, the role of *bcl-2* in blocking CTL-mediated cell death is controversial (Chiu et al., 1995; Schroter et al., 1995; Sutton et al., 1997). To assess the effect of *bcl-2* on GrnA-mediated cytolysis, GrnA was loaded into Jurkat cells transfected to overexpress *bcl-2* or *neo* control. Overexpression of *bcl-2* did not affect GrnA-induced cytolysis (Figure 5D). The GrnA substrate PHAP II was cleaved in both *neo* and *bcl-2* overexpressing cells (Figure 5E). However, the caspase substrate Rho-GTPase was not cleaved after GrnA loading, irrespective of *bcl-2* overexpression. In contrast, Rho-GTPase was cleaved after GrnB loading in *neo*-transfected Jurkat cells but not in Jurkat cells transfected with *bcl-2*. The lack of any effect of *bcl-2* overexpression on PHAP II

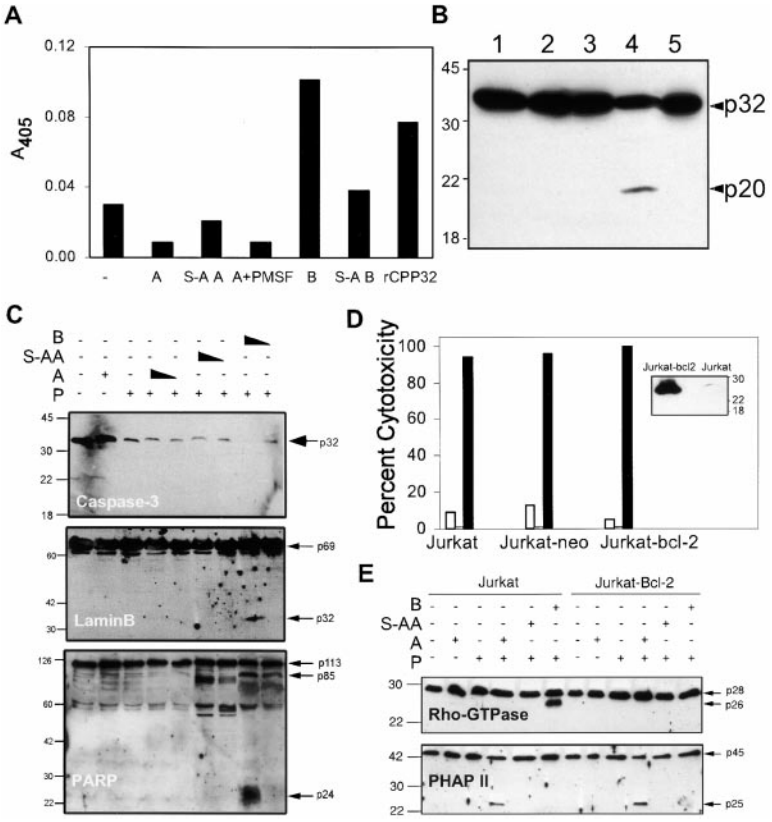

Figure 5. GrnA Does Not Activate the Caspase Pathway

(A) Caspase-3 (CPP32) is activated in Jurkat cell lysates by GrnB but not by GrnA. Caspase-3 activity was measured by cleavage of the colorimetric substrate DEVD-pNA. Recombinant CPP32 was used as a positive control.

(B) Caspase-3 is cleaved to p20 in Jurkat lysates by GrnB (lane 4) but not by S-AGrnA (lane 1), GrnA (lane 2), S-AGrnB (lane 3), or medium (lane 5).

(C) GrnA loading of Jurkat cells with perforin does not result in cleavage of the caspase pathway substrates caspase-3, lamin B, or PARP; however, loading with GrnB does result in cleavage of these substrates. Cells loaded with GrnA (5, 1 μM), S-AGrnA (5, 1 μM) or GrnB (3, 1 μM) were lysed in SDS-PAGE sample buffer and analyzed by immunoblot with antibodies to caspase-3, lamin B, and PARP. (The 20 kDa caspase-3 cleavage product was not visualized in loaded cells but was detected in treated cell lysates [B].)

(D) *bcl-2* overexpression (*bcl-2* immunoblot in inset) does not inhibit GrnA-mediated cytolysis. ^{51}Cr-labeled Jurkat cell lines were treated with GrnA (white bars), perforin (shaded bars), or GrnA and perforin (black bars).

(E) GrnA loading of Jurkat cells leads to cleavage of PHAP II but not of Rho-GTPase, independent of *bcl-2* overexpression. GrnB (1 μM) loading leads to cleavage of the caspase pathway substrate Rho-GTPase, which is blocked in cells that overexpress *bcl-2*. PHAP II, however, is not cleaved in GrnB-loaded cells.

cleavage or GrnA-induced cytolysis further supports the caspase independence of the GrnA pathway.

Granzyme A Esterase Activity Is Not Needed for Cytolytic Activity

Surprisingly, ^{51}Cr release was induced by perforin-loading enzymatically inactive S-AGrnA into Jurkat cells (Figure 6). To verify this unexpected finding, cells were loaded with GrnA pretreated with PMSF. PMSF was chosen because it is unstable in aqueous solution and would have been completely inactivated prior to loading. S-AGrnA and PMSF-treated enzyme had no BLT esterase activity. Enzymatically inactive and active GrnA and enzymatically active GrnB induced cytolysis. However, S-AGrnB did not induce cytolysis. Susceptibility to inactive GrnA was not limited to Jurkat cells, since K562 cells were also lysed by inactive GrnA (data not shown). Inactive GrnA induced cytolysis but was unable to bring about DNA damage (Figure 3).

Discussion

In this paper we used perforin loading of recombinant granzymes to distinguish and define some of the properties of cell death induced by GrnA and GrnB. We found that the requirements for cytolysis are distinct from those for DNA damage induced by each of these enzymes. For both enzymes, membrane integrity was disrupted in a caspase-independent manner, as has previously been reported for CTL-induced cytolysis (Sarin et al., 1997, 1998). We were surprised to find that GrnA induces cytolysis even when serine protease activity is abrogated either by PMSF treatment or by replacing the active site serine to produce an inactive mutant. DNA degradation into oligonucleosomal fragments by GrnB is completely caspase dependent, while no aspect of GrnA-induced cell death involves caspase activation. The caspase independence of the GrnA pathway was verified by showing that neither the key common final pathway caspase (caspase-3) nor key caspase substrates, including PARP, lamin B, and Rho-GTPase, are activated after GrnA loading and that GrnA-induced cell death and DNA damage is not blocked by caspase inhibitors or *bcl-2* overexpression.

Whether or not GrnA by itself induces DNA damage had been uncertain, since only delayed release of small DNA fragments is detected after CTL attack of caspase-treated targets, after attack by CTL with genetically inactivated GrnB, or after loading the rat homolog of GrnA (Shi et al., 1992a; Heusel et al., 1994; Anel et al., 1997). These findings were confirmed here with loading recombinant GrnA. We found, however, that GrnA induces rapid DNA damage detectable 2–4 hr later in the form of single-strand nicks that cannot be visualized by agarose gel electrophoresis or detected by release of small soluble DNA fragments, but it can be seen in denaturing alkaline gels or by radiolabeled nucleotide incorporation by Klenow polymerase. Moreover, oligonucleosomal DNA breakdown by GrnB is synergistically enhanced by

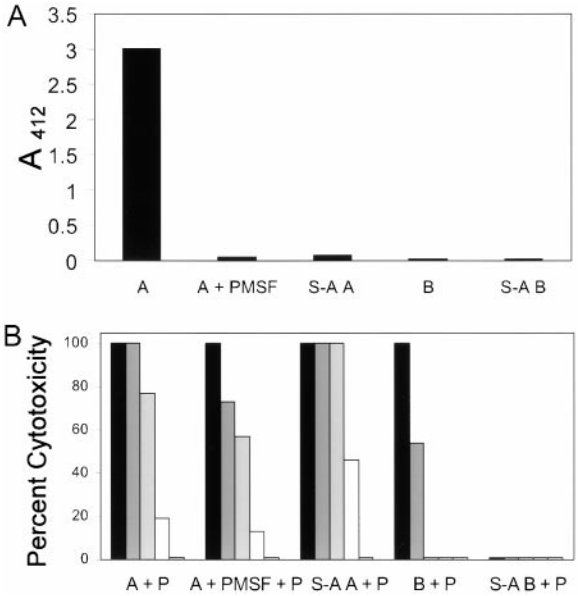

Figure 6. rGrnA Induces Cytolysis Independent of Esterase Activity

(A) GrnA, but not PMSF-treated GrnA, S-AGrnA, GrnB, or S-AGrnB, has BLT esterase activity.

(B) Both active and enzymatically inactive forms of GrnA induce cytolysis. ^{51}Cr-labeled Jurkat cells were loaded with 2-fold serial dilutions of GrnA, PMSF-inactivated GrnA (A + PMSF), S-AGrnA (S-A A), GrnB, or S-AGrnB (S-A B), beginning with initial concentrations of 3 μM GrnA and 1 μM GrnB. Grn or perforin alone induced less than 10% specific cytotoxicity (data not shown).

the addition of GrnA, as has been previously reported (Nakajima et al., 1995). Recent evidence suggests that chromatin degradation in apoptosis may involve a multistep process with sequential degradation of genomic DNA initially to 50–300 kb fragments, followed by its ultimate degradation to 180–200 bp oligonucleosomal fragments, which produces a characteristic apoptotic ladder on agarose gels (Kokileva, 1994; Lagarkova et al., 1995). It is likely that the sequential pattern of DNA cleavage during apoptosis is due to the activation of more than one endogenous DNase. The caspase-activated DNase (CAD), which becomes activated when its inhibitor (ICAD) is cleaved by caspase-3 during caspase-mediated apoptosis, is likely the effector DNase for oligonucleosomal DNA damage induced by GrnB (Enari et al., 1998; Sakahira et al., 1998). The caspase-independent GranA-induced DNA damage, however, should involve activation of an additional endogenous DNase, perhaps important in the initial steps of DNA breakdown into large fragments. Whether the GrnA-induced cell death pathway can be truly labeled apoptotic without the direct induction of oligonucleosomal DNA fragmentation is mainly a matter of semantics, since the other hallmark changes of apoptosis and DNA damage are induced.

GrnA might also facilitate GrnB-induced oligonucleosomal DNA breakdown by other mechanisms. GrnA cleaves histone H1, the anchoring histone that keeps DNA wrapped around the histone core (M. Pasternack, personal communication). We recently showed that PHAP II, a cytoplasmic protein also known as set, TAFI-β, and I_2^{PP2A} and with homology to evolutionally conserved nucleosome assembly proteins, is also a GrnA substrate (Adachi et al., 1994; Vaesen et al., 1994; Kawase et al., 1996; Li et al., 1996; Beresford et al., 1997). In this paper we show that GrnA loading induces PHAP II cleavage to the 25 kDa N-terminal fragment previously seen in in vitro experiments (Beresford et al., 1997). GrnA cleavage of PHAP II is caspase independent, since it occurs with recombinant GrnA and PHAP II and, as shown here, occurs in vivo in cells transfected to overexpress *bcl-2*. PHAP II has nucleosome assembly function and can remodel chromatin to make it accessible to exogenous nucleases (Kawase et al., 1996; Okuwaki and Nagata, 1998) and (D. Z. et al., unpublished data). Therefore, GrnA, through its interactions with histone H1 and PHAP II, may facilitate DNA damage by initiating the unwinding of chromatin as a first step to enhance DNA accessibility to DNases.

These results help us to understand the results of loading and knockout experiments. Since GrnA does not initiate oligonucleosomal DNA damage, there is no early release of soluble DNA fragments after purified rat GrnA loading or exposure to GrnB knockout CTL (Shi et al., 1992a; Heusel et al., 1994). The delayed release of soluble fragments could be due to delayed caspase activation, perhaps through loss of plasma membrane integrity, oxidative changes, disruption of the mitochondrial potential, or change in DNA damage-sensing proteins such as p53 well after the GrnA-treated cell has committed to death. Although mitochondrial changes have been shown in GrnB-mediated death (MacDonald et al., 1999), whether GrnA disturbs mitochondrial integrity and function remains to be explored. However, other GrnA loading and transfection studies did detect some release, albeit at reduced levels, of detergent soluble DNA fragments within 2–4 hr in murine targets (Hayes et al., 1989; Nakajima and Henkart, 1994). This apparent difference may be explained by the fact that mouse cell lines are known to more readily undergo oligonucleosomal DNA degradation in response to inducers of apoptosis than most human cell lines (Sellins and Cohen, 1991). In fact, K562 cells, which have the *bcr-abl* translocation and were used in many of our experiments, are unusually resistant to oligonucleosomal fragmentation in response to UV and γ irradiation, chemical inducers, or protein synthesis inhibitors (Bedi et al., 1994; McGahon et al., 1994; Roger et al., 1996). Therefore, this cell line may be particularly useful for delineating other forms of DNA damage. The synergy in oligonucleosomal damage induced when GrnA and GrnB act together in RBL-double transfectants (Nakajima et al., 1995) was also found in our loading experiments and may be explained by the distinct pathways of DNA damage induced by each of these CTL enzymes. The fact that DNA degradation induced by GrnA- plus GrnB-transfected RBL is comparable to that of CTL clones suggests that both enzymes together, but neither alone, activate the major DNases involved in apoptosis.

Several features of the cytolysis after GrnA loading merit discussion. The lack of dependence on granzyme protease activity is surprising and distinct from GrnB. This finding is consistent with the results of Hayes et al. (1989) in which only DNA degradation, but not cytolysis,

induced by CTL clones was abrogated by CTL pretreatment with PMSF. However, Hudig (Hudig et al., 1987) found that treatment of granule proteins with long-lasting serine protease inhibitors completely blocked cytolysis. An explanation for this apparent discrepancy might be inactivation of a nongranzyme target cell serine protease required for cytolysis. Since GrnA has been found in the serum in conditions of chronic CTL activation (Spaeny-Dekking et al., 1998), it may be more difficult to inhibit cytolysis induced by circulating GrnA, since its induction of cytolysis is protease independent, than by GrnB, whose cytolysis is protease dependent. Although GrnB-specific serpins, which bind to and inactivate the active site serine, have been described, none have yet been found for GrnA (Sun et al., 1996).

We previously identified a strong interaction between GrnA and hsp27, a protein that regulates actin polymerization (Beresford et al., 1998). We found that hsp27 is not a substrate for GrnA but redistributes after CTL attack to associate with newly formed actin filaments, and we postulated that hsp27 is a key actor in the morphological apoptotic changes induced by GrnA. If this is the case, then these changes may occur without serine protease activity, since hsp27 is not a substrate of GrnA. Another prominent feature of GrnA loading is the appearance of RNA-containing membrane blebs. PI-staining blebs have previously been described after cell treatment with cytotoxic granules from an NK cell line (Andrade et al., 1998). Since RNA is not generally thought to be localized in the cell periphery where blebs might be expected to bud off, this RNA localization must involve a specific mechanism. During heat stress, hsp27- and RNA-containing membrane-bound stress granules have been described (Welch and Suhan, 1985; Collier et al., 1988). It is possible that the small heat shock protein hsp27 might have a role in this process. It remains to be shown whether hsp27 is in the PI staining blebs induced by GrnA loading. We did not detect these blebs after loading comparable amounts of GrnB.

The induction of complementary pathways of cytolysis and DNA damage by GrnA and GrnB may help provide protection from pathogens that have evolved mechanisms to evade one pathway or the other, such as those described for the caspase pathway in poxviruses, and from mutations in transformed cells that render them more or less sensitive to apoptosis (Torigoe et al., 1994; Tewari et al., 1995; Roger et al., 1996; Sutton et al., 1997). Just as *bcl-2* overexpression renders some tumor cell lines relatively resistant to the caspase pathway (Torigoe et al., 1994; Schroter et al., 1995), the cell lines we tested varied in their sensitivity to cytolysis by GrnA loading. Although for many infections either granzyme is sufficient for CTL elimination of virally infected cells via granule mediated lysis, in some situations, whether because of more pathogenic virus, greater infectious dose, or less potent immune response, the activation of both death pathways may be critical for protection.

Experimental Procedures

Cell Lines

K562, Jurkat, HL-60, Raji, and HeLa cells were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% FCS, 2mM glutamine, 2 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. *bcl-2* and *neo*-transfected Jurkat cells were kindly provided by John Reed (Torigoe et al., 1994).

Granzymes and Perforin

Recombinant GrnA and enzymatically inactive S-AGrnA were produced and purified as previously reported (Beresford et al., 1997) and further purified over a cation exchange S column (BioRad). Grn-containing fractions in 500 mM NaCl and 50 mM Bis-Tris (pH 5.8) eluted as a single peak at approximately 1 M NaCl with a 0.5–2.0 M NaCl gradient. This material was concentrated to 3 mg/ml using a YM10 Centricon concentrator (Amicon) and stored frozen at −20°C. BLT esterase activity was assayed as previously described (Pasternack and Eisen, 1985). Recombinant GrnB and S-AGrnB were produced and purified as previously described (Xia et al., 1998; MacDonald et al., 1999). Perforin was purified from the rat RNK-16 cell line as described (Shi et al., 1992b). Sublytic concentrations of perforin that induce between 2% and 10% cytolysis in a 2 hr assay were determined independently for each cell line.

Granzyme Loading with Perforin

Cells ($2 \times 10^3/\mu l$) in 30–60 μl HBSS with 1 mg/ml BSA, 1 mM $CaCl_2$ and 1mM $MgCl_2$ were incubated between 1–6 hr at 37°C with granzymes (generally 3 μM GrnA or S-AGrnA or 1 μM GrnB or S-AGrnB, unless otherwise specified) and sublytic concentrations of perforin. For PI staining, 5 μg/ml PI was added after Grn incubation and cells were visualized by backlit fluorescence microscopy (Zeiss Axiophot). For cytotoxicity assays, cells were radiolabeled with ^{51}Cr for 1 hr and washed before GrnA loading. ^{51}Cr release in the supernatant of pelleted cells was counted on a Packard Topcount. Specific cytotoxicity was calculated using the formula [(sample release) − (spontaneous release)] / [(total release) − (spontaneous release)] × 100. For caspase inhibition experiments, radiolabeling occurred in the presence or absence of 100 μM DEVD-FMK, alone or with 100 μM zVAD-FMK (Calbiochem). For immunoblots, cell lysates were prepared from Grn-loaded cells by preincubating cells for an additional 30 min in 1 mM PMSF before resuspending the cell pellet in 2× SDS-PAGE sample buffer and boiling for 5 min.

Detergent Permeabilization

HL60 cells were incubated at 37°C for 2 hr with 3 μM GrnA or 3 μM thrombin (Sigma) in HBSS with 0.3% Triton X-100 (Sigma), 0.5 mg/ml BSA, 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$. Cells were fixed in 3 mg/ml formaldehyde for 20 min at 37°C, cytospun onto glass slides, and treated with 10 μg/ml RNase A (Sigma) and 10 μg/ml RQ1 DNase (Sigma) in PBS or PBS alone for 1 hr at 37°C. Slides were stained for 10 min with 50 μg/ml propidium iodide (PI) in PBS, washed, and visualized by fluorescence microscopy.

DNA Fragmentation Assay

DNA was extracted by proteinase K treatment and EtOH precipitation from K562 cells that were perforin-loaded with Grn, as above, for 6 hr and analyzed by electrophoresis and EtBr staining on 1% agarose gels. In addition, K562 cells were preincubated for 2 hr with 50 μCi ^{125}IUdR (Amersham) with or without caspase inhibitors and loaded, as above, with perforin and granzymes. Four hours later, cells were lysed in 0.3% Triton X-100 and centrifuged at 2580 × g. Supernatants were counted to quantitate release of detergent soluble ^{125}IUdR DNA fragments. Percent specific release was calculated using the formula [(sample release) − (spontaneous release)] / [(total release) − (spontaneous release)] × 100. Radiolabeled DNA was extracted by proteinase K-EtOH treatment in parallel samples for analysis on 1% agarose gels.

Klenow Incorporation Assay

To assess more subtle forms of DNA damage, the Klenow fragment of DNA polymerase was used to label DNA breaks. Two-to-six hours after Grn loading with perforin, pelleted cells were lysed in an equal volume of NP40 lysis buffer (0.5% NP40, 5 mM $MgCl_2$, 25 mM KCl, and 10 mM Tris HCl [pH 7.5]) and incubated with 5 U Klenow (New England Biolabs) and 10 μCi ^{32}P-dCTP (NEN) for 1 hr at 37°C. Radiolabeled nuclei, pelleted by centrifuging for 5 min at 2580 × g and washed twice in 5 ml NP40 lysis buffer, were counted after adding

scintillation fluid (Beckman). The DNA labeling index was calculated by dividing the disintegrations per minute of loaded cells by the disintegrations per minute of mock treated cells. In parallel samples, DNA was extracted by proteinase K treatment and EtOH precipitation from pelleted nuclei and analyzed by alkaline agarose gel electrophoresis to assess single-strand breaks as described (Sambrook et al., 1989). Samples prepared in alkaline loading buffer were electrophoresed on a 1% gel at 0.25 V/cm. The gel was renatured in 1 M Tris (pH 8.5), stained with EtBr to visualize the standards, and fixed in 7% TCA. The dried gel was imaged using a Storm 860 Molecular Dynamics PhosphorImager.

Immunoblot

Cell lysates were electrophoresed through reducing SDS-PAGE, transferred to nitrocellulose, and probed as in Beresford et al. (1997) with the following antibodies: caspase-3 (Transduction Labs), Rho-GTPase (Pharmingen), PARP (Boehringer Mannheim), laminB (Calbiochem), PHAP II (protein A–purified rabbit polyclonal sera to an N-terminal peptide of PHAP II as described [Adachi et al., 1994]).

Caspase-3 Assay

Jurkat cell lysates prepared from 10^5 cells lysed in 50 μl of NP40 lysis buffer were incubated with 2 μM GrnA, S-AGrnA, or GrnA pretreated for 24 hr with 1 mM PMSF, or 1 μM GrnB or S-AGrnB pretreated for 2 hr at 37°C. Treated lysates were assayed for caspase-3 activity in 50 mM Tris (pH 7.5), 1 mM DTT, and 0.2 mM Ac-DEVD-pNa (Calbiochem) as described (Xia et al., 1998). Recombinant caspase-3 was used as a positive control, a kind gift of Dr. J. Yuan (Zhang et al., 1998). Cell lysates treated as above but incubated for 4 hr were also analyzed by SDS-PAGE and immunoblot for caspase-3.

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