THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 5, Issue of February 2, pp. 3683–3690, 2001

Printed in U.S.A.

Induction of Rapid Histone Degradation by the Cytotoxic T Lymphocyte Protease Granzyme A*

Received for publication, June 20, 2000, and in revised form, September 5, 2000
Published, JBC Papers in Press, November 1, 2000, DOI 10.1074/jbc.M005390200

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The cytotoxic T lymphocyte protease granzyme A induces caspase-independent cell death in which DNA single-strand nicking is observed instead of oligonucleosomal fragmentation. Granzyme A is a specific tryptase that concentrates in the nucleus of targeted cells and synergistically enhances DNA fragmentation induced by the caspase activator granzyme B. Here we show that granzyme A treatment of isolated nuclei enhances DNA accessibility to exogenous endonucleases. *In vitro* and after cell loading with perforin, GrnA completely degrades histone H1 and cleaves core histones into ~16-kDa fragments. Histone digestion provides a mechanism for unfolding compacted chromatin and facilitating endogenous DNase access to DNA during T cell and natural killer cell granule-mediated apoptosis.

Cytotoxic T lymphocytes (CTL)[1] induce apoptosis by engagement of cell surface death domain-bearing receptors, such as Fas, or by exocytosis of cytolytic granules containing perforin and serine proteases called granzymes (Grn) (1–4). Experiments with mice in which the perforin gene is genetically disrupted suggest that the granule exocytosis pathway is the primary mechanism for CTL protection from viral infection and tumors (5). GrnA and GrnB, the most abundant granzymes, are delivered to the cytosol of targeted cells via perforin, concentrate in target cell nuclei, and independently and synergistically induce apoptosis (6–13). Death receptor engagement and GrnB activate the caspase apoptotic pathway (14). However, CTL induce target cell death in the presence of caspase blockade and in some cases in target cells that overexpress Bcl-2 (13, 15–19). This suggests that CTL also activate caspase-independent cell death pathways.

The most abundant CTL protease, GrnA, induces rapid cell death that has the features of apoptosis, except for oligonucleosomal DNA degradation (13). Until recently it was unclear whether GrnA induces any DNA damage since small fragments of DNA are not released, DNA degradation is not visualized on agarose gels, and DNA breaks are not generally detected with terminal deoxynucleotidyltransferase labeling. Induction of cell death by GrnA was considered slow, based on assays that measure the release of small radiolabeled DNA fragments into the supernatant. However, other apoptotic changes occur as rapidly with GrnA loading as with GrnB loading (Ref. 13, data not shown). DNA damage in the form of single-strand nicks occurs within 2 h in cells loaded with GrnA. These breaks can be labeled with Klenow polymerase and visualized on denaturing alkaline agarose gels. None of the features of GrnA-induced death are blocked by caspase inhibition or Bcl-2 overexpression. Moreover, effector caspases are not activated by GrnA loading. Therefore, the GrnA pathway, including the DNA damage it induces, is caspase-independent. However, perforin loading of GrnA facilitates the oligonucleosomal DNA damage of the caspase-dependent DNase (CAD/DFF40) (20–22) activated by loading GrnB.

Although many of the features of GrnB-induced cell death have been elucidated with the definition of caspase activation pathways, the story is incomplete since GrnB induces cell death but not DNA fragmentation when caspase activation is blocked (13, 19). However, still less is known about the molecular events triggered by GrnA. Although GrnA has tryptic activity, it is a highly specific protease. When comparable concentrations of GrnA and trypsin with equivalent benzyloxycarbonyl-L-lysine-thiobenzyl esterase activity are incubated at 37 °C with nuclear lysates, trypsin completely degrades the sample within 1 h, so that no bands are visible on SDS-PAGE gels. GrnA, on the other hand, only visibly degrades 1 of 22 bands after 25 h (23). Moreover, only a few candidate GrnA substrates, including interleukin-1β, fibronectin, type IV collagen, the thrombin receptor, pro-urokinase-type plasminogen activator, and nucleolin have been identified (23–27). In most cases, the biological significance of these substrates remains to be demonstrated. In fact, nucleolin does not appear to be a direct substrate of GrnA.[2] We recently identified the nucleosome assembly protein PHAP II/set/TAF-Iβ/I_2^{PP2A} as a substrate of GrnA during CTL lysis and GrnA loading with perforin (13, 28–34).

While looking for potential nuclear substrates of GrnA, we found that immobilized histone H1 binds GrnA on far-Western blots and that histone H1 is cleaved in nuclear lysates by purified CTL granule extracts (23).[3] Recombinant GrnA degrades purified histone H1 *in vitro* but requires the presence of heparan sulfate (HS).[3] The HS dependence of the *in vitro*

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[1] The abbreviations used are: CTL, cytolytic T lymphocyte; Grn, granzyme; GrnA, granzyme A; Ser → Ala GrnA, enzymatically inactive GrnA with Ser → Ala mutation of the active site; GrnB, granzyme B; CAD/DFF, caspase-dependent DNase; PHAP, putative HLA-associated protein; HS, heparan sulfate; PMSF, phenylmethylsulfonyl fluoride; z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; PAGE, polyacrylamide gel electrophoresis.

[2] M. Pasternack, unpublished information.

[3] L. Wagner, W. Gartner, P. J. Beresford, L. Bensinger, Y. Ge, A. H. Greenberg, J. Lieberman, M. S. Pasternack, submitted for publication.

cleavage reaction may not be surprising since the granzymes released from cytolytic granules are complexed to sulfated proteoglycans and because both GrnA and histone H1 are very basic (calculated pI values, 9.1 and 11.3, respectively).

Histone H1 is an attractive substrate for a cell death pathway in which DNA is targeted. The linker histone H1 plays a critical role in chromatin hypercondensation (35–37), which may protect genomic DNA from endonuclease digestion. Lys- and Arg-rich histones might be especially good targets of a tryptase like GrnA. Therefore, we investigated whether histones might be physiologically relevant targets of GrnA. Here we show that recombinant GrnA *in vitro* and *in vivo*, after cell loading with perforin, degrades not only histone H1 but also cleaves core histones. The cleavage sites recognized by GrnA are distinct from those cut by trypsin; in fact histone H1 is initially cut in a folded core region that is protected from trypsin digestion. We also find that GrnA loading of isolated nuclei disrupts chromatin and facilitates exogenous DNase access to DNA. Chromatin disruption by histone modification may be essential to GrnA-induced DNA nicking and may also contribute to the synergy of GrnA and GrnB in inducing oligonucleosomal DNA fragmentation.

EXPERIMENTAL PROCEDURES

Cell Lines—K562, Jurkat, COS, and HL60 cells were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol.

Granzymes and Perforin—Recombinant GrnA and enzymatically inactive Ser → Ala GrnA were produced and purified as previously reported (13, 34). Recombinant GrnB was produced and purified as described previously (38). Perforin was purified from the rat RNK-16 cell line as described (39) and used at a sublytic concentration that induced <10% cytolysis in a 2-h assay, as determined independently for each cell line.

Recombinant PHAP II—PHAP II cDNA (30) was amplified by polymerase chain reaction from an Epstein-Barr virus-transformed B cell line library using primers containing *Bam*HI and *Xho*I restriction sites (5′-atc gga tcc gat gtc ggc gcc ggc ggc caa-3′ and 5′-atg ctc gag gtc atc ttc tcc ttc atc ctc ctc t-3′). The polymerase chain reaction product was inserted into pET-25b(+) (Novagen) through the *Bam*HI and *Xho*I cloning sites. The PHAP II plasmid was transformed into BL21 cells (Novagen) and PHAP II expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma). His-tagged PHAP II was sequentially purified by nickel resin (Novagen) and anion exchange chromatography. The identity and proper folding of the recombinant protein was verified by reactivity with a rabbit antiserum raised to an N-terminal PHAP II peptide as described (29) and by its function in a nucleosome assembly assay.[4]

Preparation of Mononucleosomes and Purified Histones—Mononucleosomes and histones were purified from HeLa or HL60 cells as described (40). Briefly, 10^9 cells were lysed in Nonidet P-40 lysis buffer (20 mM HEPES (pH 7.5), 0.25 M sucrose, 3 mM $MgCl_2$, 0.2% Nonidet P-40, 3 mM β-mercaptoethanol, and 0.4 mM PMSF). Pelleted nuclei were washed twice with lysis buffer and once with buffer A (20 mM HEPES (pH 7.5), 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM β-mercaptoethanol, and 0.4 mM PMSF). The nuclei were resuspended in buffer A to which was added dropwise with gentle stirring one-third of the total volume of buffer A containing 0.6 M KCl and 10% glycerol. After stirring for 10 min at 4 °C, the nuclear pellet was recovered by centrifugation for 30 min at 17,500 × *g*. To prepare mononucleosomes, chromatin was isolated after Dounce homogenization, treated for 5 min at 37 °C with micrococcal nuclease, and applied to a Sepharose CL-6B column. The fractions were analyzed on SDS-PAGE and native agarose gels after deproteinization. Fractions containing mononucleosomes stripped of histone H1 were pooled. To purify histones, nuclear pellets containing ~12 mg of DNA were resuspended in 50 ml of HAP buffer (50 mM sodium phosphate (pH 6.8), 0.6 M NaCl, 1 mM β-mercaptoethanol, and 0.4 mM PMSF) and stirred gently for 30 min at 4 °C. To this mixture was added 20 g of dry Bio-Gel HTP Hydroxylapatite powder (Bio-Rad) with stirring. The resin was packed into a column, and the flow-through containing partially pure histone H1 was collected. After further washing with 600 ml of HAP buffer, core histones were eluted from the column with HAP buffer containing 2.5 M NaCl. Histone H1 was further purified by gel filtration.

In Vitro GrnA Cleavage Reaction—Purified histones or H1-stripped HeLa cell nucleosomes (0.5 μg of DNA) were incubated with various amounts of recombinant granzymes in the presence or absence of heparan sulfate (Sigma), recombinant PHAP II, or random plasmid DNA (prepared by plasmid DNA purification kit (Qiagen)) in 25-μl reaction buffer (100 mM Tris HCl (pH 8.0), 50 mM NaCl) at 37 °C for indicated times. The cleavage reaction was stopped by adding 5× SDS-loading buffer containing a mixture of protease inhibitors (50 μg/ml antipain, 2 μg/ml aprotinin, 40 μg/ml bestatin, 60 μg/ml chymostatin, 10 μg/ml E-64, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma) to which was added 250 μM GrnA specific inhibitor Ph-NHCONH-CiEtOIC (a kind gift of J.C. Powers, Georgia Institute of Technology, Atlanta, GA) (34)). Boiled samples were electrophoresed on 15% SDS-PAGE gels (for core histones) or 12% SDS-PAGE gels (for histone H1) and analyzed by immunoblot using polyclonal antisera against histone H1, H2B, and H3 (Biodesign International, Inc.) or staining with GelCode® Blue (Pierce). For N-terminal sequencing of the cleavage products, electrophoresed samples were transferred to a polyvinylidene difluoride membrane (ProBlott™, Applied Biosystems), and the desired Coomassie-stained bands were cut and sent for protein sequencing by the Tufts Core Facility.

Cleavage of Histones in Isolated Nuclei—Nuclei were isolated from HL-60 cells by Nonidet P-40 lysis as described above and washed twice with buffer B (20 mM Tris (pH 7.2), 0.25 M sucrose, 3 mM $CaCl_2$). Nuclei (1×10^6) in 100 μl of buffer B containing 50 mM NaCl were incubated with the indicated amounts of GrnA with or without HS at 37 °C for indicated times. The reaction was stopped by adding 5× SDS-loading buffer and the protease inhibitor mixture. Boiled samples were analyzed by SDS-PAGE and immunoblot.

Limited Nuclease Digestion Assay—Isolated nuclei as above were washed twice with lysis buffer, resuspended in buffer B containing 1% Triton X-100 and then incubated on ice for 5 min. After further washing with buffer B, 1×10^6 nuclei in 100 μl were incubated with 400 nM GrnA or PMSF-inactivated GrnA and/or 400 nM PHAP II at 37 °C for 2 h. Micrococcal nuclease (20 units, Roche Molecular Biochemicals) or RQ1 DNase I (10 units, Promega) was added for 5 min or 10 min, respectively, before the addition of stopping buffer (10 mM EDTA, 200 μg/ml protease K, and 2% SDS in 20 mM Tris (pH 8.0)). After overnight deproteinization, DNA was extracted with phenol and ethanol precipitation and analyzed by ethidium bromide staining on 1.5% agarose gels.

Granzyme Loading with Perforin—Recombinant GrnA, Ser → Ala GrnA, or GrnB were loaded with perforin into K562 or Jurkat cells as described (13). For each reaction 1×10^5 cells in 60 μl of Hanks' balanced salt solution with 1 mg/ml bovine serum albumin, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ were incubated with an indicated amount of recombinant granzymes and sublytic concentrations of perforin at 37 °C for indicated times. In some experiments, caspase activation was blocked by preincubating effector and target cells for 30–60 min with 100 μM z-VAD-FMK and 100 μM z-DEVD-FMK (Calbiochem), concentrations that were maintained throughout the experiment. Quantitation of cytolysis induced by loading was assayed in parallel experiments by treating ^{51}Cr-labeled cells with GrnA and perforin, incubating at 37 °C for 2 h, and counting ^{51}Cr released into the supernatant on a Packard Topcount as described (13). Specific cytotoxicity was calculated by the formula: ((sample release) − (spontaneous release))/((total release) − (spontaneous release)) × 100. Cells were lysed by adding 5× SDS-loading buffer and a mixture of described previously protease inhibitors. Samples were boiled for 5 min and then electrophoresed on 15% SDS-PAGE and immunoblotted with antibodies against histone H1, H2B, and H3 and Rho-GDI (Pharmingen).

Klenow Incorporation Assay—To assess single-stranded DNA damage induced by GrnA loading, which is not detectable by terminal dUTP nick-end labeling assay, the Klenow fragment of DNA polymerase was used to label DNA breaks as described (13). Six h after Grn loading with perforin, pelleted cells were lysed in an equal volume of Nonidet P-40 lysis buffer and incubated with 5 units of Klenow (New England Biolabs) and 10 μCi of ^{32}P-dCTP (PerkinElmer Life Sciences) for 1 h at 37 °C. Radiolabeled nuclei, pelleted by centrifuging for 5 min at 2580 × *g* and washed twice in 5 ml of Nonidet P-40 lysis buffer, were counted after adding scintillation fluid (Beckman). The DNA labeling index was calculated by dividing the dpm of loaded cells by the dpm of mock-treated cells.

[4] P. J. Beresford, D. Zhang, D. Oh, Z. Fan, E. L. Greer, M. Jaju, and J. Lieberman, manuscript in preparation.

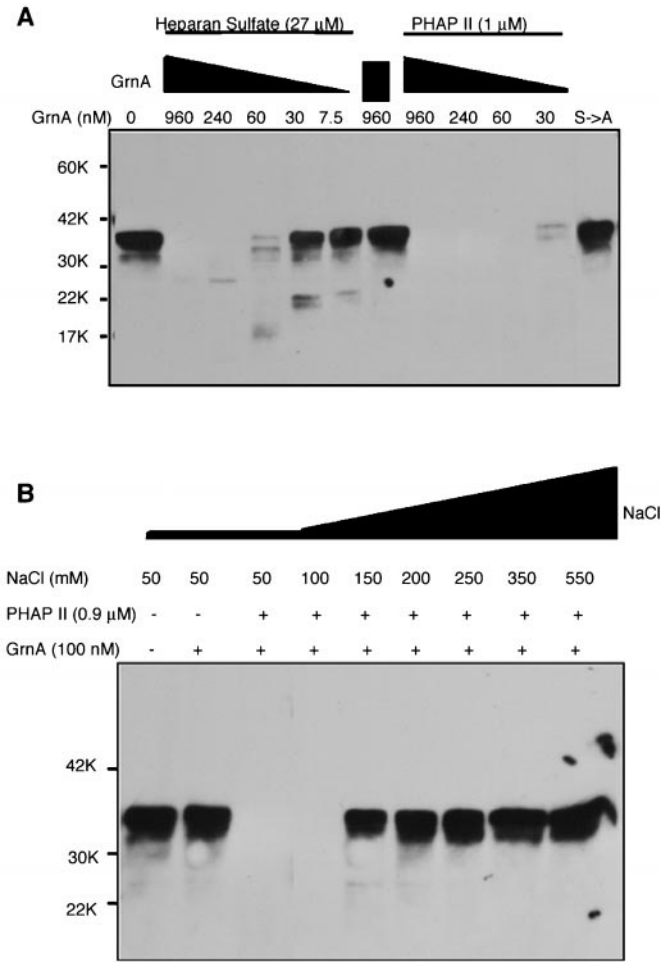

FIG. 1. **GrnA cleaves histone H1 *in vitro* in the presence of PHAP II.** *A*, PHAP II is more efficient on a molar basis than HS in promoting the *in vitro* cleavage of purified histone H1 by GrnA. Histone H1 (200 ng) was treated for 4 h at 37 °C with serial dilutions of GrnA (beginning with 960 nM at the highest concentration) in the presence of 1 μM recombinant PHAP II or 27 μM HS. Without either PHAP II or HS, no histone cleavage occurs; neither does it occur with enzymatically inactive Ser → Ala GrnA (960 nM). *B*, GrnA cleavage of histone H1 in the presence of PHAP II is inhibited by increasing concentrations of salt beginning at 150 mM. Immunoblots probed for histone H1 are shown. *K*, ×1000.

RESULTS

Histone H1 Is an In Vitro Substrate of GrnA in the Presence of Negatively Charged Heparin Sulfate or PHAP Proteins—Histone H1 is an *in vitro* substrate of GrnA complexed to HS[3] (Fig. 1*A*). We recently identified two highly acidic proteins PHAP II/set/TAF-Iβ/I_2^{PP2A} (calculated pI ~ 3.9) and PHAP I/I_1^{PP2A} (calculated pI ~ 3.8) as candidate participants in the GrnA cell death pathway based on their binding to recombinant GrnA (30, 34, 41). PHAP II, which has nucleosome assembly protein activity (32), is a GrnA substrate *in vitro* after GrnA loading with perforin and after CTL attack (13, 34). Moreover, PHAP II binds to purified core histones in an ionic interaction (data not shown and Ref. 42). We therefore tested whether this negatively charged molecule could substitute for HS in the histone H1 cleavage reaction (Fig. 1*A*). Histone H1 is cleaved by GrnA more efficiently on a molar basis in the presence of recombinant PHAP II compared with HS. Histone H1 is cleaved in the presence of HS to a fragment with an apparent molecular mass of 25 kDa at the lowest concentration of GrnA tested (7 nM). At higher concentrations of GrnA, smaller fragments of ~22- and 18-kDa are often seen; at the highest concentrations, no immunoreactive band is visible. It should be noted that all the histones migrate aberrantly on SDS-PAGE gels because of their charge. Molecular masses given in this paper are the apparent molecular masses. The digestion in the presence of PHAP II is more rapid and complete; none of the intermediate H1 cleavage products are detected at any of the concentrations tested. Histone H1 isoforms of differing mobilities, due to differences in backbone sequence or post-translational modifications (43), are all susceptible to GrnA digestion. Histone H1 *in vitro* cleavage is inhibited by increasing concentrations of salt beginning at physiological concentrations of ~150 mM. (Fig. 1*B*) The PHAP II-associated acidic protein PHAP I is comparable with PHAP II as a cofactor for GrnA in the histone H1 cleavage reaction (data not shown). The lack of specificity for the negatively charged cofactor and the inhibition by salt suggest that ionic interactions between these charged molecules are important.

GrnA Cleaves Histone H1 and Core Histones in Isolated Nuclei—To determine whether histone H1 cleavage also occurs *in situ*, we treated isolated HL60 nuclei with GrnA in the presence or absence of HS. (Fig. 2*A*) Within 1 h of incubation at 37 °C with 180 nM GrnA, histone H1 (visualized as a group of bands of molecular mass of ~35 kDa) is cleaved to ~25 kDa as seen on immunoblot. At higher concentrations, smaller fragments are sometimes visualized, as in *in vitro* assays. At higher enzyme concentrations or longer incubation times, no immunoreactive band is visualized, suggesting complete digestion (Fig. 2, *B* and *C*). Similar results are found with nuclei isolated from Jurkat and HeLa cells (data not shown). The addition of HS does not enhance the cleavage, suggesting that native molecules in the nucleus can replace negatively charged HS. Cleavage occurs as early as 15 min after adding GrnA at concentrations of GrnA as low as 240 nM. To determine whether core histones might also be targets of GrnA, GrnA-treated nuclei were also analyzed by immunoblot for histones H2B and H3 (Fig. 2*D*). Specific antibodies to the other core histones are not available. Both of these core histones were also cleaved to smaller fragments, with kinetics similar to the histone H1 cleavage reaction. Cleavage of the core histones is specific, since it does not occur with mutant enzyme or in the presence of GrnA inhibitor.

GrnA Treatment of Nuclei Enhances Chromatin Susceptibility to Exogenous Nucleases—Disruption of the histones in chromatin might alter the sensitivity of DNA to degradation by endonucleases activated during apoptosis. GrnA treatment of isolated nuclei without the addition of exogenous nucleases shows no apparent change in DNA length on agarose gels (data not shown). To determine whether GrnA treatment enhances the nuclease susceptibility of chromatin, isolated nuclei were permeabilized with 1% Triton X-100 and pretreated with GrnA with and without recombinant PHAP II before brief incubation with micrococcal nuclease (Fig. 3*A*) Without GrnA pretreatment, genomic DNA is digested into oligonucleosomes; after GrnA pretreatment, only mononucleosome-sized fragments remain. Although PHAP II is a nucleosome assembly protein that might be expected to alter DNA accessibility, the addition of recombinant PHAP II, either alone or with GrnA, does not alter susceptibility to micrococcal nuclease. After limited RQ1 DNase I digestion of isolated nuclei (Fig. 3*B*), DNA fragmentation is also more complete after GrnA pretreatment. DNA from nuclei that were mock-treated or treated with PMSF-inactivated GrnA contains fragments greater than 1.2 kilobases in size; after GrnA treatment, the largest visualized fragments are only 300 base pairs. Therefore, GrnA loading of intact nuclei enhances DNA nuclease digestion. GrnA enhancement of endonuclease susceptibility requires its serine protease activity.

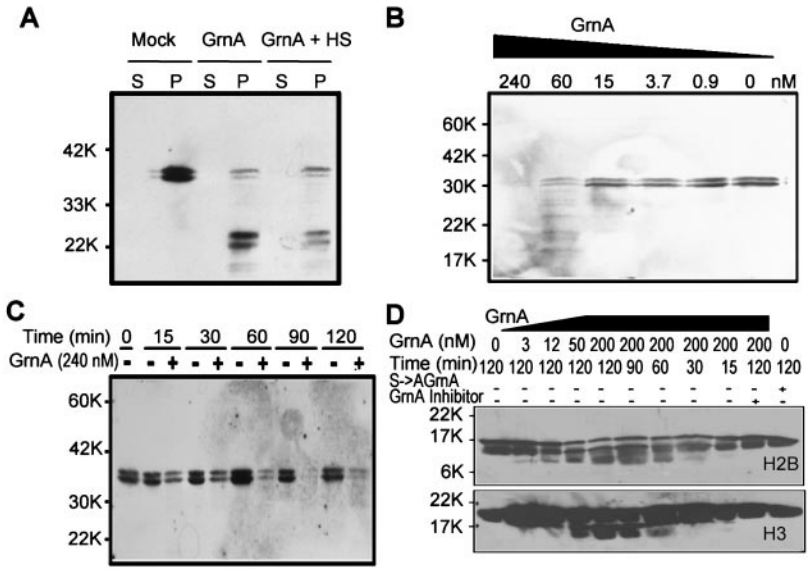

FIG. 2. **GrnA cleaves histone H1 and core histones in isolated nuclei without HS.** *A*, HL60 nuclei were incubated at 37 °C with 180 nM GrnA for 1 h in the presence or absence of HS, and the nuclear pellet (*P*) and supernatant (*S*) were analyzed by SDS-PAGE and histone H1 immunoblot. Histone fragmentation does not require HS *in situ* in isolated nuclei. There was no detectable whole or fragmented histone H1 released into the nuclear supernatants. *B* and *C*, dose response for a 1-h incubation of intact nuclei and kinetics of cleavage *in situ*. *D*, core histones H2B and H3, visualized by immunoblot, are also cleaved in isolated HeLa nuclei treated with GrnA. *S*→AGrnA, Ser → Ala GrnA.

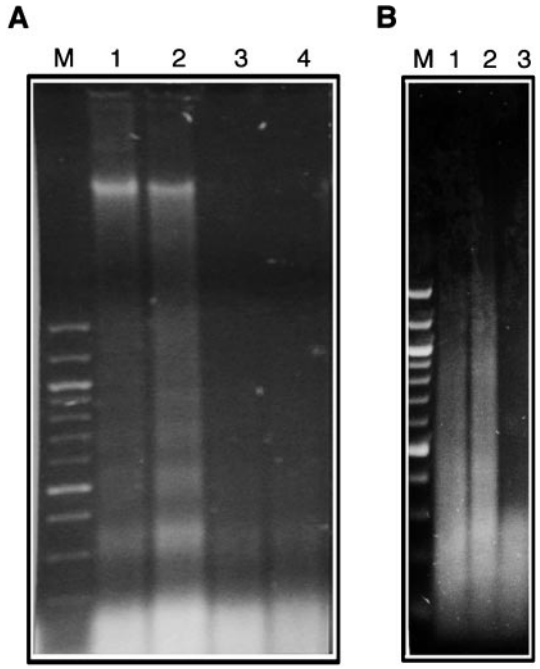

FIG. 3. **GrnA treatment enhances chromatin accessibility to exogenous nucleases in isolated premeabilized nuclei.** *A*, COS nuclei were incubated for 2 h with medium (mock digestion; *lane 1*), 400 nM PHAP II (*2*), 400 nM GrnA (*3*), or 400 nM GrnA and 400 nM PHAP II (*4*) and then incubated for 5 min with micrococcal nuclease before stopping the reaction. *B*, HL60 nuclei were incubated at 37 °C for 3 h with medium (mock digestion; *lane 1*), 400 nM PMSF-treated GrnA (*2*), or 400 nM active GrnA (*3*) before digestion with RQ1 DNase I for 10 min. Extracted DNA visualized by ethidium bromide staining. *Lane M*, 100-base pair DNA ladder (New England Biolabs).

DNA Is an in Vitro Cofactor for GrnA Histone Cleavage—Although the PHAP proteins can be found in either the nucleus or cytoplasm, they do not localize to the nucleus by immunofluorescence microscopy and immunoblot under the exponential growth conditions used here (data not shown). Because DNA is a prominent acidic nuclear molecule associated with histones and because DNA binds in a sequence-independent manner to GrnA (data not shown), we determined whether plasmid DNA can substitute *in vitro* as the negatively charged moiety in the histone H1 cleavage reaction. When plasmid DNA is added, histone H1 is completely degraded (Fig. 4*A*). Adding approximately equal amounts by weight of plasmid DNA and histone H1 substrate optimizes cleavage in solution; either more or less leads to less rapid proteolysis (Fig. 4*A* and data not shown). Histone H1 begins to be cleaved *in vitro* within 2 min and is completely degraded by 1 h (Fig. 4*B*). Core histones contained in purified nucleosomes stripped of linker histones, prepared as described by Cote *et al.* (40), were also cut by GrnA *in vitro* (Fig. 4*C*). When the core histones were separated from DNA, their *in vitro* cleavage was facilitated by addition of plasmid DNA (Fig. 4*D*). Stained polyacrylamide gels of GrnA-treated purified core histones also demonstrates that all the core histones are GrnA targets *in vitro*.

Histones Are Cleaved by GrnA in Perforin-loaded Cells in the Presence of Caspase Inhibition at GrnA Concentrations Required to Induce Cytolysis—The concentrations of GrnA and kinetics of histone H1 cleavage suggest that histone H1 and possibly the core histones might be physiologically relevant GrnA substrates. We tested this by loading GrnA into K562, HeLa, and Jurkat cells with a sublytic concentration of perforin, the CTL granule protein required for granzyme concentration in the nucleus (6, 12, 39) (Fig. 5*A*). In GrnA-loaded cells, but not in cells treated with GrnA or perforin alone, histone H1 was completely degraded by 400 nM GrnA within 2 h. Moreover, histone H1 was not cleaved in cells loaded with either the enzymatically inactive Ser → Ala mutant GrnA or the homologous GrnB. In a dose response assay, histone H1 cleavage began to be detected at GrnA concentrations of 25–100 nM and within 30–60 min of loading GrnA. Although GrnA loading mimicked the conditions of CTL lysis, it is difficult to know what effective concentrations of perforin and GrnA are present in the synaptic cleft formed by the apposition of a CTL with its specifically recognized target cell. However, the concentration of GrnA, which induces histone H1 cleavage, was comparable with that which induces cell death measured by ^{51}Cr release assay (Fig. 5*B*) or single-stranded DNA damage measured by Klenow incorporation (13) (Fig. 5*C*). To determine whether GrnA digestion of the core histones is physiologically relevant, immunoblots of GrnA-loaded cells were also probed for histones H2B and H3 (Fig. 5*D*). Histone H2B and H3 were cleaved to ~16-kDa fragments with kinetics and concentration requirements similar to that for histone H1. Therefore, the core histones, like linker histone H1, are likely to be cleaved under physiological conditions of cytolysis induced by CTL. Although histone H1 digestion went to completion *in vivo*, the core histones were not completely degraded. Histone H1 and core histone cleavage after perforin loading of GrnA was specific. Some

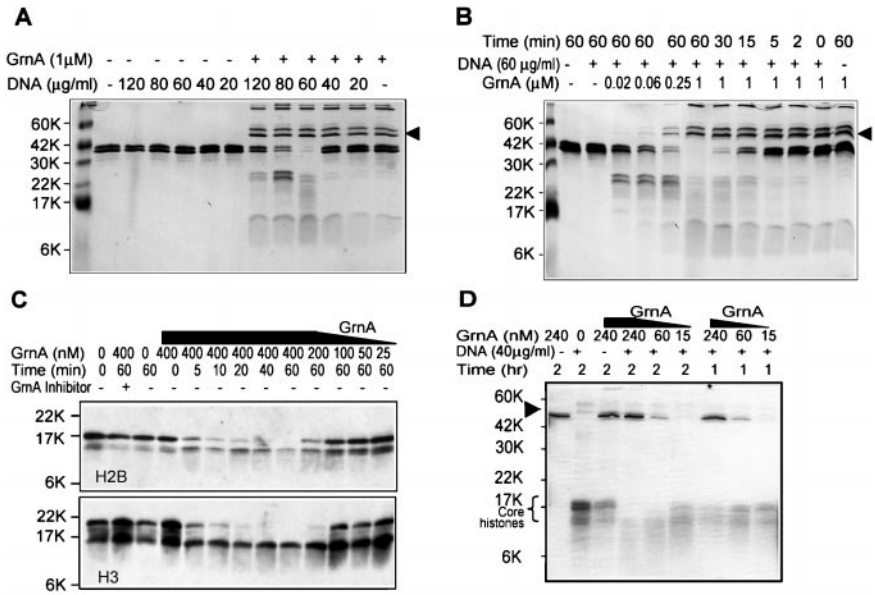

FIG. 4. *A* and *B*, DNA substitutes for HS or PHAP II in the *in vitro* cleavage reaction of purified histone H1. Purified histone H1 (1 μg) was digested in a 25-μl reaction volume, and the reaction was analyzed by GelCode® staining of SDS-PAGE gels. The reaction time for *A* was 1 h. *C*, core histones H2B and H3 in linker histone-stripped mononucleosomes are cut by GrnA. Samples analyzed by immunoblot. *D*, purified core histones (1 μg) were also digested by GrnA *in vitro*. The addition of DNA enhances the *in vitro* reaction. Core histones were visualized by GelCode® staining. The *first lane* contains no histones. The *closed arrows* in *A*, *B*, and *D* indicate GrnA. *K*, ×1000.

other protein substrates of the caspase pathway, such as Rho-GDI and the catalytic subunit of DNA-dependent protein kinase (data not shown), were not cleaved after GrnA loading but were cleaved after GrnB loading (not shown and Ref. 13). Moreover, histone H1 and the core histones were not degraded after loading GrnB or enzymatically inactive GrnA. Furthermore, GrnA cleavage of histones H1, H2B, and H3 was independent of caspase activation since it occurred in the presence of caspase blockade with z-VAD-FMK and z-DEVD-FMK (Fig. 5*D*). Histone H1 was also not degraded in Jurkat cells treated with anti-Fas antibody, an activator of the caspase pathway (data not shown).

GrnA Cuts Histone H1 in a Core Folded Region and Cleaves the N-terminal Tail of Histone H2B at Cleavage Sites Distinct from Trypsin—Although GrnA is a tryptase, it cleaved the arginine- and lysine-rich histones at specific sites that are different from the described previously trypsin sites (Fig. 6). The N-terminal sequence SLVSKGTLVQ of the GrnA-produced ~25-kDa histone H1 fragment places the GrnA cleavage after Lys-86–90 of the various H1 subtypes (GenBank™ accession numbers P10412 (histone H1.4), P16401 (H1.1), P16403 (H1.2), P16402 (H1.3) CAB11421 (H1.5), P22492 (H1T)). This is in a folded core region of histone H1 (amino acids 36–121) that is normally protected from trypsin digestion (36), supporting the notion that the histone H1 cleavage by GrnA is specific. The major GrnA cleavage band of purified core histones was also analyzed by N-terminal sequencing. The N-terminal sequence KGSKKAVTK of this band corresponds to cleavage after Lys-12 in the N-terminal tail of Histone H2B. This GrnA cleavage site also differs from the trypsin digests, which occur after Lys-21 and Lys-24 of H2B (44). Although the cleavage site is different, removal of part of the N-terminal tail of the core histones may have a similar effect as trypsin at unfolding chromatin. A minor component of protein in this band contained the N-terminal sequence of histone H3, suggesting that this histone may be cut by GrnA near the C terminus.

DISCUSSION

We have shown that GrnA, a key CTL and natural killer cell enzyme that induces cell death of virally infected or transformed cells, can disrupt chromatin and make it more susceptible to nuclease digestion. This may be due to the specific activity of this tryptase for arginine- and lysine-rich histones. Just as GrnA enhances DNA susceptibility to exogenous endonucleases, it is also likely to make DNA more susceptible to the caspase-activated DNase CAD triggered via GrnB during cell-mediated lysis.

GrnA cleavage of histones may be an important factor in the synergistic enhancement of DNA degradation by GrnA and -B (6, 7, 13). Although we have not seen evidence for histone degradation in apoptosis induced by GrnB loading or activation of the Fas pathway, other modifications of histones may also open up chromatin accessibility to the caspase-activated DNase. In fact, histone H1 is poly(ADP-ribosyl)ated during apoptosis induced by chemotherapeutic drugs or UV irradiation (45). Furthermore, inhibition of poly(ADP-ribosyl)ation in cells treated with these apoptosis inducers blocks both the enhanced accessibility of chromatin to exogenous DNases and internucleosomal DNA fragmentation caused by activation of endogenous DNases. A role for histone H1 interaction with CAD has also been suggested by the fact that histone H1 facilitates the DNase activity of CAD/DFF (46). It has also been suggested that chromatin with higher degrees of histone acetylation is more susceptible to apoptotic nucleases (58). Since DNA fragmentation is a hallmark of apoptosis, it should come as no surprise that the histone proteins that maintain chromatin in a condensed configuration and make it inaccessible need to be modified to allow access to endonucleases, just as they are modified to open up chromatin for transcriptional activation.

The compact structure of DNA complexed to core histones and hypercondensation induced by histone H1 (35–37) may protect genomic DNA from endonuclease digestion. When chromatin is stripped of linker histones (of which histone H1 is the predominant example), chromatin is released from a compacted solenoid to an extended state. When mononucleosomes stripped of linker histones are then treated with trypsin, the N-terminal highly basic tails of the core histones, which are the sites of histone acetylation for transcriptional regulation, are removed, leaving fragments similar in size to what we observe with GrnA treatment (44). The basic tails have little to do with the formation of the nucleosome core but are important in stabilizing the chromatin solenoid (47). Trypsinized polynucleosomes unfold into a more extended structure and are degraded more rapidly by exogenous nucleases than their untreated counterparts (47). Tryptic digestion of chromatin cleaves small N-terminal peptides of at most 27 amino acids in length from the free N-terminal tails of histones H2A, 2B, 3, and 4 and even smaller fragments (10 or fewer amino acids) from the C termini of only histones H2A and H3 (44, 48–50).

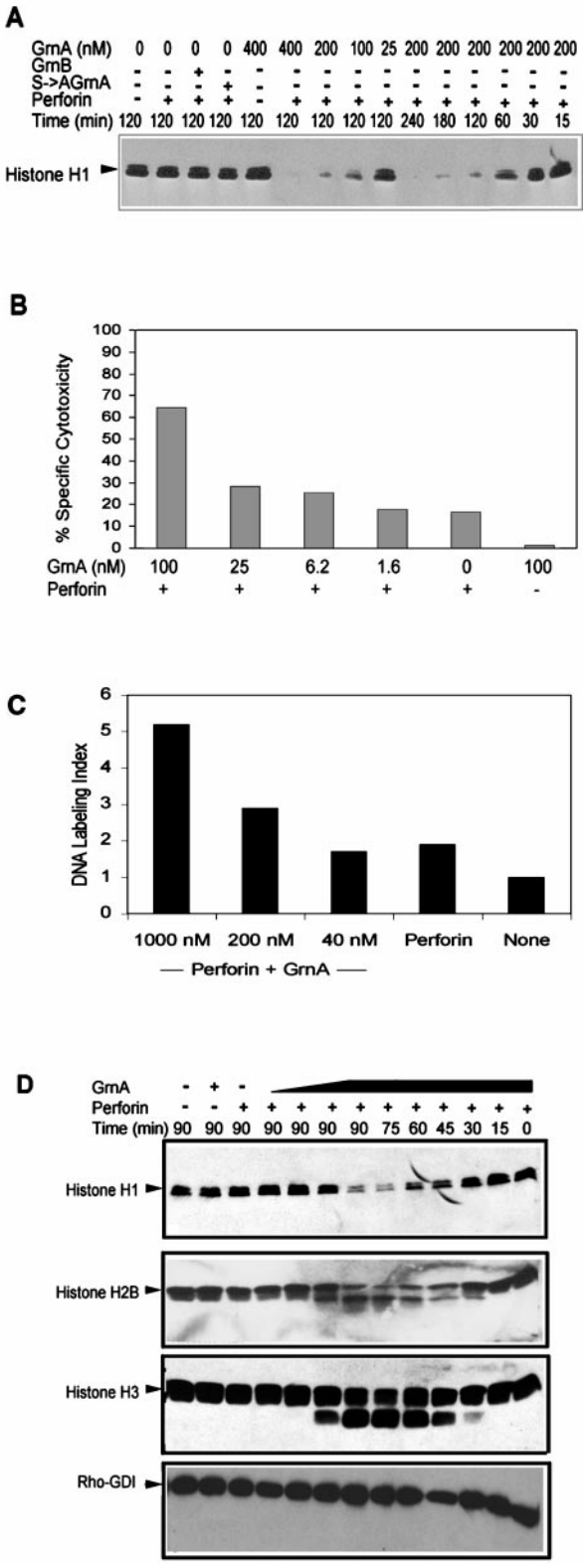

FIG. 5. **GrnA loading of K562 cells with perforin induces cleavage of histone H1 and core histones, even in the presence of caspase blockade.** *A*, GrnA, but not GrnB or Ser → Ala GrnA, loading induces histone H1 degradation. Granzymes were loaded with a sublytic concentration of perforin into K562 cells. The granzyme loading was performed by incubation at 37 °C for 2 h with indicated concentrations of GrnA or 400 nM Ser → Ala GrnA or 500 nM GrnB or by incubation for indicated times with 200 nM GrnA. *B*, dose response for cytotoxicity induced by GrnA loading, measured by a 2 h ^{51}Cr release assay, shows that the dose of GrnA required for cutting histone H1 parallels that for induction of cell death. *C*, dose response for single-stranded nicking induced by GrnA loading, measured by Klenow incorporation 6 h after loading, shows that the dose of GrnA required for cutting histone H1 parallels that for DNA damage. The DNA-labeling index compares the amount of labeling induced after treatment compared with mock-treated cells. *D*, GrnA loading leads to cleavage of histone H1 and core histones H2B and H3 in the presence of complete caspase blockade with z-DEVD-FMK and z-VAD-FMK. Blots of control samples, performed in parallel but not treated with caspase inhibitors, are indistinguishable (not shown). K562 cells were preincubated for 1 h with caspase inhibitors before perforin-loading GrnA (200 nM and 2-fold serial dilutions) for the indicated times. Cell lysates were analyzed by immunoblot for the indicated proteins. The caspase substrate Rho-GDI was not cleaved. *S*→AGrnA, Ser → Ala GrnA.

This digestion leaves intact the central nucleosome core. Although GrnA cleavage of the core histones differs in detail from the action of trypsin, both enzymes cleave fragments at the free termini, leaving the core of the core histones intact. The net effect of removing the basic tails of the core histones on chromatin structure may be similar. However, structural studies of GrnA-treated chromatin are required to prove this. In light of our finding, it is interesting that loading trypsin into cells induces all the nuclear features of apoptosis, including oligonucleosomal DNA fragmentation (51).

An indirect piece of evidence of histone modification during apoptosis comes from the prevalence of anti-histone antibodies in mice and humans with systemic lupus erythematosis (52). Recent studies suggest that many autoantigens are directed at proteins modified during apoptosis (53). In fact lupus autoantibodies recognize histone H1 and the N-terminal released fragments of the core histones, which are not the major antigenic regions when whole core histones are injected into animals (54).

Although initial *in vitro* studies suggested that proteoglycan was required for histone H1 cleavage by GrnA,[3] we found that a variety of negatively charged molecules (PHAP I, PHAP II, and DNA) could facilitate the *in vitro* reaction. Moreover, loading free GrnA into intact cells or isolated nuclei efficiently led to histone cleavage, which suggests that DNA provides the balancing negative charge *in vivo*. The granzymes are released from cytolytic granules complexed to serglycans, which may help enhance *in vivo* stability (55).[3] It is unknown whether the granzymes dissociate from the proteoglycan complex before uptake into the target cell, in an endocytic vesicle during uptake, (56) or sometime thereafter. A recent study showed that proteoglycan-complexed GrnB was less active than free GrnB in cleaving caspases-3 and -7 *in vitro* but was comparable in caspase activation when loaded into intact cells (55). This suggests that the granzyme-proteoglycan complexes probably dissociate before the granzymes encounter their cytosolic or nuclear substrates.

It is intriguing that all the substrates of GrnA that we have identified (PHAP II, histone H1, and core histones) are known to associate with and modify chromatin structure. However, it is unclear what the role of PHAP II is in making DNA more accessible for degradation or in the histone digestion we describe here. PHAP II was first identified as a translocated gene (*set*) linked to the nucleoporin *can* in undifferentiated leukemia (29). It was postulated that the nucleoporin linkage facilitated the nuclear localization of the set protein (57). The nucleosome assembly protein function of set/PHAP II (32) could then function to unwind as well as assemble chromatin, thereby enhancing transcription for leukemogenesis. We found that PHAP II also translocates to the nucleus of target cells during CTL lysis.[4] Similarly, we postulated that nucleosome disassembly by PHAP II might be the first step for DNA degradation. However, the addition of recombinant PHAP II with GrnA to isolated nuclei does not enhance endonuclease digestion compared with adding GrnA alone. Moreover, we also found that GrnA cleavage of PHAP II disrupts its nucleosome assembly

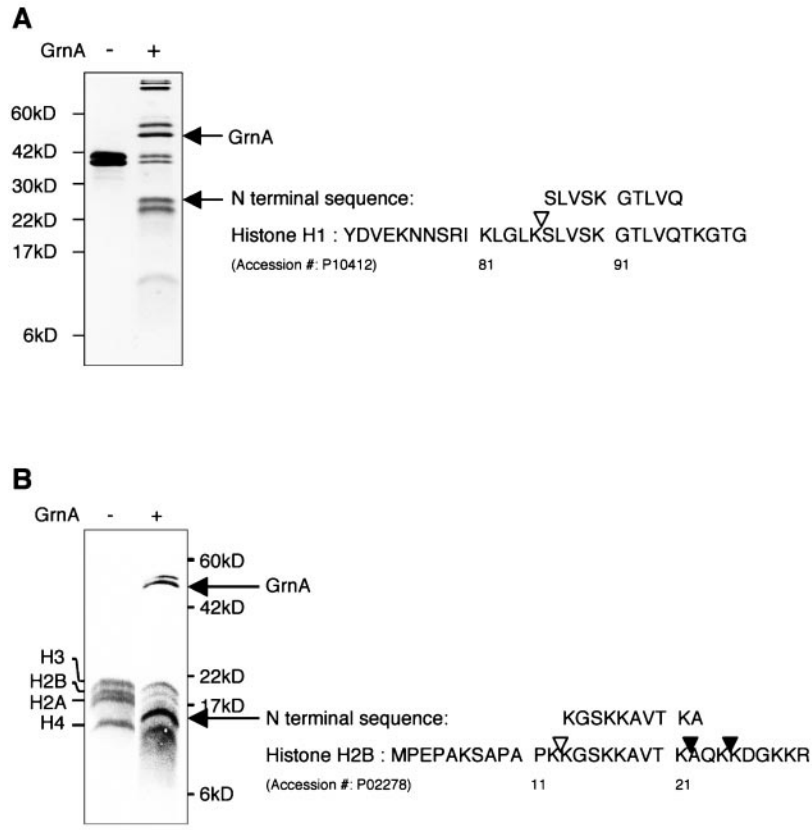

FIG. 6. **GrnA cleaves histone H1 and H2B at different sites than trypsin.** *A*, the N-terminal sequence of the initial cleavage product of histone H1 identifies the GrnA cleavage site after Lys-85. This is in a core region of histone H1 (amino acids 36–121) that is protected from trypsin digestion (36). The doublet visualized in the uncleaved and cleaved histone H1 bands corresponds to post-translational modifications of histone H1. *B*, when purified core histones are digested by GrnA in the presence of plasmid DNA, N-terminal sequencing of the dominant band demonstrates GrnA cleavage of histone H2B after Lys-12, as indicated by the *open arrow*. The *closed arrows* identify the previously reported trypsin cleavage sites (44).

function.[4] This makes this hypothesis for PHAP II function in GrnA-mediated cell death less likely.

GrnA degradation of histones and subsequent enhancement of chromatin sensitivity to DNases is likely to be an important component of GrnA-induced DNA fragmentation and the synergistic induction of DNA fragmentation by the two predominant granzymes (6, 7, 13). Post-translational modifications of the histones, which modulate chromatin structure and DNA accessibility for transcription, do not appear to grossly alter histone susceptibility to GrnA cleavage, but this needs to be explored further.

Acknowledgments—We thank J. Powers for the GrnA inhibitor and R. Kingston, J. Th'ng and F. Rosen for useful suggestions.

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