Purification of Three Cytotoxic Lymphocyte Granule Serine Proteases That Induce Apoptosis through Distinct Substrate and Target Cell Interactions

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Summary

We recently reported the purification of a lymphocyte granule protein called "fragmentin," which was identified as a serine protease with the ability to induce oligonucleosomal DNA fragmentation and apoptosis (Shi, L., R. P. Kraut, R. Aebersold, and A. H. Greenberg. 1992. *J. Exp. Med.* 175:553). We have now purified two additional proteases with fragmentin activity from lymphocyte granules. The three proteases are of two types; one has the unusual ability to cleave a tripeptide thiobenzyl ester substrate after aspartic acid, similar to murine cytotoxic cell protease I/granzyme B, while two are tryptase-like, preferentially hydrolyzing after arginine, and bear some homology to human T cell granule tryptases, granzyme 3, and Hanukah factor/granzyme A. Using tripeptide chloromethyl ketones, the pattern of inhibition of DNA fragmentation corresponded to the inhibition of peptide hydrolysis. The Asp-ase fragmentin was blocked by aspartic acid–containing tripeptide chloromethyl ketones, while the tryptase fragmentins were inhibited by arginine-containing chloromethyl ketones. The two tryptase fragmentins were slow acting and were partly suppressed by blocking proteins synthesis with cycloheximide in the YAC-1 target cell. In contrast, the Asp-ase fragmentin was fast acting and produced DNA damage in the absence of protein synthesis. Using a panel of unrelated target cells of lymphoma, thymoma, and melanoma origin, distinct patterns of sensitivity to the three fragmentins were observed. Thus, these three granule proteases make up a family of fragmentins that activate DNA fragmentation and apoptosis by acting on unique substrates in different target cells.

The molecular mediators of lymphocyte-induced cytolysis are thought to reside within the cytoplasmic granules of CTL and NK cells (1–4). The delivery of these molecules to the plasma membrane of the target cells occurs by exocytosis (5) and has been detected by the release of granule constituents into the intercellular space between the killer cell and target (6, 7). The destruction of the target cells appears to be of two distinct forms: the dissolution of the plasma membrane and apoptosis, including the characteristic cleavage of DNA into oligonucleosomal fragments (for review see reference 8). One of the constituents of the cytoplasmic granules is the pore-forming protein called either perforin in the T cell (3) or cytolysin in the NK cell (2), which is capable of disrupting the target cell membrane but is unable to produce the DNA damage identified after CTL- and NK cell–mediated killing (1, 8, 9). In addition to perforin/cytolysin, a subfamily of serine proteases has been identified in lymphocytes (10, 11) that is stored in cytoplasmic granules (12, 13). Exocytosis of granules results in the release of serine proteases into the extracellular environment (14). The lymphocyte serine proteases, sometimes called "granzymes," have been identified in human (15–17), murine (10, 18, 19), and rat (20) homologues, with as many as seven distinct genes now identified in mice and three in humans. The specificity of only two gene products has been determined. Granzyme A/Hanukah factor (HF)[1] was originally discovered by its ability to cleave the Nα-CBZ-L-lysine thiobenzyl ester (BLT) substrate (14), and was subsequently identified as a tryptase, while murine cytotoxic protease I (CCPI)/granzyme B was found to be an asp-ase (21–23). Other protease activities have been detected in the rat NK granules, although the proteins mediating the hydrolysis have not been identified (24).

In an earlier publication, we reported the purification of a granule protein called fragmentin, which, in the presence

[1] *Abbreviations used in this paper:* BLT, Nα-CBZ-L-lysine thiobenzyl ester; CCPI, murine cytotoxic cell protease I; HF, Hanukah factor.

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of cytolysin/perforin, was capable of rapidly inducing DNA fragmentation and apoptosis in intact target cells (1). Amino acid sequencing of internal peptides indicated that the protein was similar to the rat natural killer protease 1 (RNKP-1), which is likely the species homologue of murine CCPI/granzyme B (1). This observation suggested that granule serine proteases may play a direct role in mediating cell-mediated cytotoxicity and apoptosis. In the present study, we have identified two additional lymphocyte granule proteases that can induce target cells to undergo DNA fragmentation with different efficiencies and through unique substrate interactions. Of the three proteases, one is fast acting and induces DNA damage through its asp-ase activity, while the other two are slow-acting tryptases. The asp-ase was the protease showing homology to RNKP-1 and CCPI/granzyme B (1), while one tryptase reported in this paper resembled HF/granzyme A (15, 16, 19) and the second tryptase was homologous to human granzyme 3 (17).

Materials and Methods

Cells. The RNK-16 tumor cell line used as the source of granules has been described previously (1). It was serially passaged intraperitoneally in pristine-primed F344 rats (Fisher Scientific Co., Pittsburgh, PA). YAC-1 T cell lymphoma and A1.1 thymoma target cells were maintained in RPMI 1640 and B16 melanoma in α-MEM supplemented with 10% FCS, penicillin-streptomycin, and 2 mM L-glutamine.

Purification of Fragmentin Proteins. The purifications of the 31–32-kD fragmentin 2 and the 27-kD BLT-esterase, now called fragmentin 3, have been described in detail (1). Fragmentin 1 was purified from the same granule preparation. The mono-Q fractions were screened for both ^{125}I-UdR release activity in a 2-h cytotoxicity assay in the presence of a constant amount of cytolysin, and for BLT-esterase activity. Fractions containing cytotoxic activity were pooled and used for purification of fragmentins 2 and 3. The remaining fractions containing BLT-esterase activity were pooled and concentrated with a Centricon 30 microconcentrator (Amicon Corp., Danvers, MA) to a final volume of 1 ml, then diluted to 5 ml in 20 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 0.02% NaN_3 (TEB) to give a final NaCl concentration of 0.1 M. This was applied to a 1-ml Heparin Hi-trap (Pharmacia LKB Biotechnology, Piscataway, NJ) connected to the FPLC system. Fractions were eluted with a linear gradient of 0–1 M NaCl in TBE for 100 min, at a flow rate of 0.2 ml/min, and 1-ml fractions were collected and screened in the BLT-esterase assay. The fractions containing BLT-esterase activity were pooled, concentrated, and buffer changed to 10 mM bis-Tris, pH 6.0, containing 50 mM NaCl (BT), using a Centricon 30 microconcentrator (Amicon Corp.). This pool in a 12-ml volume was applied to mono-S beads (HR 5/5) for 12 min, and the column was washed with BT for 8 min. Fractions were eluted with two gradients, 5 min of 0–0.5 M NaCl and 20 min of 0.5–1 M NaCl in BT. The flow rate was 1 ml/min, and 1-ml fractions were collected and screened with the BLT-esterase assay. The purity of each fraction from the peak of BLT-esterase was assessed by SDS-PAGE under reducing and nonreducing conditions.

Purification of Cytolysin. The method for purification of cytolysin has been described previously (1).

Assay of DNA Damage. Tumor cells were labeled as described by Shi et al. (1). Briefly, 5–10 μCi ^{125}I-UdR was added to 2–5 $\times$ 10^6 YAC-1 cells in 0.5 ml supplemented RPMI 1640. The cells were incubated at 37°C in a 5% CO_2 atmosphere for 90 min, washed twice in HBSS, incubated an additional 30 min, then washed once and resuspended at 1.25 $\times$ 10^5 cell/ml in HBSS containing 2 mM $CaCl_2$, 10 mM Hepes, 4 mg/ml BSA, pH 7.2 (HH-BSA). Tumor cells (10^4 cells in 80 μl) in HH-BSA and 80 μl of test protein in HE (10 mM Hepes, pH 7.2 containing 140 mM NaCl and 1 mM EGTA) were incubated in 96-well V-bottomed or flat-bottomed microtiter plates at 37°C for 2 h or as indicated. Then, 40 μl of 1% Triton X-100 in 50 mM EGTA and 50 mM Tris-HCl, pH 7.2, was added to the reaction well. The contents were mixed, and the plates centrifuged at 800 *g* for 5 min. Half of the supernatant was harvested from each well and soluble ^{125}I-UdR was determined in a Compugamma universal gamma counter (1282; Wallac LKB). The specific isotope release was determined as described (1).

In assays using protease inhibitors, inhibitors were dissolved in DMSO at a concentration of 200 mM and stored at 4°C. Inhibitors were diluted in HE medium and incubated with a constant amount of fragmentin or cytolysin at room temperature for 15 min in the assay wells, then target cells were added. The tripeptide chloromethyl ketones were synthesized as described previously (25).

In assays using cycloheximide (Sigma Chemical Co., St. Louis, MO), the drug was freshly prepared in HH-BSA at a concentration of 50 μg/ml and incubated with ^{125}I-UdR-labeled YAC-1 cells for 2 h at 37°C before the cytotoxicity assay.

SDS-PAGE. Proteins were run on 6-cm 12% gels according to Laemmli (26). An additional 1 cm of 4% gel was added over the running gel to resolve high molecular mass bands. Gels were developed with a commercial silver stain kit (Bio-Rad Laboratories, Richmond, CA).

NH_2-terminal Amino Acid Sequencing of Fragmentin 3. For NH_2-terminal sequence analysis, proteins were separated by PAGE, electroblotted onto Immobilone P membrane (Millipore Continental Water Systems, Bedford, MA), and detected by coomassie blue staining as described (27). Selected bands were cut out and sequenced in a protein sequencer (477A; Applied Biosystems, Inc., Foster City, CA) using standard protocols. Resulting phenylthiohydantoin derivatives were separated in a HPLC system (120A; Applied Biosystems, Inc.) and identified by visual inspection.

Substrate Kinetics. The enzymatic hydrolysis of peptide thioester substrates by fragmentins 2 (batches 1 and 2) and 3 were measured in 0.1 M Hepes, 0.5 M NaCl, pH 7.5, buffer containing 8% Me_2SO and at 25°C in the presence of 4,4′-dithiodipyridine (PDS) (27), and hydrolysis rates of thioester substrates by fragmentins 1 and 2 (batch 3) were measured in 0.1 M Hepes, 0.5 M NaCl, 1 mM EDTA, pH 7.5, buffer containing 13% Me_2SO and at 21°C in the presence of 5,5′-dithio-bis(2-nitrobenzoic acid) (DNTB) (28, 29). Stock solution of substrate was prepared in Me_2SO and stored at −20°C. For fragmentins 2 and 3, the initial rates were measured at 324 nm (ϵ_{324} = 19800 M^{-1}/cm^{-1}) using a spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) when 5 or 10 μl of an enzyme stock solution was added to a cuvette containing 2.0 ml of buffer, 0.15 ml of 5 mM PDS, and 25 μl of substrate. The same volumes of substrate and PDS were added to the reference cell to compensate for the background hydrolysis rate of the substrates. For fragmentins 1 and 2, the initial rates were measured at 405 nm (ϵ_{405} = 14,000 M^{-1}/cm^{-1}) using a microplate reader (Termomax) when 2 μl of an enzyme stock solution was added to a well containing 0.2 ml of buffer, 0.02 ml of 5 mM DTNB, and 10 μl of substrate. The same volumes of substrate and DTNB were added to the blank well to compensate for the background hydrolysis rate of the substrates. Initial rates were measured in duplicate for each substrate concentration and were averaged in each case.

Inhibition Kinetics: Incubation Method. The inactivation reaction was initiated at 21°C by adding a 10-μl aliquot of inhibitor in Me_2SO; concentration was 8%. Aliquots were removed at various time intervals, diluted into substrate solution (90-fold dilution), and the residual activity was measured spectrophotometrically with a microplate reader. Fragmentins 1 and 3 were assayed with Z-Arg-SBzl, and fragmentin 2 was assayed with Boc-Ala-Ala-Asp-SBzl. First-order inactivation rate constants (k_{obs}) were obtained from plots in In substrate hydrolysis rate at time t/substrate hydrolysis rate at time 0 vs. time. Several inhibitors did not show any inhibition toward fragmentins after a 10-min incubation of enzyme with inhibitor.

Results

Identification of Three Granule Proteases (Fragmentins) with DNA Fragmenting Activity. The 31–32-kD fragmentin described in our earlier publication, along with a 27-kD BLT-esterase, were prepared by FPLC chromatography (1) and identified as single bands on SDS-PAGE under reducing and nonreducing conditions (Fig. 1, *A*, *B*, *D*, and *E*). While the 27-kD BLT-esterase was highly abundant, we noted a second smaller BLT-esterase peak on Mono-Q chromatography (Fig. 2 *A*), which had been excluded from the first purification protocol, and subjected this peak to sequential heparin agarose (Fig. 2 *B*) and Mono-S (Fig. 2 *C*) columns, as described in Materials and Methods. The Mono-S protein peak containing this second BLT-esterase ran as a single band at ~47 kD under nonreducing conditions on SDS-PAGE and at 30 kD after reduction (Fig. 1, *C* and *F*). The molecular mass was similar to human HF/granzyme A (15, 17), which is also a BLT-esterase that runs as a 43-kD homodimer and a 30-kD monomer under nonreducing and reducing conditions, respectively. This distinguishes it from the more abundant BLT-esterase, which ran at 27 kD under nonreducing conditions (Fig. 1 *B*).

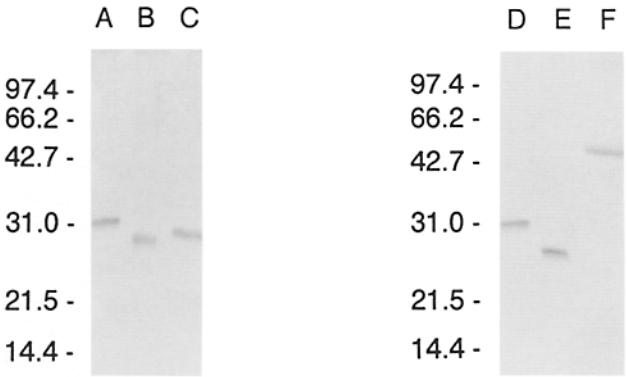

Figure 1. SDS-PAGE of purified lymphocyte granule proteases. FPLC-purified proteases were loaded on a 12% gel run according to Laemmli (26) under reducing (lanes *A–C*) and nonreducing conditions (lanes *D–F*), then silver stained. Lanes *A* and *D* contain 0.6 μg of the 31–32-kD fragmentin protein (1), lanes *B* and *E* contain 0.6 μg of the major BLT-esterase that copurified with fragmentin (1), and lanes *C* and *F* contain 0.16 μg of the second minor BLT-esterase purified from the same granules as described in Materials and Methods and shown in Fig. 2. Molecular mass markers are shown to the left of the stained gels.

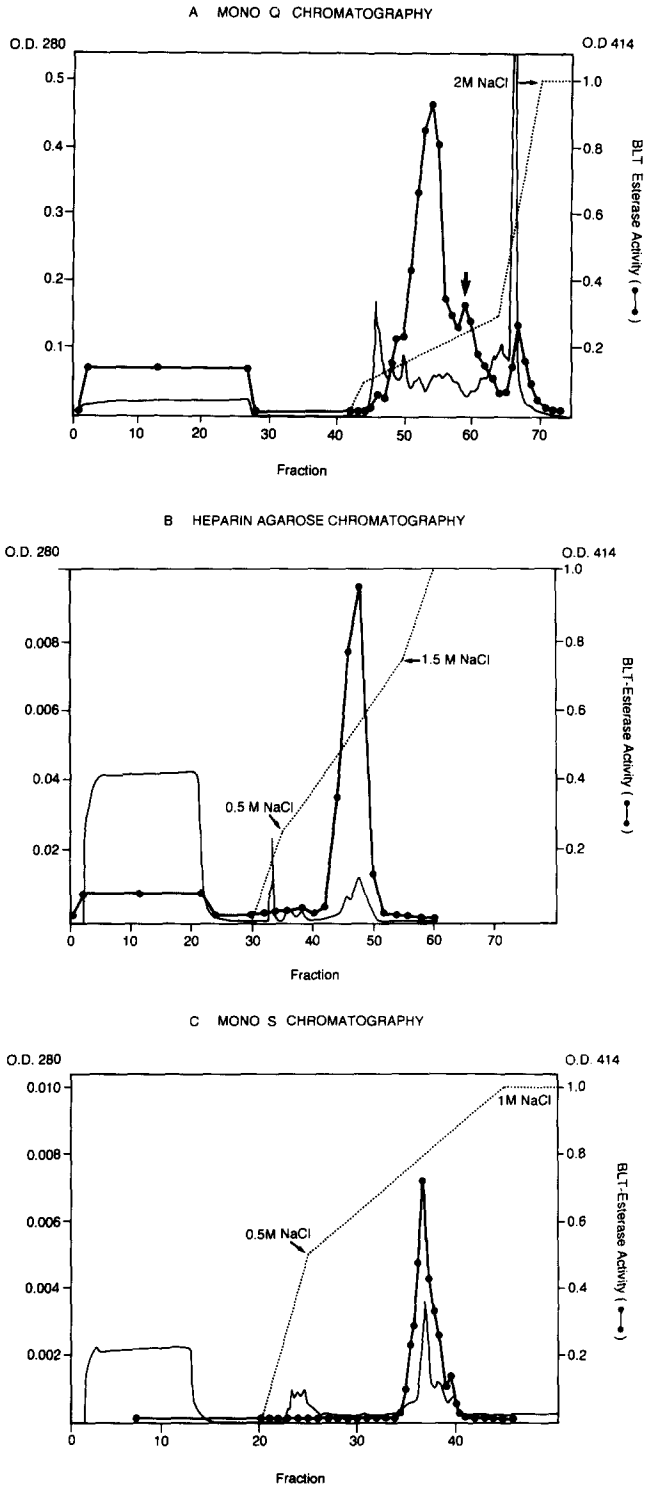

Figure 2. Purification of an RNK granule BLT-esterase. After an initial step of phenyl superose chromatography to remove cytolysin (see Materials and Methods), fractions containing BLT-esterase activity were applied to a Mono-Q column (*A*). Two BLT-esterase peaks were identified. The fractions containing the minor peak (*arrow*) were pooled, concentrated, and applied to a heparin agarose column (*B*). Fractions containing the peak of BLT-esterase were pooled, concentrated, and buffer changed to 10 mM bis-Tris, pH 6.0, containing 50 mM NaCl, and applied to Mono-S beads (*C*). Fractions were eluted, and the BLT-esterase peak was concentrated, desalted, and run on SDS-PAGE (see Fig. 1, *C* and *F*).

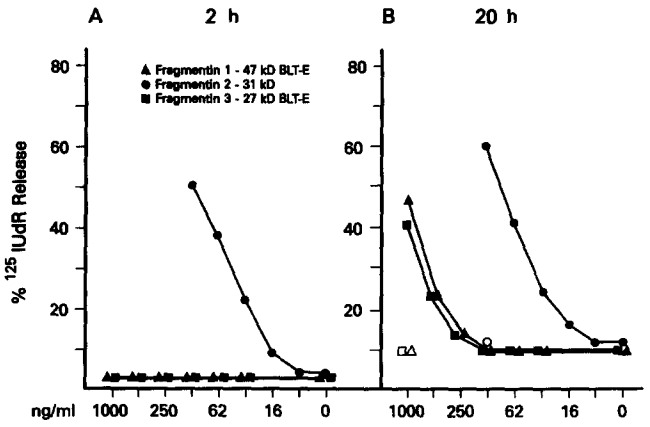

Figure 3. DNA fragmentation of YAC-1 cells by purified granule proteases. Fragmentins 1 (▲), 2 (●), and 3 (■) were examined in a 2-h (*A*) and 20-h (*B*) cytotoxicity assay. None of the proteases were active in the absence of cytolysin (*open symbols*).

	1	2	3	4	5	6	7	8	9	10	11
FRAGMENTIN 3	Xxx	Ile	Gly	Gly	Xxx	Glu	Val	Gln	Pro	His	Ser
HuGRANZYME 3	Ile	*	*	*	Lys	*	*	Ser	*	*	*
HuGRANZYME A	Ile	*	*	*	Asn	*	*	Thr	*	*	*
MuGRANZYME A	Ile	*	*	*	Asp	Thr	*	Val	*	*	*

	12	13	14	15	16	17	18	19	20	21	22
FRAGMENTIN 3	Xxx	Pro	Phe	Met	Ala	Ala	Ile	Gln	Tyr	Xxx	Gly
HuGRANZYME 3	Arg	*	*	*	*	*					
HuGRANZYME A	Arg	*	Tyr	*	Val	Leu	Leu	Ser	Leu	Asp	Arg
MuGRANZYME A	Arg	*	Tyr	*	*	Leu	Leu	Lys	Leu	Leu	Ser

Figure 4. NH_2-terminal amino acid sequence of the 27-kD BLT-esterase fragmentin 3. The sequence is compared to the other known BLT-esterases, granzyme 3 (17), and human and murine Hanukah factor/granzyme A (15, 16, 19).

All three proteins were able to induce DNA fragmentation of YAC-1 cells (Fig. 3). However, after a 2-h incubation, DNA damage of YAC-1 cells was only observed with the 31-kD fragmentin in the presence of a constant amount of cytolysin (Fig. 3 *A*). By 20 h, both the 47- and 27-kD BLT-esterases induced significant DNA damage, but only when cytolysin was present and at a specific activity that was lower than the 31-kD fragmentin (Fig. 3 *B*). Thus, two additional fragmentins with considerably slower kinetics and lower specific activity are present in the RNK granule. We have named these three proteins fragmentin 1 (47 kD, unreduced apparent molecular mass), fragmentin 2 (31 kD), and fragmentin 3 (27 kD) in our further discussions.

We sequenced the NH_2-terminal amino acids of the 27-kD BLT-esterase fragmentin 3 to further identify this protein. The sequence contained the conserved regions of the granzyme family and was most homologous to human granzyme 3 (17), which is one of only two known granule BLT-esterases (Fig. 4). It differed significantly from the other BLT-esterase, human and murine HF/granzyme A (Fig. 4), particularly in

Table 1. *Enzymatic Hydrolysis Rate of Peptide Thiobenzyl Esters by Fragmentins*

		Hydrolysis rate		
Substrates	[S]	Fragmentin 1*	Fragmentin 2‡	Fragmentin 3‡
	μM		*nM/s*	
Boc-Ala-Ala-Asp-SBzl	115	0.6	51	0
	211		27.2*	
Boc-Ala-Ala-Met-SBzl	116	0	40	0
	144		4.7*	
Boc-Ala-Ala-Ser-SBzl	115			0
	133	0	8.4	
Boc-Ala-Ala-Asn-SBzl	144		4.2	
Boc-Ala-Ala-Glu-SBzl			0	
Suc-Phe-Leu-Phe-SBzl	115	0.1	0.8	0
Z-Arg-SBzl	106	135		
	115		8.2	
	131		3.0*	11.7

* Hydrolysis rates were measured in 0.1 M Hepes, 0.5 M NaCl, 1 mM EDTA, pH 7.5, and 13% Me_2SO at 21°C. An aliquot of 2 μl of the enzyme was added to the assay mixture containing 0.2 ml of buffer, 0.02 ml of 5 mM DTNB, and 10 μl of substrate. Enzyme concentrations were: fragmentin 1, 1.7 nM; fragmentin 2, 8.4 nM (batch 2).

‡ Hydrolysis rates were measured in 0.1 M Hepes, 0.5 M NaCl, pH 7.5, and 8% Me_2SO at 25°C. An aliquot of 5 or 10 μl of the enzyme was added to the assay mixture containing 2 ml of buffer, 0.15 ml of 5 mM PDS, and 25 μl of substrate. Enzyme concentrations were: fragmentin 2, 7.3 nM (batch 1); fragmentin 3, 6.2 nM.

Table 2. *Inhibition of DNA Fragmentation (ID_{50}) by Chloromethyl Ketones*

	Molecular mass (nonreduced)	Chloromethyl ketones*				
		Asp	Nle	Phe	Arg	Trp
	kD			*μM*		
Fragmentin 1	47	>250	NT‡	NT	0.25	NT
Fragmentin 2	31	0.3	50	22	155	12
Fragmentin 3	27	>250	>250	125	0.18	15

* Amino acid refers to the third position in a tripeptide chloromethyl ketone. Asp, Boc-Ala-Ala-Asp-CH_2Cl; Nle, Boc-Ala-Ala-Nle-CH_2Cl; Phe, Boc-Gly-Leu-Phe-CH_2Cl; Arg, D-Phe-Pro-Arg-CH_2Cl; Trp, Z-Trp-CH_2Cl.
‡ NT, not tested.

position 14, which is a conserved tyrosine, and positions 17, 18, and 20, which are conserved leucines.

Substrate and Inhibitor Studies of the Fragmentins. To identify the specificity of peptide hydrolysis by fragmentins 1, 2, and 3, we utilized thiobenzyl ester substrates (Table 1). Fragmentin 1 had high enzymatic activity toward Z-Arg-SBzl, but had very low or no activity toward other substrates. Fragmentin 2 had primarily Boc-Ala-Ala-Asp-SBzl esterase activity, but contained Boc-Ala-Ala-Met-SBzl activity in some enzyme batches. Fragmentin 2 also slowly hydrolyzed Ser-, Asn-, Phe-, and Arg-containing substrates. Fragmentin 3, like fragmentin 1, was active toward Z-Arg-SBzl, but had no activity against other thioester substrates. The asp-ase activity of fragmentin 2 was not enhanced by fragmentin 3. Fragmentin 2 (23 nM) was incubated with fragmentin 3 (52 nM) for 0.5, 10, and 30 min; the asp-ase activity was not increased when compared to fragmentin 2 in buffer for the same period of time. Similarly, the tryptase activity of fragmentin 3 was not enhanced by fragmentin 2. Fragmentin 3 (52 nM) was incubated with fragmentin 2 (23 nM) for 0.5, 10, and 30 min, and the tryptase activity was not greater than the sum of fragmentins 3 and 2 alone in buffer for the same period of time.

To identify the substrates that were important for DNA fragmentation, a series of tripeptide chloromethyl ketones were tested as inhibitors. These compounds are able to form a tetrahedral complex with protease-active site residues and permanently inactivate serine proteases (30). By comparing chloromethyl ketones with various amino acid sequences, unique inhibitors were identified that have allowed us to distinguish the DNA fragmentation induced by these three fragmentin proteins (Table 2). The fast-acting fragmentin 2 was completely blocked by Boc-Ala-Ala-Asp-CH_2Cl (ID_{50}, 0.3 μM) but was poorly inhibited by the other chloromethyl ketones (ID_{50}, 12–155 μM). In contrast, the slow-acting fragmentins 1 and 3 were both highly inhibited by D-Phe-Pro-Arg-CH_2Cl (ID_{50}, >250 μM). Fragmentin 3 was not inhibited by Nle- or Phe-containing chloromethyl ketones, but was moderately inhibited by Z-Trp-CH_2Cl. The inhibition of enzymatic activities of fragmentins by peptide chloromethyl ketones was also examined (Table 3). Fragmentins 1 and 3, which have trypsin-like activity, were only inhibited by a potent thrombin inhibitor, D-Phe-Pro-Arg-CH_2Cl, but not inhibited by the other four chloromethyl ketones. Fragmentin 2, which contained asp-ase and met-ase activities, was inhibited by Boc-Ala-Ala-Asp-CH_2Cl and Z-Trp-CH_2Cl. Generally, the inhibition of enzymatic activities of these frag-

Table 3. *Inhibition of Fragmentins by Peptide Chloromethyl Ketones*

Inhibitor	[I]	$K_{obs}/[I]$		
		Fragmentin 1	Fragmentin 2	Fragmentin 3
	μM		*M/s*	
Boc-Ala-Ala-Asp-CH_2Cl	380–446	NI*	3.0	NI
Boc-Ala-Ala-Nle-CH_2Cl	405–439	NI	NI	NI
Boc-Gly-Leu-Phe-CH_2Cl	408–442	NI	NI	NI
D-Phe-Pro-Arg-CH_2Cl	400–430	9.0	NI	77
Z-Trp-CH_2Cl	385–410	NI	36	NI

Inhibition was measured in 0.1 M Hepes, 0.5 M NaCl, 1 mM EDTA, pH 7.5, and 8% Me_2SO at 21°C. Z-Arg-SBzl (120 μM), Boc-Ala-Ala-Asp-SBzl (210 μM), and Z-Arg-SBzl (300 μM) were used as the substrates for fragmentins 1, 2, and 3, respectively.
* No inhibition after 10 min of incubation of enzyme and inhibitor.

mentins by peptide chloromethyl ketones was consistent with the inhibition results of the DNA fragmentation.

Requirement for Protein Synthesis for DNA Damage by Fragmentins. The slow kinetics of DNA damage produced by the tryptase fragmentins 1 and 3 contrast sharply with the rapid action of the asp-ase fragmentin 2. Induction of DNA fragmentation and apoptosis by many agents is slow and requires protein synthesis (31, 32). We examined the relative DNA damage produced by the fragmentins, with or without pretreatment of YAC-1 cells with 50 μg/ml cycloheximide. The DNA damage produced by fragmentin 2 is unaffected by cycloheximide in either a rapid 2-h assay or after a longer 14-h incubation (Fig. 5). In contrast, fragmentin 1 and 3 activity in 14–18-h assays was reduced by 30–40% after cycloheximide treatment, indicating that at least part of their activity was dependent upon protein synthesis.

Differential Susceptibility of Target Cells to Granule Fragmentins. The existence of at least three granule fragmentins with unique substrate specificities suggested that they may induce DNA fragmentation at varying rates in different target cells. We have examined several target cells, including the YAC-1 lymphoma, A1.1 thymoma, and B16 melanoma, and found unique patterns of DNA fragmentation in each cell line when assessed after a 20-h assay (Fig. 6). YAC-1 tumor cells are preferentially damaged by fragmentin 2, while the A1.1 thymoma is barely affected by this protease. In contrast, fragmentin 1 efficiently induces DNA damage in the A1.1 thymoma, while being the least effective against the B16 melanoma. Fragmentin 3 is active against all of the tumor cell lines.

Discussion

In an earlier publication we described a rat lymphocyte granule protease that rapidly induced DNA fragmentation and apoptosis in YAC-1 cells in the presence of perforin/cytolysin (1). This molecule was homologous to the rat NK protease 1, and its activity could be blocked by the general serine protease inhibitor, 3,4-dichloroisocoumarin. In this manuscript, we have identified the protease as an asp-ase and describe two additional granule serine proteases with fragmentin activity that are tryptases. These proteases differ from the asp-ase fragmentin in their substrate specificity, inhibitory activity by chloromethyl ketones, kinetics of action, requirement for protein synthesis to mediate DNA fragmentation, and target cell preference. A summary of these characteristics is given in Table 4.

One of the tryptase fragmentins appeared to be a dimer that ran at 47 kD under nonreducing conditions, and at 30 kD as monomers under reducing conditions. This is similar to the molecular mass described for human granzyme A (15), but differs from the 60-kD dimeric BLT-esterase affinity purified from the RNK leukemia cell line, which was identified as granzyme A (4). The reason for this discrepancy is not immediately apparent. The second BLT-esterase we identified was 27 kD under nonreducing conditions, and had NH_2-terminal sequence homology to human granzyme 3 (17), the only other BLT-esterase that has been identified in lymphocyte granules. The 27-kD BLT-esterase was more abundant than the 47-kD granzyme A–like BLT-esterase in the RNK-16 granules. This is not the case for murine and human CTL granules, where granzyme A is the major BLT esterase (15, 17). It is not clear if this is a difference between NK cell and CTL lines, a species difference, or a peculiarity of the RNK-16 tumor. However, in the studies of substrate specificity and inhibition of qualitative activity of these proteins, we find little functional difference between them. The three proteases, therefore, fall into two groups based on substrate specificities. Fragmentins 1 and 3 are tryptases and preferential hydrolyze the Z-Lys-SBzl and Z-Arg-SBzl substrates, similar to granzyme A (21–23), and fragmentin 2 is an asp-ase, cleaving most efficiently Boc-Ala-Ala-Asp-SBzl and, to a lesser extent, the Boc-Ala-Ala-Met-SBzl substrate. Granzyme B also has a preference for the Ala-Ala-Asp-SBzl substrate with some activity against Ala-Ala-AA-SBzl where AA is either Met, Asn, or Ser (21). However, the possibility that fragmentin 2 is contaminated with a met-ase also exists due to the observation that met-ase activity varies between batches of purified enzyme (Table 1). The inhibitory activities of fragmentins by chloromethyl ketones using thioester substrates are generally consistent with the inhibition results of their DNA fragmentation. D-Phe-Pro-Arg-CH_2Cl was the only chloromethyl ketone that inhibited fragmentins 1 and 3, although the in-

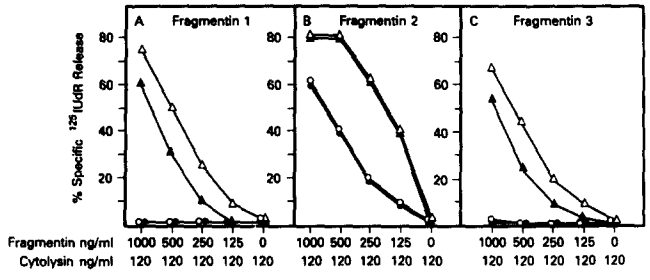

Figure 5. Requirement for protein synthesis to induce DNA damage by the fragmentins. DNA damage induced by fragmentins after treatment of YAC-1 cells with 50 μg/ml of cycloheximide (CHX) (*filled symbols*) or control medium (*open symbols*) in a 2-h (*circles*) or a 14–18-h (*triangles*) cytotoxicity assay. Inhibition of [^{3}H]methionine incorporation was 92% at 50 μg/ml CHX. In two experiments, the ID_{40} for fragmentin 1 was 37% and 32%, and fragmentin 3 was 44% and 31%, respectively.

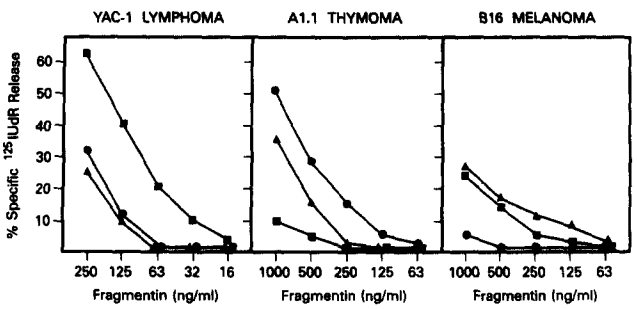

Figure 6. Differential susceptibility of target cells to fragmentins. Using a 20-h assay, fragmentins 1 (●), 2 (■), and 3 (▲) induced DNA damage in YAC-1 lymphoma, A1.1 thymoma, and B16 melanoma with varying efficiency.

Table 4. *Characteristics of Apoptosis-inducing Granule Proteases*

	Homologous granule serine protease	Molecular mass		Kinetics of DNA damage	Inhibition of DNA damage by cycloheximide	Inhibition of DNA damage by chloromethyl ketones	Peptide substrate for hydrolysis	Inhibition of peptide hydrolysis by chloromethyl ketones
		reduced	nonreduced					
		kD						
Fragmentin 1	HF/granzyme A	30	47	Slow	Partial	D-Phe-Pro-Arg-CH_2Cl	Z-Arg-SBzl	D-Phe-Pro-Arg-CH_2Cl
Fragmentin 2	RNKP-1/CCPI/ granzyme B	32	31	Fast	None	Boc-Ala-Ala-Asp-CH_2Cl	Boc-Ala-Ala-Asp-SBzl	Boc-Ala-Ala-Asp-CH_2Cl Z-Trp-CH_2Cl
Fragmentin 3	Granzyme 3	29	27	Slow	Partial	D-Phe-Pro-Arg-CH_2Cl	Z-Arg-SBzl	D-Phe-Pro-Arg-CH_2Cl

hibition rates were slow. Surprisingly, Z-Trp-CH_2Cl was a more potent inhibitor than Boc-Ala-Ala-Asp-CH_2Cl for fragmentin 2 on the inhibition of its enzymatic hydrolysis of Boc-Ala-Ala-Asp-SBzl. This contrasts with the preferential inhibition of DNA damage by Boc-Ala-Ala-Asp-CH_2Cl (Table 2). The reasons for this are unclear, but might reflect substrate availability in vivo, where the asp-ase activity would be more important for inducing DNA fragmentation.

The asp-ase and tryptase fragmentins differ considerably in their kinetics and specific activity. The asp-ase fragmentin 2 was very fast acting, producing DNA damage in <2 h (1), while fragmentins 1 and 3 required longer assays and much higher protein concentrations to be detected (1) (Fig. 3). The reasons for this difference may be partly explained by the observation that at least some of the fragmentin 1 and 3 activity required new protein synthesis. This is similar to apoptosis induced by agents such as anti-CD3 antibody (32) and glucocorticoids (31), where DNA damage can be abrogated by inhibitors of protein synthesis. Thus, both protein synthesis–dependent and –independent pathways of killing can be mediated by the same fragmentin protease. An explanation for this observation will need a better understanding of the molecular mechanisms of apoptosis.

One of the important questions that needs to be addressed is why more than one fragmentin exists in the lymphocyte granule. If these proteases mediate the apoptosis induced by NK cells and CTL, then a diversity of effector molecules must give some advantage in killing. Serine proteases have been shown to activate enzymes of the clotting cascade (33, 34), which might suggest that activating interactions may occur between fragmentins. However, we have been unable to demonstrate fragmentin 3 enhancement of fragmentin 2 hydrolysis of Boc-Ala-Ala-Asp-SBzl, or fragmentin 2 enhancement of fragmentin 3 hydrolysis of Z-Arg-SBzl. Another possibility is that the proteases cleave distinct proteins and each is capable of activating the apoptotic pathway independently, resulting in synergistic amplification of DNA damage in a target cell. This hypothesis is supported by our observation that fragmentin 3 synergistically amplifies DNA damage of YAC-1 cells by fragmentin 2 (1). Finally, since each fragmentin is apparently capable of acting as an effector molecule, then perhaps their ability to cleave different substrates will allow preferential activation of the apoptotic pathway in different target cells. This possibility was confirmed by identifying unique patterns of DNA damage induced by the three fragmentins in different target cells (Fig. 6). The differential ability to induce target cell damage by the fragmentins has potential implications for the interpretation of the specificity of killing by granulated cytotoxic lymphocytes. That is, susceptibility to killing may be determined not only by membrane receptor interactions, but by the susceptibility of the target cell to and the abundance of a given fragmentin in the cytotoxic cell granule.

In conclusion, we have identified a family of lymphocyte granule protease fragmentins that induce apoptosis through unique substrate and target cell interactions, and suggest that they are important mediators of lymphocyte cytotoxicity.

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