A Natural Killer Cell Granule Protein That Induces DNA Fragmentation and Apoptosis

By Lianfa Shi,* Ricky P. Kraut,* Ruedi Aebersold,‡ and Arnold H. Greenberg*

From the *Manitoba Institute of Cell Biology, Departments of Pediatrics and Immunology, University of Manitoba, Winnipeg, Manitoba, R3E 0V9; and the ‡Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, V6T 1W5 Canada

Summary

We report the purification from a rat natural killer (RNK) large granular lymphocyte leukemia of a 32-kD granule protein that induces rapid DNA fragmentation and apoptosis. The protein, which we have called "fragmentin," was capable of causing DNA from intact YAC-1 cells to be cleaved into oligonucleosomal-sized fragments and producing severe chromatin condensation within 1 h. Amino acid sequence of tryptic peptides indicated that fragmentin was highly homologous to the NK and T cell granule serine proteases RNK protease 1 and mouse cytotoxic T cell protease I (CCPI)/granzyme B. Preincubation with the serine esterase inhibitor 3,4-dichloroisocoumarin blocked fragmentin-induced DNA damage, but had no effect on cytolysin. Fragmentin activity against four lymphoma target cells was completely dependent on the presence of cytolysin. Fragmentin produced rapid membrane damage as well as DNA fragmentation at nonlytic cytolysin doses, suggesting that fragmentin activity was not limited to its effects on the nucleus. Fragmentin and cytolysin activity were completely inhibited by EGTA, indicating the process was Ca^{2+} dependent. A role for cytolysin in endocytosis of fragmentin was suggested by the observation that treatment of YAC-1 with cytochalasin B or sodium azide and 2-deoxyglucose blocked DNA fragmentation but not cytolysin activity. A 30-kD Nα-CBZ-L-lysine thiobenzyl esterase, which copurified with fragmentin, was inactive on its own but was able to synergistically amplify the DNA damage induced by fragmentin in the presence of cytolysin. Fragmentin activity was not dependent on protein synthesis, as cycloheximide treatment of YAC-1 cells did not prevent DNA damage. We postulate that fragmentin is the molecular mediator of NK cell–mediated DNA fragmentation and apoptosis.

Previous studies have shown that during CTL- and NK cell–mediated killing, the DNA of target cells undergoes rapid breakdown into oligonucleosomal fragments and cells die with morphological changes typical of apoptosis (1–3). No single molecule produced by these cells can account for this form of cell death. Granule exocytosis is a mechanism that has been proposed for the delivery of the lethal hit by NK cells and activated CTL (4, 5). Among the various granule components that have been identified and molecularly cloned are the homologous pore-forming proteins perforin of CTL (6) and cytolysin of NK cells (7). Perforin and cytolysin form transmembrane ionic pores that produce membrane damage (6–8) but do not cause DNA fragmentation (9). The existence of some other granule components that might play a role in target cell DNA damage has been suggested. However, investigations of the DNA-fragmenting ability of isolated granules from CTL lines and NK cells have given conflicting results (10–14). We report here the purification

from the granules of a rat NK (RNK)[1] LGL leukemia of a 32-kD protein that induces very rapid DNA fragmentation with nuclear collapse and apoptosis in tumor target cells. We have named the protein "fragmentin."

Materials and Methods

Cells. The RNK-16 tumor cell line, used as a source of granules, has been described previously (15). It was serially passaged intraperitoneally in pristine-primed Fischer (F344) rats. The murine T cell lymphoma target cells, YAC-1, SL2, EL4, and L5178Y, were

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[1] *Abbreviations used in this paper:* BLT, Nα-CBZ-L-lysine thiobenzyl ester; CCPI, mouse cytotoxic protease I; DCI, 3,4-dichloroisocoumarin; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); FPLC, fast protein liquid chromatography; HF, Hanukah Factor; [125]IUdR, [^{125}I]iododeoxyuridine; RNK, rat NK; RNKP-1, RNK protease 1.

maintained in RPMI 1640 supplemented with 10% FCS, penicillin-streptomycin, and 2 mM L-glutamine.

Isolation of Granules. The method for isolation and solubilization of granules of RNK cells has been described in detail (7, 16). In brief, RNK cells were washed out from the peritoneum using cold HBSS with heparin, pelleted by centrifugation, and resuspended at 10^8 cells/ml in disruption buffer (0.25 M sucrose, 10 mM Hepes, 4 mM EGTA, and 160 U/ml heparin, pH 7.4). They were equilibrated at 0°C and at 450 psi in a nitrogen cavitation bomb for 20 min, then lysed by decompression. The resulting homogenate was made up to 8.8 mM $MgCl_2$ and incubated at room temperature for 25 min with 1,000 U/ml DNase I (Sigma Chemical Co., St. Louis, MO). Nuclei were removed by successive filtration through 5- and 3-μm nucleopore filters (Nucleopore Corp., Pleasanton, CA). The resulting homogenate was cooled at 4°C. 5–6-ml aliquots were layered on 19 ml of 48% (wt/vol) Percoll in disruption buffer without heparin and centrifuged at 20,000 rpm at 4°C for 10 min with 0 deceleration using a 70-Ti rotor in a centrifuge (L8-70M; Beckman Instruments, Inc., Fullerton, CA). The bottom-most 5 ml from the resulting gradients was harvested. This granule-containing fraction was centrifuged at 34,000 rpm for 16 h at 4°C using the same rotor, and the granules, a loose white layer over a hard Percoll pellet, were collected by Pasteur pipette and stored at −80°C. The granules were solubilized in 2 M NaCl with three freeze/thaw cycles. This material was centrifuged at 34,000 rpm for 1 h in a 70-Ti rotor (Beckman Instruments, Inc.). The supernatant was harvested and filtered through a sterile Millex GV₄ 0.22-μm filter (Millipore Corp., Bedford, MA) to remove membrane fragments.

Purification of Granule Proteins. The filtered solubilized granule preparation (7.5 × 10^9 RNK cell equivalents in a 4.5-ml volume) were loaded on a 1-ml phenyl superose column (HR 5/5; Pharmacia LKB Biotechnology, Uppsala, Sweden) connected to a fast protein liquid chromatography (FPLC) system. All buffers and fractions were kept on ice. Fractions were eluted with a linear gradient of 2 to 0 M NaCl in 20 mM Tris-HCl (pH 7.2), 0.1 mM EGTA, and 0.02% NaN₃ buffer (TEB) as follows. Sample was loaded in 4.5 ml for 9 min, then the column was washed for 24 min. A 2 to 0 M NaCl linear gradient in TEB was then run for 20 min. The elution rate was 0.5 ml/min, 1-ml fractions were collected, and the eluate was monitored by measurement of OD_{280}. The column was run twice (1.5 × 10^{10} RNK cell equivalents total), and identical fractions were pooled. DNA-fragmenting and cytolysin activities were screened in [^{125}I]iododeoxyuridine (^{125}IUdR) and ^{51}Cr release assays using double-labeled YAC-1 cells as described below. All ^{125}IUdR assays contained a constant amount of cytolysin, which was necessary to detect activity.

DNA-Fragmenting Protein. Fractions containing activity, which were eluted with the first protein peak of the phenyl superose column, were pooled and concentrated using a Centriprep 10 concentrator (Amicon, Beverly, MA) to a final volume of 2.4 ml. This material was diluted to 36 ml in TEB to give a final [NaCl] of ∼0.145 M and applied on a Mono Q column (HR 5/5; Pharmacia LKB Biotechnology). The sample was loaded for 36 min and washed for 6 min in TEB. Fractions were eluted from the Mono Q column with a linear gradient of 0.2 to 0.6 M NaCl in TEB for 20 min, then a steeper gradient of 0.6 to 2 M NaCl in TEB for 6 min. The flow rate was 1 ml/min, and 1-ml fractions were collected and screened with the ^{125}IUdR release assay. Active fractions were pooled, and concentrated with a Centricon 10 microconcentrator (Amicon) to a final volume of 1 ml and then diluted to 5 ml in TEB to give a final NaCl of ∼0.1 M for subsequent heparin chromatography. We used an HR 5/5 column (Pharmacia LKB Bio-

technology) that was packed with 1 ml of heparin-agarose gel (Pierce Chemical Co., Rockford, IL) and connected to the FPLC system. Samples were loaded for 25 min, and the column was washed for 25 min with TEB. Fractions were eluted with a linear gradient of 0 to 1 M NaCl in TEB for 100 min. The flow rate was 0.2 ml/min, and 1 ml fractions were collected and screened for activity. Active fractions from the heparin-agarose chromatography were pooled and concentrated; the buffer was changed to 10 mM bis-Tris, pH 6.0, containing 50 mM NaCl; then 12 ml was applied to Mono S beads (HR 5/5) for 12 min, and then the column was washed with 10 mM bis-Tris for 8 min. Fractions were eluted with a linear gradient of 10 mM bis-Tris, pH 6.0, containing 0–1 M NaCl with a flow rate of 1 ml/min for 20 min. DNA-fragmenting activity was detected in the first protein peak at 0.75 M NaCl. The complete purification protocol was run continuously over a 2–3-d period, storing samples at 4°C between chromatographic steps.

Cytolysin. A simple and rapid method for purifying cytolysin was developed. The cytolysin-containing fractions eluted from the phenyl superose column were usually stored at −80°C. Samples were then thawed, pooled, and concentrated with Centricon 30 microcentrators (Amicon) to a final volume of 1.0 ml. This material was diluted to 10 ml with TEB to give a final [NaCl] of ∼0.1 M and loaded on the FPLC heparin-agarose column. Fractions were eluted with three gradients, 6 min of 0–0.3 M NaCl, 40 min of 0.3–0.5 M NaCl, and 30 min of 0.5–1.0 M NaCl in TEB. The flow rate was 0.5 ml/min, and 1-ml fractions were collected and screened with a ^{51}Cr-release assay and Nα-CBZ-L-lysine thiobenzyl ester (BLT) esterase assay. The purity of each fraction from the peak of cytolytic activity was assessed by SDS-PAGE. Experimentally useful amounts of pure cytolysin could be obtained from an initial loading on the phenyl superose column of as little as 4 × 10^9 cell equivalents of solubilized granule preparation.

Tryptic Peptide Sequencing. Tryptic peptide cleavage fragments for internal sequence analysis were prepared as described (17). Separated proteins were electroblotted onto nitrocellulose and detected on the membrane by Amido black staining. The region where the 32-kD protein was bound was sliced out, and the protein was digested on the matrix with trypsin. The resulting peptides were separated by reverse-phase HPLC on a C₄ column (150 × 2.1 mm; Vydac Separations Group, Hesperia, CA) in a peptide analyzer (Waters, Inc., Rochester, MN) using a trifluoroacetic acid/acetonitrile buffer system. Prominent peptides were collected manually and frozen immediately. Sequence analysis was performed on a sequencer (477; Applied Biosystems, Inc., Foster City, CA) using standard protocols.

^{51}Cr and ^{125}IUdR Release Assay. Target cells in the log phase of growth (2.5 × 10^5/ml) were labeled as described by Russell et al. (3) with some modifications. Briefly, 20–40 μCi ^{125}IUdR and in some experiments also 100 μCi sodium ^{51}Cr-chromate were added to 2–5 × 10^6 target cells in 0.5 ml of supplemented RPMI, the cells were incubated at 37°C in a 5% CO_2 atmosphere for 90 min, washed once in HBSS, incubated an additional 45 min, then washed twice and resuspended at 1–2 × 10^5 cells/ml in HBSS containing 2 mM $CaCl_2$ 10 mM Hepes, 4 mg/ml BSA, pH 7.4. We only used double-labeled cells for which ^{125}IUdR cpm and ^{51}Cr cpm did not differ by more than a factor of 2. Aliquots of 10^4 cells in 100 μl were added to 100 μl of granule-derived material (with [NaCl] adjusted to 140 mM) that had been diluted in normal saline with 10 mM Hepes and 1 mM EGTA (at pH 7.4) in 96-well, V-bottomed microtiter plates kept on ice. Assays in which the effect of various chemicals were analyzed utilized a similar procedure except that 10^4 cells were delivered in 50 μl and the chemical in 50 μl. Cytochalasin B (Sigma Chemical Co.) was dissolved in DMSO

(Mallinckrodt Canada Inc., Point-Claire, Quebec) for a stock solution of 20 mM and stored at 4°C. Sodium azide (Sigma Chemical Co.) and 2-deoxyglucose (Sigma Chemical Co.) were made fresh in assay medium. Target cells were incubated 6–8 h at 37°C in cytochalasin B, and 5 h with sodium azide or 2-deoxyglucose before addition of granule proteins. Cycloheximide (Sigma Chemical Co.) was prepared in assay medium. Target cells were incubated for 2 h at 37°C with cycloheximide before the cytotoxicity assay.

The plates were then incubated at 37°C for the times indicated and subsequently centrifuged at 800 g for 5 min. In the ^{125}IUdR release experiments, 80 μl of target cells and 80 μl of test protein were incubated, then 40 μl of 1% Triton X-100, 5 mM EGTA, and 50 mM Tris-HCl (pH 7.2) was added to reaction wells, the well contents were mixed, and the plates were centrifuged. In both sets of experiments, 100 μl of supernatant was harvested from each well, and the corresponding ^{51}Cr cpm and ^{125}IUdR cpm were determined in a Wallac LKB 1282 Compugamma universal gamma counter (Pharmacia LKB Biotechnology), correcting for overlapping energy spectra. The specific isotope release was determined as described previously (16).

Calculation of Lytic Units. For ^{125}IUdR and ^{51}Cr release assays, we defined one lytic unit as the amount of protein that gave 50% specific ^{125}IUdR or ^{51}Cr release from 10^4 YAC-1 cells. For calculations of specific activity in lytic units per milligram, protein concentrations were determined using a bicinchoninic acid protein assay kit (Micro BCA; Pierce Chemical Co., Rockford, IL) adapted to 96-well plates. Readings were made in an ELISA reader (Titertek, Multiskan MCC/340 MK II; Flow Laboratories Inc., Mississauga, Ontario) with a 540-nm filter. For the hemolysis assay, we defined 1 hemolytic unit of cytolysin as the amount of material (μl) that gave 50% hemoglobin release. Hemolytic units were used as a measure of activity when cytolysin protein concentration was below detection limits.

BLT Esterase Microassay. We determined the serine esterase activity of individual fractions from a Percoll gradient of RNK cell homogenate using a modification of the colorimetric BLT thioesterase assay of Green and Shaw (18).

The stock reagents were 10 mM BLT (Sigma Chemical Co.) in H₂O, and 10 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma Chemical Co.) in dimethyl formamide. These were further diluted in HBSS-Hepes (10 mM) to 1 mM BLT and to 0.5 mM DTNB, respectively. Each well of a 96-well flat- or round-bottomed microtiter plate received dilute DTNB solution. To the reaction wells, we added 50 μl of dilute BLT solution, whereas to their "blank" counterparts we added 50 μl of HBSS-Hepes. We added 100 μl of appropriately diluted (in HBSS-Hepes) Percoll gradient fractions to both sets of wells, mixed well contents on a plate shaker for 10 min, and incubated a further 30 min at room temperature. The BLT esterase activity in each well was determined by measuring the absorbance at 414 on a Titertek Multiskan plate reader. It is assumed that OD is linear with enzyme dose.

SDS-Polyacrylamide Gel Electrophoresis. Proteins were run on 6-cm-long 12% gel slabs according to Laemmli (19). An additional 2 cm of 4% gel was added over the running gel to resolve high M_r bands. Gels were developed using a commercial silver stain kit (Bio-Rad Laboratories, Richmond, CA).

Results

Purification of a 32-kD Protein with DNA Fragmenting Activity. To identify and purify the putative granule DNA-fragmenting molecule, we isolated cytoplasmic granules from

RNK cells as described previously (7, 16), and two aliquots of 0.75 × 10^{10} cell equivalents were solubilized in 2 M NaCl and subjected to FPLC chromatography (see Materials and Methods). After an initial step of phenyl superose chromatography, we found two protein peaks (Fig. 1 A). The second peak had cytolysin activity but caused no DNA damage in double-labeled (^{51}Cr and ^{125}IUdR) YAC-1 target cells. The first protein peak induced ^{125}IUdR release only after combining it with the cytolysin containing fractions (Fig. 1 a). The possibility that the DNA fragmentation required the presence of cytolysin from the second peak was confirmed utilizing purified cytolysin prepared by sequential Ultragel AcA54 and heparin-agarose chromatography as described earlier (16) or phenyl superose and heparin-agarose as described in Materials and Methods. In all subsequent chromatographic steps, we screened for DNA-fragmenting activity in the presence of cytolysin. The first protein peak from the phenyl superose chromatography was pooled, concentrated, and desalted, and then applied to a Mono-Q column. The fractions containing the DNA-fragmenting activity (Fig. 1 B) were pooled and concentrated, then applied to a heparin-agarose affinity column. Following heparin chromatography, a single peak of protein containing DNA-damaging activity was observed (Fig. 1 C), which ran as two major bands of 30- and 32-kD and a minor 31-kD band on SDS-PAGE after reduction (Fig. 1 E). The active fractions of the heparin-agarose chromatography were pooled, concentrated, and subjected to Mono S chromatography. Two protein peaks were identified (Fig. 1 D); the first contained DNA-fragmenting activity, and the second was a BLT esterase. The first peak contained the major 32-kD protein, which ran at 31 kD under nonreducing conditions (Fig. 1 E). The second protein peak that had BLT esterase activity was 30 kD under reducing and 28 kD under nonreducing conditions (Fig. 1 E). The minor 31-kD protein eluted between the two peaks. Recovery and purification for each step are presented in Table 1. The 32-kD protein was named "fragmentin" after its ability to induce DNA fragmentation.

Cytolysin was purified by a new two-step method using phenyl superose and heparin-agarose chromatography (see Materials and Methods). The initial phenyl superose step separated the cytolysin from most granules proteins including fragmentin (Fig. 1 A). The heparin-agarose step removed a contaminating BLT esterase (Fig. 2) and yielded a single band of 65-kD on SDS-PAGE under reducing conditions (Fig. 2) with a recovery of 12–15% and purification of >300–fold (Table 1).

Fragmentin Is Homologous to RNKP-1 and CCPI. Four tryptic peptides of fragmentin were prepared and sequenced (17). The amino acid sequence is given in Fig. 3 and compared with the deduced amino acid sequences of three granule proteases: the RNK protease 1 (RNKP-1), which was cloned from the same RNK-16 tumor used to purify fragmentin (20), the murine T cell granule proteases CCPI/Granzyme B (21), and Hanukah Factor (HF)/Granzyme A (22). Fragmentin was highly homologous to RNKP-1, differing in only two of 41 identifiable amino acids. CCPI/Granzyme B was also closely related as it differed in seven amino acids, whereas
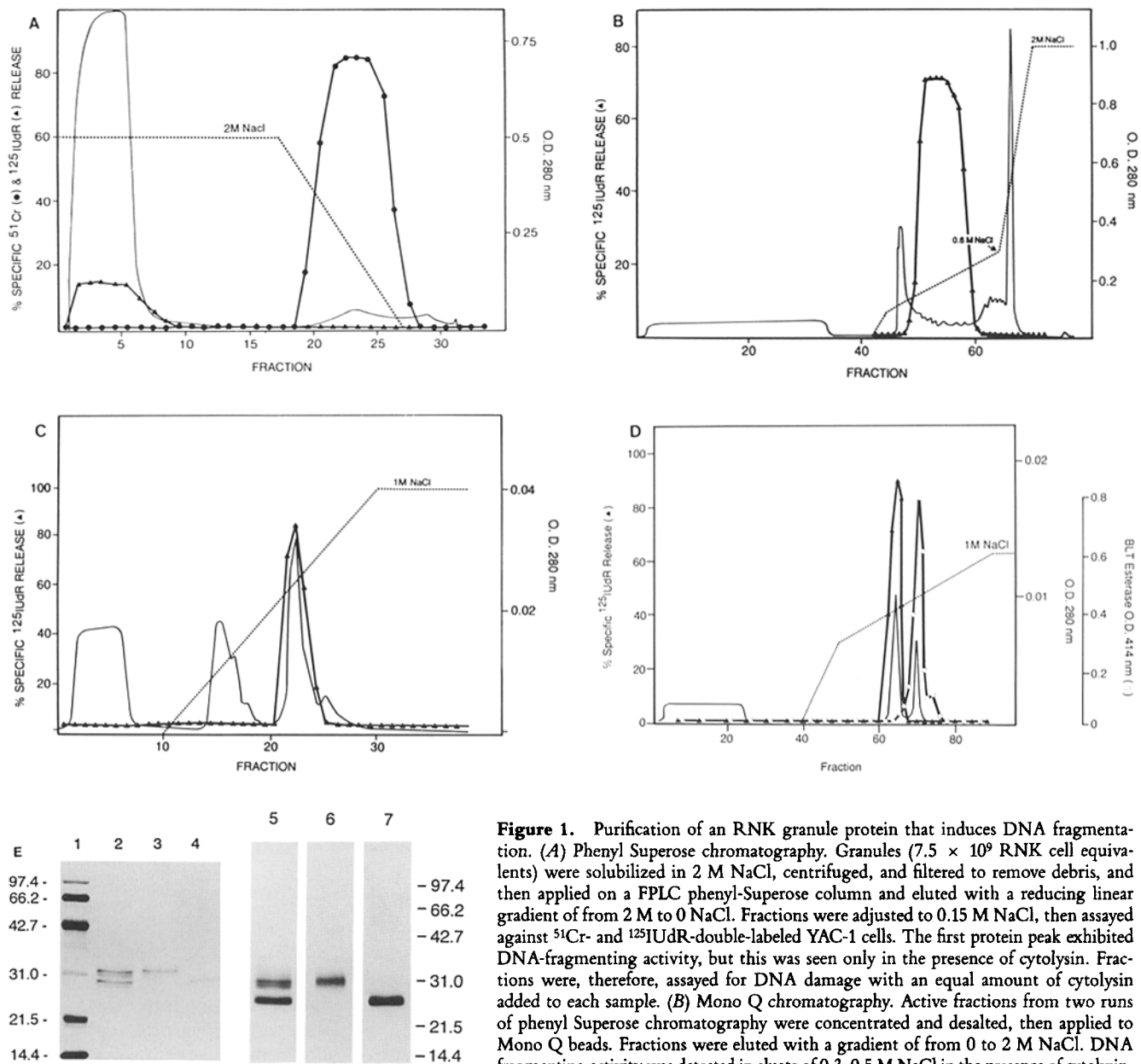
Figure 1. Purification of an RNK granule protein that induces DNA fragmentation. (*A*) Phenyl Superose chromatography. Granules (7.5 × 10⁹ RNK cell equivalents) were solubilized in 2 M NaCl, centrifuged, and filtered to remove debris, and then applied on a FPLC phenyl-Superose column and eluted with a reducing linear gradient of from 2 M to 0 NaCl. Fractions were adjusted to 0.15 M NaCl, then assayed against ^{51}Cr- and ^{125}IUdR-double-labeled YAC-1 cells. The first protein peak exhibited DNA-fragmenting activity, but this was seen only in the presence of cytolysin. Fractions were, therefore, assayed for DNA damage with an equal amount of cytolysin added to each sample. (*B*) Mono Q chromatography. Active fractions from two runs of phenyl Superose chromatography were concentrated and desalted, then applied to Mono Q beads. Fractions were eluted with a gradient of from 0 to 2 M NaCl. DNA fragmenting activity was detected in eluate of 0.3–0.5 M NaCl in the presence of cytolysin. (*C*) Heparin-agarose chromatography. Active fractions from the Mono Q column were pooled, concentrated, and applied to a heparin-agarose column. Fractions were eluted with a gradient of 0 to 1 M NaCl. DNA-fragmenting activity was eluted at 0.5–0.6 M NaCl in a single protein peak. (*D*) Mono S chromatography. Active fractions from the heparin-agarose chromatography were pooled, concentrated, and buffer changed to 50 mM NaCl, and then applied to Mono S beads. Fractions were eluted with a linear gradient of 0 to 1 M NaCl. DNA-fragmenting activity was detected in the first protein peak at 0.75 M NaCl. The second peak was a BLT esterase. (*E*) SDS-PAGE of heparin agarose and Mono S protein peaks. 15 μl of sample containing 800 ng of protein was loaded on a 12% gel run according to Laemmli (19) under reducing conditions (100 mM dithiothreitol at 95°C for 3 min). Lane *1*, molecular mass standards; lane *2*, heparin-agarose fraction 22; lane *3*, Mono S fraction 65 containing DNA-damaging activity; and lane *4*, Mono S fraction 71. Lanes *5–7* correspond to the same protein peaks run under nonreducing conditions using 1,600 ng of protein per lane. Molecular mass standards for gels run under nonreducing conditions are given on the right. Gels were silver stained according to manufacturers instruction's (BioRad, Mississauga, Ontario).

Table 1. *Purification of Fragmentin and Cytolysin from RNK Granules*

	Protein	Total activity[*]	Activity yield	Specific activity	Purification
	mg	*U*	*%*	*U/mg*	*fold*
Fragmentin					
Granules	18	3.2×10^4	100	1.78×10^3	1.0
Phenyl Superose	16.5	2.7×10^4	84	1.64×10^3	0.92
Mono Q	0.546	1.3×10^4	41	2.38×10^4	13.4
Heparin Agarose	0.171	5.8×10^3	18	3.39×10^4	19.0
Mono S	0.06	3.5×10^3	11	5.83×10^4	32.8
Cytolysin					
Granules	54	1.5×10^5	100	2.8×10^3	1.0
Phenyl Superose	1.28	1.2×10^5	80	9.4×10^4	33.6
Heparin Agarose	0.022	1.9×10^4	12.7	8.6×10^5	307.0

[*] A unit of fragmentin activity was defined as the amount required to produce 50% release of ^{125}IUdR from 10^4 YAC-1 cells. A unit of cytolysin activity was the amount producing 50% release of ^{51}Cr from 10^4 YAC-1 cells.

19 amino acids did not match the corresponding HF/Granzyme A sequence.

Fragmentin Induces DNA Fragmentation and Apoptosis. Duke et al. (1, 2) found that NK cells could cleave YAC-1 target cell DNA into oligonucleosomal-sized fragments that gave a ladder-like pattern on gel electrophoresis. We incubated 10^6 unlabeled YAC-1 cells with the purified protein and/or cytolysin for 4 h at 37°C, then extracted the DNA, and subjected it to agarose gel electrophoresis and ethidium bromide staining. Fig. 4 shows that the protein and cytolysin together caused a DNA ladder, whereas each alone was inactive.

Time-lapse morphological examination of YAC-1 over a 2-h period following incubation with the protein and cytolysin revealed extensive blebbing of the cell surface and convolution of the nuclear membrane, which are typical of CTL-induced damage and apoptosis (23–25). Cell death was associated with nuclear collapse and severe chromatin condensation within 1 h, which was visualized as apoptotic bodies (24, 25) by transmission electron microscopy (Fig. 5 *D*). Cytolysin alone produced membrane damage without chromatin condensation (Fig. 5 *C*), whereas the fragmentin by itself was without effect (Fig. 5 *B*).
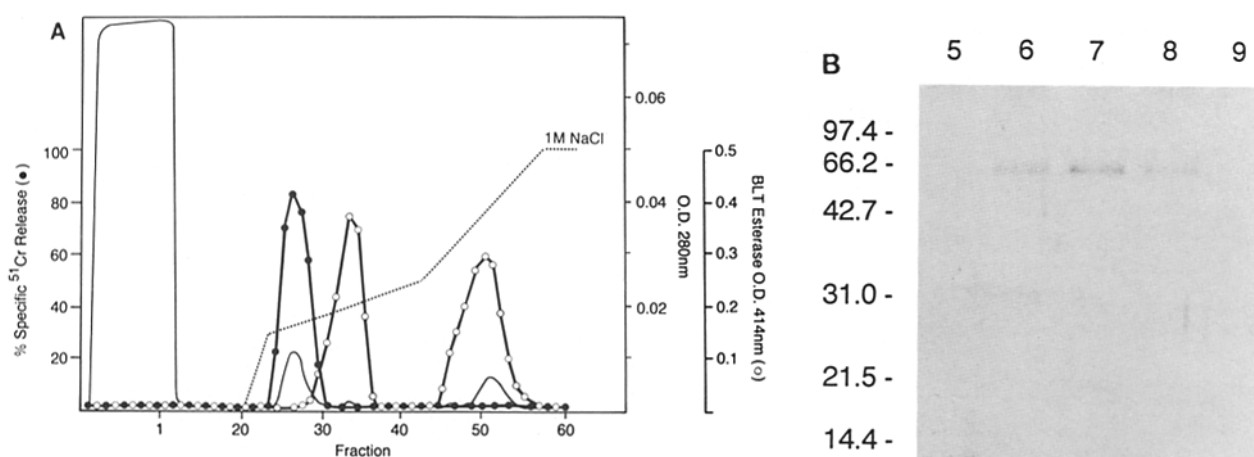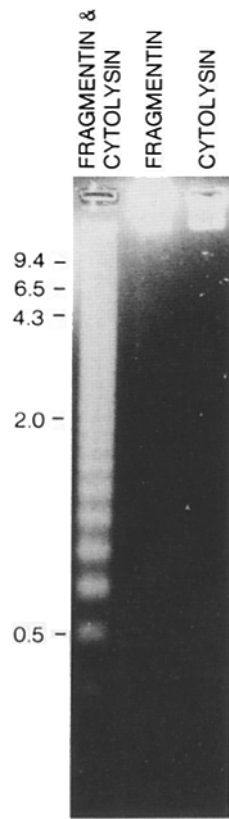

Figure 2. Cytolysin purification. (*A*) Heparin-agarose chromatography. Cytolysin-containing fractions from the phenyl-Superose column (Fig. 1 *a*) were pooled, concentrated, applied to the heparin agarose column, and eluted with three gradients: 0–0.3, 0.3–0.5, and 0.5–1.0 M NaCl. The first protein peak was cytolysin, and two subsequent peaks had BLT esterase activity. (*B*) SDS-PAGE of cytolysin. Samples (8 μl) of cytolysin eluted from heparin-agarose column were loaded on a 12% gel run according to Laemmli (19) under nonreducing conditions. Lanes *5–9* were heparin-agarose fraction 25–29 containing cytolysin activity. The protein concentration of the peak at fraction 27 was 25 μg/ml, and 0.2 μg was loaded in lane 7 of the SDS-PAGE. Molecular mass standards are given on the left. Gels were silver stained.

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Peptide 1

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Fragmentin        Tyr-Ser-Asp-Thr-Leu-Gln-Glu-Val-Glu-Leu-Thr-Val-Gln-Glu-Asp-Gln

RNKP-1            * - * - * - * - * - * . * . * - * - * . * - * - * . * - * . * - *

CCPI/Granzyme B   * - * -Asn- * - * - * - * - * - * - * - * - * - * - * -Lys- * -Arg

HF/Granzyme A     Pro- * -Glu- * - * -Arg- * - * -Asn-Ile- * - * - * -Ile-Asp-Arg-Lys
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Peptide 2

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Fragmentin        Ala-Asn-Glu-Ile-Xxx-Ala-Gly-Asp

RNKP-1            * - * - * - * - * -Cys- * - * - *

CCPI/Granzyme B   Thr- * -Gln- * -Cys- * - * - *

HF/Granzyme A     Leu- * -Met- * -Cys- * - * - *
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Peptide 3

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Fragmentin        Pro-Phe-Pro-Ala-Tyr-Asn-Ser

RNKP-1            * -His- * - * . * . * . * - *

CCPI/Granzyme B   * -His- * -Asp- * - * -Pro

HF/Granzyme A     * -Tyr- * -Cys- * -Asp-Glu
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Peptide 4

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Fragmentin        Val-Asp-Pro-Gly-Asp-Val-Xxx-Tyr-Val-Ala-Gly-Xxx-Gly

RNKP-1            * -Lys- * - * - * - * - * -Cys- * - * - * - * - * -Trp- *

CCPI/Granzyme B   * -Lys- * - * - * - * - * -Cys- * - * - * - * - * -Trp- *

HF/Granzyme A     * -Lys- * - * -Thr-Arg-Cys-Arg- * - * - * - * -Trp- *
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Figure 3. Amino acid sequence of tryptic peptides of fragmentin.

Figure 4. DNA fragmentation in YAC-1 cells incubated with fragmentin and cytolysin. Unlabeled YAC-1 cells (10^6) were incubated with 8 μg fragmentin and/or 0.4 μg cytolysin in 1.0 ml HBSS, 10 mM Hepes, and 0.2% BSA for 4 h at 37°C. The cells were then incubated in sodium-N-lauroyl sarcosine and proteinase K at 50°C for 1 h. RNase A was added, and the sample was incubated for a further 1 h, then loaded on a 1% agarose gel and electrophoresed. The gel was stained with ethidium bromide and visualized under UV light.

Cytolysin Dependence of Fragmentin-Induced DNA Damage. In the presence of a constant amount of cytolysin, fragmentin produced a dose-dependent increase in DNA damage as measured by ^{125}IUdR release and membrane damage as measured by ^{51}Cr release, but was inactive when presented to target cells on its own (Fig. 6 A). Fragmentin could induce DNA damage at a dose of cytolysin that produced no detectable membrane damage (Fig. 6 A). Over the dose range of fragmentin, we first observed an increase in membrane damage at lower doses and this was followed by ^{125}IUdR release at higher fragmentin concentrations. Fig. 6 B shows that fragmentin activity was also dependent on cytolysin dose, with both DNA and membrane damage increasing with cytolysin dose. However, membrane damage in the presence of fragmentin was always higher than that induced by cytolysin alone. DNA and membrane damage were inhibited by EGTA, indicating that the process was Ca^{2+}-dependent (Fig. 6 A). In a kinetic analysis, DNA and membrane damage were seen as early as 15 min after treatment with fragmentin and cytolysin, and were maximal by 60 min (Fig. 6 C). Membrane damage preceded the DNA damage induced by fragmentin. When examining fragmentin activity against other lymphoma targets, activity was detected using EL4, L5178Y, and SL2 tumor cells. DNA damage was found to be dependent on the dose of cytolysin in each tumor although the range of sensitivity varied by 10-fold (Fig. 7).

Although fragmentin has an absolute requirement for cytolysin, even at sublytic doses, the mechanism by which it promotes fragmentin activity is not immediately apparent. One possibility is that cytolysin insertion into the plasma membrane might stimulate endocytosis and thus facilitate fragmentin movement from the extracellular space to the cytoplasm. To test this hypothesis, we examined the effects of cytochalasin B, an inhibitor of microfilament assembly, and of sodium azide and 2-deoxyglucose which, respectively, inhibit mitochondrial respiration and glycolysis. Both cytochalasin B and metabolic inhibitors block receptor-mediated endocytosis (26). We found that cytochalasin B completely blocked DNA and membrane damage by fragmentin without affecting cytolysin activity (Fig. 8 A). Furthermore, sodium azide and 2-deoxyglucose when combined also effectively inhibited fragmentin activity (Fig. 8 B).

Inhibition of Fragmentin by Protease Inhibitors. The structural homology of fragmentin to granzymes RNKP-1 and CCPI suggested that its ability to induce DNA fragmentation was related to its proteolytic activity. To test this hypothesis, we preincubated 40 ng of fragmentin for 30 min at 37°C with increasing doses of 3,4-dichloroisocoumarin (DCI), a general serine protease inhibitor (27) that is a potent inhibitor of lymphocyte proteases (28), and PMSF, a serine

Figure 5. Electron micrograph of YAC-1 lymphoma following incubation with fragmentin and cytolysin. YAC-1 cells were incubated for 1 h with control medium (A) and with fragmentin only (B), cytolysin only (C), and fragmentin and cytolysin (D). The highly condensed chromatin seen in D (arrows) are apoptotic bodies.
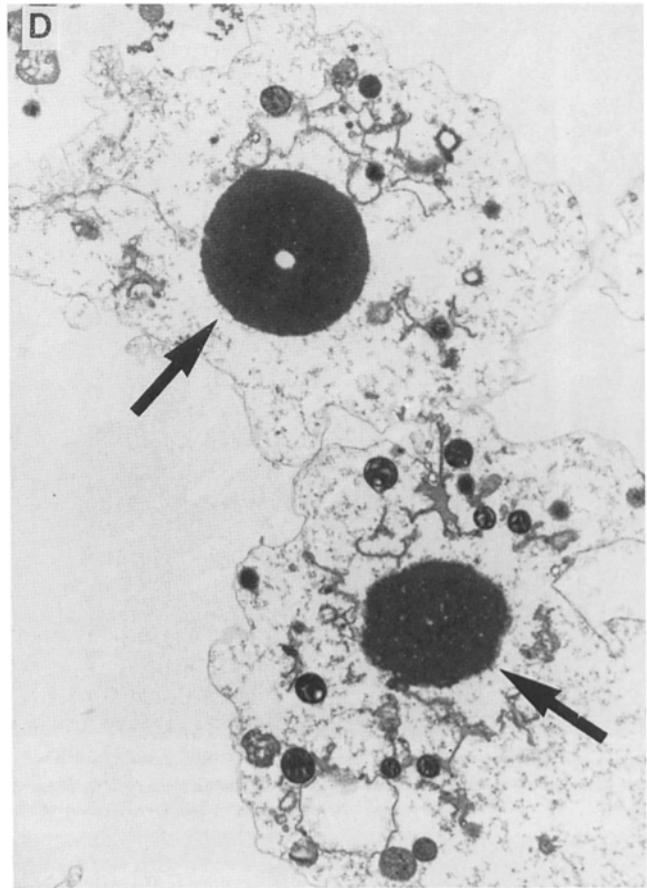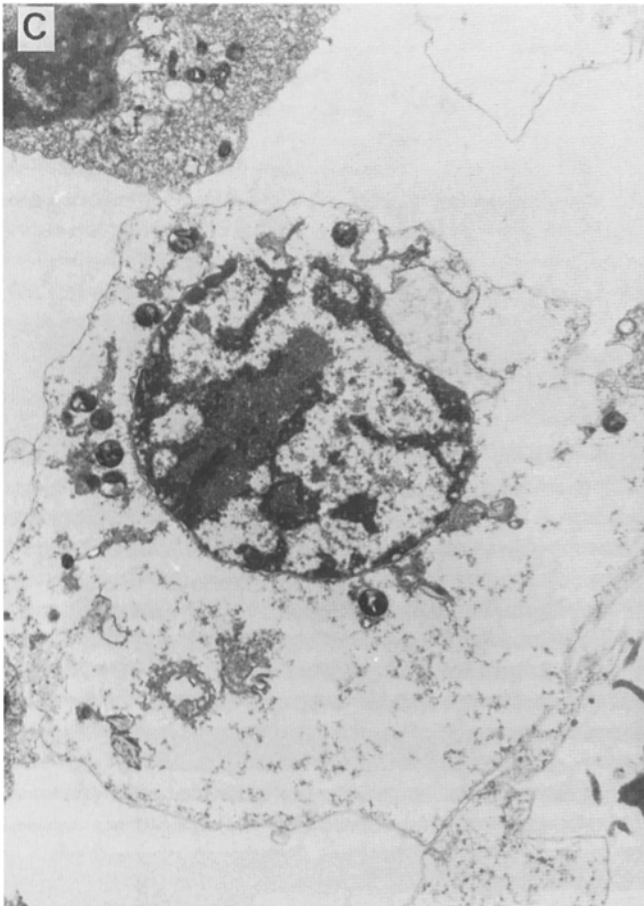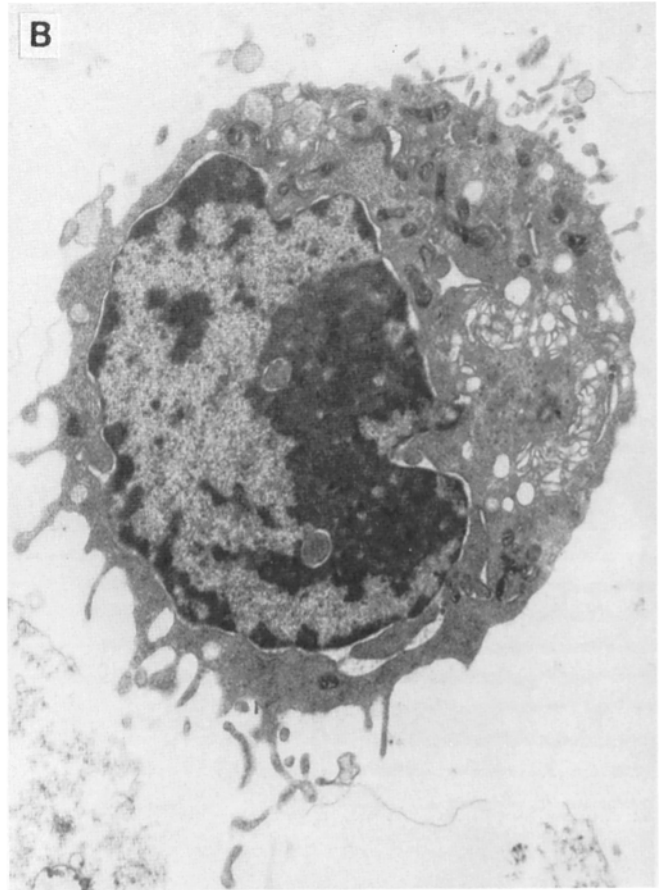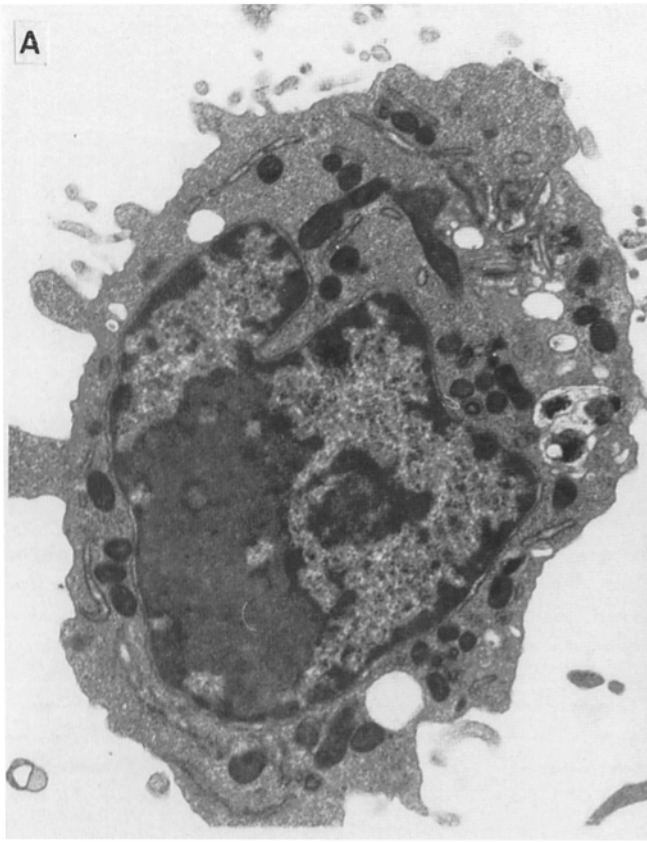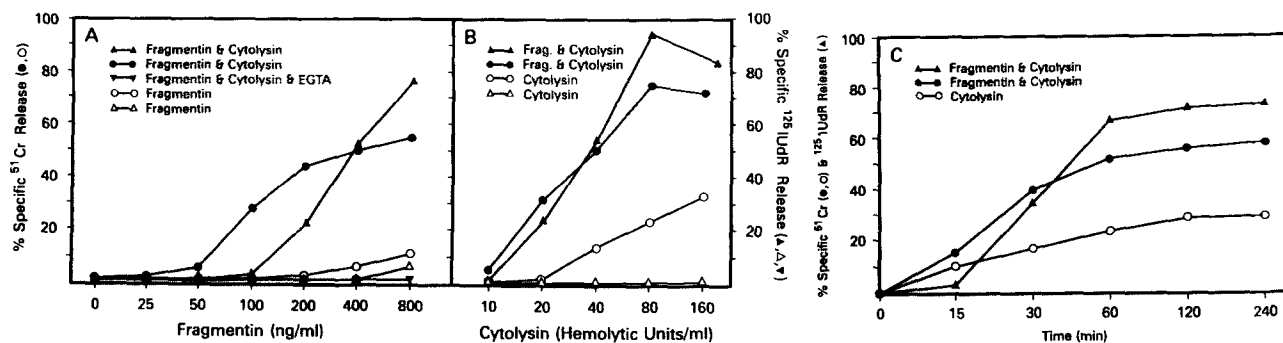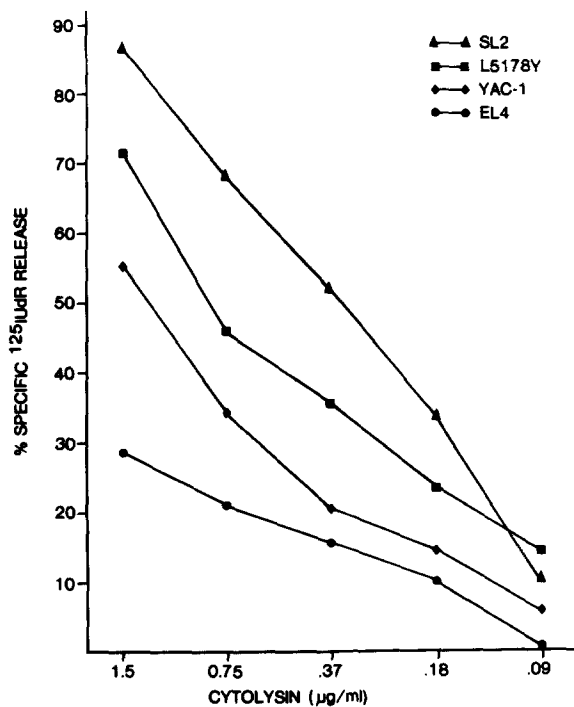
Figure 6. Fragmentin and cytolysin dose response. (*A*) Increasing fragmentin dose alone or with a constant cytolysin concentration of 20 hemolytic units per ml. (*B*) Increasing cytolysin dose alone or with a constant fragmentin concentration of 100 ng/ml. (*C*) Time course of fragmentin-induced DNA and membrane damage. Fragmentin (100 ng/ml) and cytolysin (100 hemolytic units/ml) induced rapid DNA and membrane damage of YAC-1 cells in <15 min and reached maximum effect in 60 min. Cytolysin used in these experiments was purified from RNK granules as described in Table 1 and Fig. 2.

protease inhibitor that can partially block RNK-16 granule cytotoxicity (11). DCI completely blocked fragmentin activity with an ID_{50} of 15 μM without affecting cytolysin (Table 2). The latter control was important, as loss of activity could have been due to cytolysin inactivation. PMSF, with an ID_{50} of 360 μM, on the other hand, was much less effective than DCI (Table 2).

Fragmentin Activity Is Not Dependent on Protein Synthesis. To determine whether fragmentin-induced DNA fragmentation was dependent on new protein synthesis, we examined its

activity in cells pretreated with 50 μg/ml cycloheximide for 2 h at 37°C. Fragmentin activity over a wide dose range was unaffected by cycloheximide (Fig. 9), and cycloheximide from 1 to 50 μg/ml did not alter fragmentin-induced DNA damage (not shown).

BLT Esterase Amplifies Fragmentin Activity. The relatively low fragmentin purification factor of 33-fold (see Table 1) was unexpected, and may have been the result of the lability of the molecule and loss of activity during purification. Alternatively, another molecule could have been separated during the purification that could amplify fragmentin activity. Copurification of fragmentin with a second protein with BLT esterase activity (see Fig. 1 *D*) suggested that this protein may have been the putative amplifying molecule. At a protein concentration up to 1 μg/ml, the BLT esterase was inactive. However, it was capable of enhancing fragmentin activity in a synergistic manner (Fig. 10). The amplification was ~five-fold at the ED_{50} (Fig. 10). In this experiment, we also examined the requirement for Triton X-100 solubilization of the target cell to release soluble [125]IUdR after incubation with fragmentin, cytolysin, and the BLT esterase. Although Triton X-100 enhanced the release of DNA, 20–40% of total [125]IUdR was released in the absence of the detergent (Fig. 10, *A* and *B*).

Discussion

Although the phenomenon of DNA fragmentation induced by lymphocyte-mediated killing has been known for many years, the exact molecular mechanisms have not been determined. Candidate molecules such as lymphotoxin (29, 30), TNF-α (31), Granzyme A (11, 32), and other cytotoxins (33–35) have been suggested as possible mediators. The results of the present study show that the granules of an NK cell tumor line contain both cytolysin and a second 32-kD protein, fragmentin, that together can induce rapid DNA damage in target cells.

We were able to devise purification strategies that allowed us to recover useful amounts of both fragmentin and cytolysin. A major problem of obtaining sufficient granules for large-

Figure 7. Target cell susceptibility to fragmentin-induced DNA damage is cytolysin dependent. A variable degree of DNA damage was produced by 1 μg fragmentin at increasing cytolysin doses in these cell lines. An ~10-fold difference was detected between the SL2 and EL4 lymphomas.

Table 2. *Serine Protease Inhibitors Block Fragmentin-Induced DNA Damage*

Exp.	Protease inhibitor[*]	Fragmentin	Cytolysin	Percent [125]IUdR release (percent inhibition)[‡]	Percent ^{51}Cr release (percent inhibition)
	μM	*ng/ml*	*ng/ml*		
1	DCI				
	0	250	62.5	66	–
	1	250	62.5	60 (10)	–
	4	250	62.5	52 (22)	–
	16	250	62.5	34 (51)	–
	64	250	62.5	6 (95)	–
	128	250	62.5	3 (100)	–
	128	0	0	3	–
	DCI				
	0	–	62.5	–	74
	1	–	62.5	–	72 (2)
	4	–	62.5	–	73 (1)
	16	–	62.5	–	72 (2)
	64	–	62.5	–	72 (2)
	128	–	62.5	–	73 (1)
	128	–	0	–	74
2	PMSF				
	0	250	62.5	67	–
	16	250	62.5	64 (3)	–
	32	250	62.5	58 (13)	–
	62	250	62.5	61 (9)	–
	125	250	62.5	59 (12)	–
	250	250	62.5	48 (28)	–
	500	250	62.5	20 (70)	–
	DMSO	250	62.5	65 (2)	–
	500	0	0	0	–
	PMSF				
	0	–	62.5	–	58
	16	–	62.5	–	57 (2)
	32	–	62.5	–	57 (2)
	62	–	62.5	–	57 (2)
	125	–	62.5	–	59
	250	–	62.5	–	55 (3)
	500	–	62.5	–	57 (2)
	DMSO[*]	–	62.5	–	57 (2)
	500	–	0	–	0

[*] DCI and PMSF were prepared as stock solutions of 200 mM in DMSO. The solvent control is 0.25% DMSO.
[‡] ID_{50} of DCI was 15 μM and PMSF was 360 μM.

scale purification was overcome by the selection of an RNK subline that yielded very high intraperitoneal recovery of tumor, ∼3–4 × 10⁹ cells per rat. Furthermore, the fragmentin purification protocol was designed to avoid inactiva-

tion of the molecule by repeated freezing and thawing, and to take account of its complete dependence on cytolysin to mediate effector action against YAC-1. The use of HPLC methodology was avoided because HPLC solvents inactivated frag-
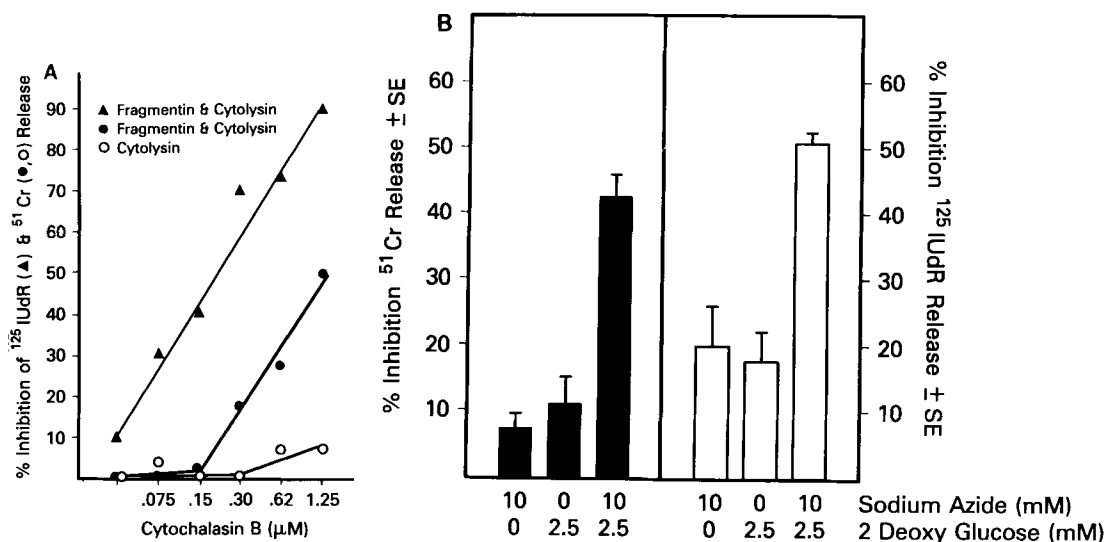
Figure 8. Inhibition of fragmentin activity by cytochalasin B, sodium azide, and 2-deoxyglucose. (A) Cytochalasin B produced a dose-dependent inhibition of fragmentin-induced DNA and membrane damage, whereas it had no effect on cytolysin activity. Specific ^{125}IUdR release was 28% and ^{51}Cr release 40% in untreated controls. (B) Sodium azide and 2-deoxyglucose had only limited effect individually, but when combined produced a significant reduction in fragmentin activity. Specific ^{125}IUdR release was 40% and ^{51}Cr release 66% in untreated controls.

mentin. The existence in T cell or NK cell granules of a molecule capable of inducing DNA fragmentation has been quite controversial (10–14). Based on our experimental observations, we suspect that the cytolysin dependence of fragmentin activity and the lability of the molecule may have thwarted previous purification attempts.

The high degree of homology of fragmentin to RNKP-1 (20) and somewhat lower degree of homology to CCPI (21) indicate that the molecule is a granzyme. The sequence data do not allow us to conclusively determine if fragmentin and RNKP-1 are identical or very closely related. Zunino et al. (20) suggested that dissimilar sequences in the S′ substrate binding site at amino acids 17–29 of RNKP-1 and CCPI indicate that they are not species homologues. We do not have

sequence from this region and therefore cannot exclude the possibility that fragmentin is a species homologue of CCPI. Further evidence that fragmentin is part of the granzyme family is the inhibition of DNA fragmentation by the serine protease inhibitor DCI. Hudig et al. (28) recently reported that RNK-16 granule lysis of YAC-1 cells measured as ^{51}Cr release was inhibited when protease activity was abolished by DCI. Our data indicate that the primary effect of DCI is on fragmentin-mediated DNA fragmentation and not on cytolysin-induced membrane damage.

Hayes et al. (32) found that immunoaffinity-purified Granzyme A from CTL granules was capable of inducing DNA damage in Triton X-100–permeabilized targets and in intact targets in the presence of cytolysin. Neither fragmentin nor the BLT esterase we have identified is Granzyme A. The molecular mass of Granzyme A in nonreducing gels is 60 kD compared with 28 kD for the BLT esterase, and the deduced amino acid sequence differs significantly from the tryptic peptides of fragmentin. Our inability to identify Granzyme A as a mediator of DNA damage in the screening assays may be due to its relatively low specific activity compared with fragmentin. Alternatively, it could have been inactivated in our purification protocol. In comparing the effects of Granzyme A reported by Hayes et al. (32) to fragmentin, we found that they are comparable in their requirement for cytolysin to induce DNA damage. However, Granzyme A appears to differ in its absolute requirement for detergent to release damaged DNA from target cells at the termination of the assay. Fragmentin liberated up to 40% of the labeled DNA in the absence of Triton X-100. This would suggest that fragmentin can damage the cell compartment in which the damaged DNA is retained. This most likely is the nuclear membrane.

The cytolysin purification protocol described here utilized sequential chromatography by hydrophobic phenyl superose
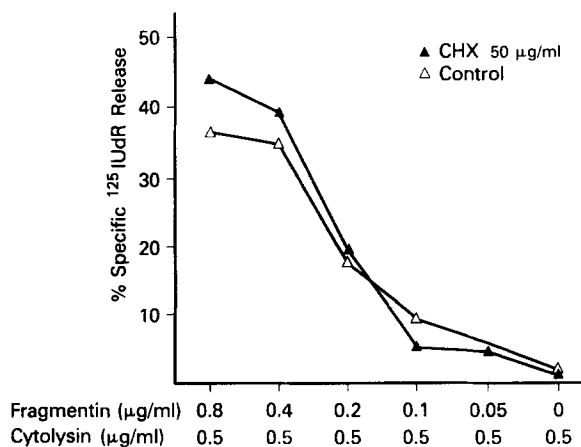
Figure 9. Fragmentin activity does not require protein synthesis. YAC-1 cells were incubated with cycloheximide (▲) (50 μg/ml) for 2 h before addition of fragmentin and cytolysin. Control, △. Similar results were obtained with a cycloheximide dose response of 1–50 μg/ml.
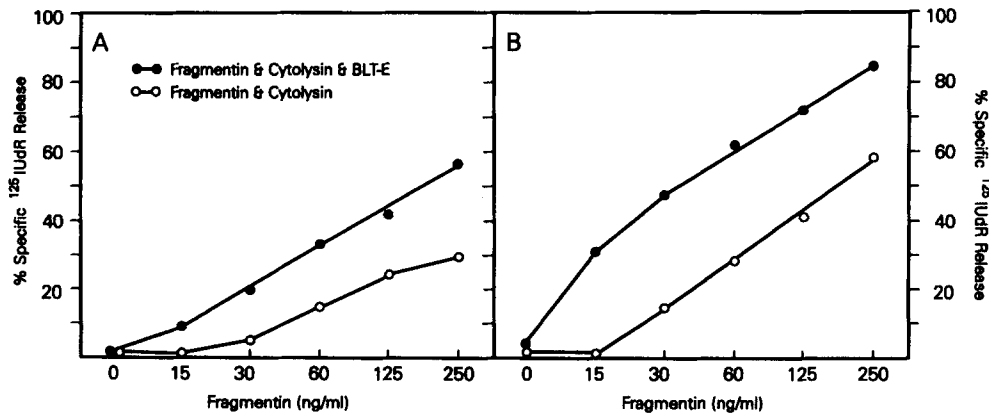
and heparin-agarose affinity columns. A hydrophobic step useful for perforin purification has been reported by Ishiura et al. (36). Although cytolysin/perforin has no obvious regions of hydrophobicity, it does have an amphipathic α-helical region that is a potential lipid-binding domain (37–39). Because cytolysin/perforin displays an overall negative surface charge and does not bind to lipid membranes in the absence of Ca^{2+}, it has been suggested that Ca^{2+} induces conformational changes which may result in exposure of lipid-binding domains (40). Our successful use of phenyl superose chromatography supports this model and indicates that a high salt environment in the absence of Ca^{2+} can reversibly induce exposure of this hydrophobic domain.

The purified cytolysin did not induce DNA damage. This disagrees with the findings of Hameed et al. (14) but supports work from Duke et al. (9). Furthermore, we found that whereas purified fragmentin was inactive on its own, DNA damage occurred in a dose-dependent manner in the presence of purified cytolysin. Interestingly, we observed membrane damage at fragmentin doses and times that showed no DNA damage. Furthermore, greater membrane damage was always observed in the presence of fragmentin than was produced by cytolysin alone, and, in some instances, cytolysin doses were chosen that produced no membrane damage. This strongly suggests that the fragmentin is either directly inducing membrane damage, or is greatly enhancing the effects of cytolysin. The inhibition of fragmentin, but not cytolysin-induced membrane damage by cytochalasin B (see Fig. 7), suggests that fragmentin may not be acting solely to amplify cytolysin activity. Because membrane damage was observed at fragmentin doses that did not produce [125]IUdR release, this also indicated that membrane disruption cannot simply be secondary to DNA damage. Overall, this argues that fragmentin may have an independent effect on the cell membrane and that nuclear damage is in some way associated with or a consequence of this action. Although this might also suggest that DNA damage may not be necessary for cell death by fragmentin, further study will be needed to clarify this issue.

The speed of DNA damage and nuclear collapse induced by fragmentin is typical of the rapid target damage induced by CTL (41). This contrasts with the extended times required for DNA fragmentation after anti-CD3 antibody, irradiation, and Ca ionophore treatments. The observation that apoptosis by these agents is blocked by inhibitors of transcription or translation suggests that time is required for protein synthesis (42–44). The requirement for protein synthesis in apoptotic CTL targets is somewhat controversial, with some reports indicating that none is required (2), whereas others suggest that it is necessary to produce DNA damage (45). Fragmentin activity does not require protein synthesis (see Fig. 9). It seems possible that a molecule having similar effects to fragmentin may be synthesized in cells triggered to undergo apoptosis by other agents. Fragmentin, on the other hand, appears to bypass this step and directly deliver the lethal hit.

Fragmentin-mediated DNA damage was Ca^{2+}-dependent, and this is consistent with the Ca^{2+}-dependent DNA fragmentation mediated by NK and lymphokine-activated killer cells (1, 14). The Ca^{2+}-dependency of fragmentin may be related to both cytolysin and fragmentin activity. Cytolysin insertion, polymerization in target membranes, and subsequent induction of membrane damage is Ca^{2+} dependent (6–8). Cytolysin-mediated increases in intracellular Ca^{2+} (46) may also be important for DNA fragmentation. However, replacing cytolysin by the calcium ionophore, ionomycin, over a wide dose range was not able to promote fragmentin-induced DNA damage (Shi, L., and A. H. Greenberg, unpublished results). Thus, increased intracellular Ca^{2+} cannot be the only way in which cytolysin contributed to fragmentin activity. This does not exclude a role for Ca^{2+} once fragmentin has entered the cell. McConkey et al. (47) found that NK cell–mediated DNA fragmentation was dependent on increased target cell intracellular Ca^{2+}. Rapid increases in cytoplasmic Ca^{2+} are often detected after contact with affected cells (10, 48). It is possible that, in addition to cytolysin membrane insertion, Ca^{2+} may be important in mediating intracellular signalling. The exact role of the increased cytosolic calcium in this type of cell death remains to be determined.

Munger et al. (11) reported that Percoll-purified dense granules from cloned mouse CTL and RNK tumor cells have the ability to release [125]IUdR from intact nuclei of detergent-permeabilized target cells. They postulated that cytolysin-induced membrane damage might play the same

role as detergent in their experiments; that is, it allows a nucleolytic factor to gain access to the target nucleus. A small molecular weight molecule may be able to pass through some cytolysin-created pores and also through nuclear pores (49, 50). Another possibility is that target cells may endocytose fragmentin as they attempt to repair the membrane damage induced by cytolysin (51). This route of entry may be similar to diphtheria toxin, which creates an ionic channel to translocate the toxic protein across the endosomal plasma membrane following endocytosis (52). In support of this hypothesis, we demonstrated that cytochalasin B, 2-deoxyglucose, and sodium azide can completely or partially block fragmentin-induced DNA and membrane damage without reducing cytolysin activity.

Granule exocytosis is a mechanism that has been proposed for NK cell–mediated killing (4, 5). The presence of both fragmentin and cytolysin in granules of the NK cell suggests that they are released simultaneously following NK cell activation and degranulation. The delivery of both molecules to the target cell membrane at sites of cell–cell contact thus would create the conditions necessary for fragmentin to mediate target cell DNA breakdown and apoptosis. The dependence of fragmentin-induced injury on the presence of cytolysin may be an effective way of restricting the range of activity of this toxic molecule.

The mechanism by which the fragmentin can produce DNA damage is not known. Its homology to RNKP-1 and CCPI indicates that it is a serine protease and as such might produce proteolytic damage to defined substrates in the cytoplasm or nucleus. The restricted substrate specificity of the granzymes (53) suggests that the target proteins are very limited. This contrasts with the generalized DNA fragmentation and release it induced in target cells. We postulate, therefore, that the DNA damage is a secondary phenomenon resulting from the degradation of the fragmentin substrate. Interestingly, a BLT esterase that copurified with fragmentin also promoted DNA damage (Fig. 1 D and Fig. 10). The BLT esterase would likely act at a different site than fragmentin. This raises the interesting possibility that they may interact in an activating cascade such that the fragmentin activated the BLT esterase, or the BLT esterase activated an inactive fragmentin precursor. An alternative possibility is that they may act intracellularly at unique sites on a common substrate producing a synergistic effect on substrate degradation. The identification of the substrate(s) will be key to developing a mechanistic model for fragmentin action.

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