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Bactericidal and Tumoricidal Activities of Synthetic Peptides Derived from Granulysin[1]

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Granulysin, a 9-kDa protein localized to human CTL and NK cell granules, is cytolytic against tumor cells and microbes. Molecular modeling predicts that granulysin is composed of five α-helices separated by short loop regions. In this report, synthetic peptides corresponding to the linear granulysin sequence were characterized for lytic activity. Peptides corresponding to the central region of granulysin lyse bacteria, human cells, and synthetic liposomes, while peptides corresponding to the amino or carboxyl regions are not lytic. Peptides corresponding to either helix 2 or helix 3 lyse bacteria, while lysis of human cells and liposomes is dependent on the helix 3 sequence. Peptides in which positively charged arginine residues are substituted with neutral glutamine exhibit reduced lysis of all three targets. While reduction of recombinant 9-kDa granulysin increases lysis of Jurkat cells, reduction of cysteine-containing granulysin peptides decreases lysis of Jurkat cells. In contrast, lysis of bacteria by recombinant granulysin or by cysteine-containing granulysin peptides is unaffected by reducing conditions. Jurkat cells transfected with either CrmA or Bcl-2 are protected from lysis by recombinant granulysin or the peptides. Differential activity of granulysin peptides against tumor cells and bacteria may be exploited to develop specific antibiotics without toxicity for mammalian cells. ***The Journal of Immunology,*** **2000, 165: 1486–1490.**

Granulysin, a member of the saposin-like protein family of lipid binding proteins, colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells (1–3). Two major protein products, 15 and 9 kDa, are detected in CTL and NK cells (1). Recombinant 9-kDa granulysin disrupts artificial liposomes and cell membranes,[3] damages mitochondria, and activates caspase 9 to induce apoptosis in nucleated cells (4). Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including tumor cells, bacteria, fungi, and parasites (3, 5). After incubation with granulysin, *Mycobacteria tuberculosis* develop surface lesions, consistent with direct interaction of granulysin with the glycolipid envelope of the bacterium (5).

Granulysin is highly homologous to NK-lysin, a cytotoxic and anti-microbial molecule expressed in porcine CTL and NK cells (6). NK-lysin has a high α-helical content and is folded tightly through three intramolecular disulfide bonds formed by six conserved cysteine residues (7, 8). By analogy to NK-lysin, granulysin is predicted to adopt a similar structure (1), although the 9-kDa form of granulysin contains only four cysteine residues that form two rather than three predicted intramolecular disulfide bonds. In contrast to NK-lysin, which loses its lytic activity against tumor cells and bacteria after reduction of disulfide bonds (9), we observed that reduction of recombinant granulysin enhanced its lytic activity against tumor targets and did not affect its activity against bacteria. This observation prompted us to design a panel of synthetic peptides corresponding to linear regions of granulysin and to assess their lytic activity. Peptides from the central region of granulysin exhibit most of the lytic activity. Substitution analysis revealed that positively charged residues in these peptides are critical for lysis of both tumor cells and bacteria. Reduction of cysteine residues affects lysis of tumor targets but not bacteria. Peptides that selectively lyse bacterial targets may provide the basis for development of antibiotics with limited toxicity for human cells.

Materials and Methods

Synthesis of peptides

Peptides were synthesized using F-moc chemistry on an Applied Biosystems (Foster City, CA) automatic peptide synthesizer, purified to >95% homogeneity by reverse-phase HPLC, and peptide composition confirmed by mass spectrometry and amino acid analysis. Stock peptide solutions (5–15 mM) were prepared in DMSO and diluted into assay medium at 1–50 μM.

Cell lines and Abs

The human EBV-transformed B lymphoma cell line JY and T cell tumor line Jurkat were maintained in RPMI 1640 (Irvine Scientific, Irvine, CA) supplemented with 10% heat-activated FCS (HyClone, Logan, UT) and 2 mM L-glutamine. Jurkat cells transfected with Bcl-2 or CrmA were maintained in medium supplemented with 0.5 mg/ml G418 as described (10). Anti-Fas mAb (CH-11) was purchased from Medical and Biological Laboratories (Nagoya, Japan).

Granulysin

Recombinant refolded granulysin was prepared as described (3–5). Briefly, recombinant 9-kDa granulysin was expressed using the vector pET28a (Novagen, Madison, WI). The fusion protein was purified in 6 M guanidine HCl on nickel affinity resin. After refolding and dialysis, the histidine tag was removed by thrombin cleavage and the material was further purified by reverse-phase HPLC. After lyophilization, the protein was suspended in

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[3] A. Kaspar, F. R. Poulain, Kelekar, D. Hanson, Hitoshi, D. E. Johnson, C. J. Froelich, C. B. Thompson, A. Anel, C. Clayberger, and A. M. Krensky. Granulysin-induced apoptosis involves membrane disruption, mitochondrial damage, and capsase 9 activation. *Submitted for publication.*

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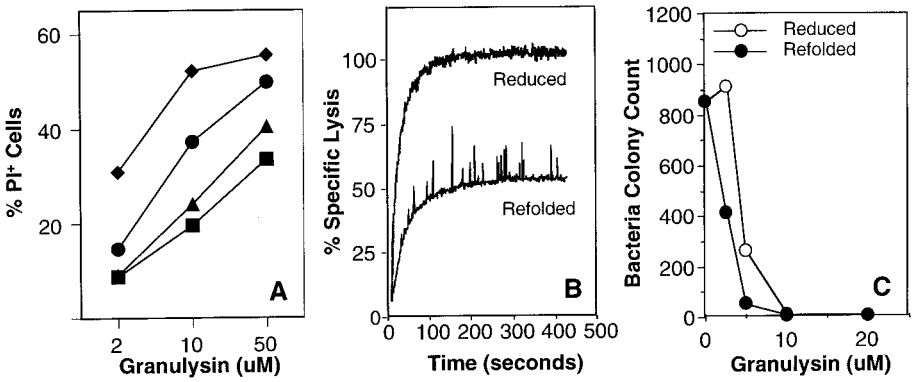

FIGURE 1. Reduced granulysin exhibits enhanced lytic activity against the Jurkat tumor cell line and liposomes, but not *Salmonella*. *A,* Recombinant granulysin alone (■), boiled for 10 min (●), treated with 1 mM DTT (▲), or boiled and treated with DTT (◆) was incubated with Jurkat cells overnight, and PI uptake was measured. *B,* Release of fluorescent probe from liposomes (25 μM total phospholipids) after addition of recombinant granulysin (0.5 μM) treated with medium (refolded) or DTT (reduced). *C,* Recombinant granulysin was treated with medium (refolded) or DTT (reduced) and tested for lysis of *Salmonella.*

PBS, and the concentration was determined using a protein assay (Bio-Rad, Hercules, CA) with lysozyme as a standard. The recombinant refolded granulysin exhibits two features that indicate that it assumes a similar tertiary structure to granulysin produced in CTL. First, both types of granulysin migrate faster in SDS-PAGE under nonreducing than reducing conditions. Second, while the granulysin-specific mAbs DH2 and DH4 recognize both recombinant and cellular granulysin, treatment of both types of granulysin with DTT abrogates recognition by DH2 but does not affect recognition by DH4 (3).

Liposome disruption assay

Granulysin-induced lysis of liposomes was measured using the fluorescent probe 1-aminonaphtlene-3,6,8-trisulfonic acid and its collisional quencher *N,N′*-(*p*-phenylenedimethylene)bis(pridiniumbromide) (Molecular Probes, Eugene, OR). Liposomes were prepared using palmitoyl-oleoyl-phosphatidyl-glycerol or a combination of dipalmitoyl-phosphatidylcholine and egg phosphatidylglycerol. Large unilamellar vesicles were prepared as previously described (11). In experiments comparing reduced and nonreduced granulysin, palmitoyl-oleoyl-phosphatidyl-glycerol liposomes (25 μM phospholipids) were treated with 550 nM granulysin. For reducing conditions, liposomes were suspended in TBS supplemented with 1 mM DTT. DTT had no effect on baseline or final fluorescence. In experiments testing granulysin peptides, dipalmitoyl-phosphatidylcholine plus egg phosphatidylglycerol liposomes (20 μM phospholipids) were treated with 0.5 μM granulysin peptides. Release of fluorescent probe was measured on a spectrophotometer as described (11). Data is plotted as percent specific lysis recorded over time.

Granulysin-mediated human cell lysis

Jurkat T cells in log-phase culture or freshly isolated PBMC were washed once with RPMI 1640 medium supplemented with 0.01% BCS and resuspended at a density of 1×10^6/ml. Cells (50 μl) were incubated with 50 μl of peptide in a 96-well plate at 37°C for 4 h. Then 50 μl of PBS containing 3 μl of 50 μg/ml of propidium iodide (PI)[4] was added to each well, and the PI-containing cells were enumerated by FACScan (Becton Dickinson). Data was analyzed with CellQuest software (Becton Dickinson). The PI-negative cell population in control sample was set as a reference to determine the percentage of PI-negative cells in all other samples. Similar results were obtained using both PBMC and Jurkat cells.

Granulysin-mediated lysis of Salmonella

A log-phase culture of *Salmonella typhimurium* was diluted to 2×10^5/ml in 10 mM sodium phosphate buffer (pH 7.4) containing 0.03% Luria-Bertani broth. A 25-μl aliquot of *Salmonella* was incubated with 25 μl of peptide at 37°C for 3 h. The bacteria and peptide mixture was diluted 100-fold with the cold phosphate buffer and plated on LB plates that were incubated at 37°C overnight. Bacterial colonies were enumerated the following day.

Treatment with DTT

Recombinant granulysin or peptide (500 μM) was incubated with 1 mM DTT and, where indicated, boiled for 10 min and then diluted to the indicated concentrations in assay medium supplemented with 250 μM DTT. This concentration of DTT had no effect on PI uptake of Jurkat cells.

[4] Abbreviation used in this paper: PI, propidium iodide.

Results

Granulysin exhibits enhanced lytic activity after reduction and boiling

A hallmark of SAPLIP family members is the characteristic spacing of conserved cysteine residues. These cysteine residues are predicted to form intramolecular disulfide bonds critical for protein structure and function. Reduction of disulfide bonds in NK-lysin, the SAPLIP member most similar to granulysin, eliminated its lytic activity against bacteria and tumor cells (9). To test whether the disulfide bonds in granulysin contribute to its function, recombinant 9-kDa granulysin was treated with 1 mM DTT and boiled for 10 min. Surprisingly, boiled and reduced granulysin is ~25 times more potent than refolded granulysin at lysing Jurkat cells, as judged by PI uptake (Fig. 1*A*). Consistent with this observation, disruption of liposomes is more complete using boiled and reduced rather than refolded granulysin (Fig. 1*B*). A mutant construct of granulysin was generated in which all four cysteine residues were changed to serine. After expression, purification, and refolding, the serine-substituted recombinant granulysin exhibited increased lytic activity relative to the cysteine-containing molecule (not shown). In contrast, boiled and reduced granulysin was equipotent to refolded granulysin in lysis of *Salmonella* (Fig. 1*C*). These findings indicate that lytic activity of recombinant granulysin does not depend on disulfide bonds or three dimensional structure and that short linear regions of the granulysin sequence might mediate lysis. Therefore, we prepared a panel of synthetic peptides corresponding to linear sequences of granulysin and assessed their lytic activity.

Helix 2 and helix 3 are important for lysis by granulysin

As a first step, five overlapping synthetic peptides (G1 to G5) and two longer peptides (G6 and G7) were synthesized (Table I). In addition, all cysteine residues were substituted with serine. Only peptides G3 and G7, which correspond to the central region of granulysin, lyse Jurkat and *Salmonella* (not shown). Peptides corresponding to the amino (G1, G2, G6) or carboxyl (G4, G5) regions of granulysin do not lyse either Jurkat or *Salmonella* at concentrations up to 100 μM (not shown).

Having narrowed our analysis to the region including helix 2 through helix 3, peptides G8 to G12 (Table I) were designed based on the predicted secondary structure of granulysin. Each of these peptides was assayed for lysis of *Salmonella*, human cells (Jurkat and PBL), and synthetic liposomes (Table II and Fig. 2). G9, which includes helix 2 through helix 3 and retains the serine for cysteine substitutions, shows potent activity against bacteria and liposomes but only intermediate activity against human cells. G8 is

Table I. *Peptide sequences*

Granulysin[a]	GR**DYRTCLTIVQKLKKMV**DKPT**QRSVSNAATRVCRT**GRSRW**RDVCRNFMRR**Y**QSRVIQGLV**AGET**AQQICED**LRLCIPST H1 H2 H3 H4 H5
G1[b] (1–19, C7 → S7)[c]	GRDYRTSLTIVQKLKKMVD
G2 (16–34, C34 → S34)	KMVDKPTQRSVSNAATRVS
G3 (30–49, C34,45 → S34,45)	ATRVSRTGRSRWRDVSRNFM
G4 (45–65, C45 → S45)	SRNFMRRYQSRVIQGLVAGET
G5 (61–80, C70,76 → S70,76)	VAGETAQQISEDLRLSIPST
G6 (1–24, C7 → S7)	GRDYRTSLTIVQKLKKMVDKPTQR
G7 (25–49, C34,45 → S34,45)	SVSNAATRVSRTGRSRWRDVSRNFM
G8 (23–51)	QRSVSNAATRVCRTGRSRWRDVCRNFMRR
G9 (23–51, C34,45 → S34,45)	QRSVSNAATRVSRTGRSRWRDVSRNFMRR
G10 (23–36)	QRSVSNAATRVCRT
G11 (42–51)	RDVCRNFMRR
G12 (42–61)	RDVCRNFMRRYQSRVIQGLV
G13 (23–41)	QRSVSNAATRVCRTGRSRW
G14 (37–51)	GRSRWRDVCRNFMRR
G15 (37–51, R38,40 → Q38,40)	GQSQWRDVCRNFMRR

[a] Predicted α helices are shown in bold.
[b] G1–G15 are the designations for each peptide.
[c] Residues included in each peptide, substitutions in sequence.

identical with G9 except that it contains the native cysteine residues at positions 34 and 45. It has high activity against tumor cells and is comparable to G9 in bacterial and liposome lysis assays. Both G8 and G9 are indistinguishable from full-length, reduced recombinant granulysin in lysing bacteria, but both are much more potent than reduced, full-length molecules in lysing tumor cells. Peptide G10, corresponding to helix 2, does not lyse bacteria, tumor cells, or liposomes, while G11, corresponding to helix 3, lyses bacteria but not tumor cells and shows intermediate activity against liposomes. Peptide G12 extends G11 at the carboxyl end through helix 4. G12 exhibits increased activity against all targets relative to G11, but is not as potent as G8. Thus, a stretch of the granulysin sequence, corresponding to a region including helix 2 and helix 3, recapitulates the lytic activity of full-length, reduced granulysin.

The positive charges in loop 2 are important for lysis

Two of the five residues in the loop region between helix 2 and helix 3 (designated loop 2) are positively charged arginine residues (Table I). In other bactericidal peptides, positive charges are critical for lytic activity (12, 13). To analyze the contribution of the loop 2 region to lysis, peptides G13, G14, and G15 (Table I) were synthesized and assayed against all three targets (Table II and Fig. 3). Peptide G13 contains helix 2 plus loop 2, while G14 contains loop 2 plus helix 3. Both peptides exhibit higher lytic activity against bacteria than their shorter counterparts, G10 and G11, which lack the loop 2 sequence. G13 does not lyse Jurkat cells or liposomes, while G14 shows intermediate activity against Jurkat targets and efficiently lyses liposomes. G15, in which the two positively charged arginine residues in loop 2 are replaced with glutamine, shows reduced activity against bacteria and liposomes and does not lyse Jurkat cells at all. Thus, lysis of Jurkat and liposomes by these peptides is dependent on the presence of helix 3, and the positively charged residues in loop 2 are critical for the lytic function of these peptides.

Activity of reduced peptides

The wild-type peptide G8 and its serine substitute counterpart, G9, both are potent anti-bacterial agents, but G9 exhibits significantly less activity against tumor cells than G8 (Table II and Fig. 2). This indicates that disulfide bonds are important for anti-tumor activity but are not required for anti-bacterial activity. To confirm this, granulysin peptides were diluted in medium containing 1 mM DTT and then incubated with bacteria or tumor cells in medium supplemented with DTT. These conditions had no detectable effect on the viability of Jurkat cells, but did slightly decrease the viability of *Salmonella* (data not shown). In the presence of DTT, G8 (cysteines at residues 34 and 45) and G14 (cysteine at residue 45) lose most of their anti-tumor activity (Fig. 4). DTT does not affect the anti-bacterial activity of G8 or G14. These findings suggest that both intra- and intermolecular disulfide bonds likely affect the activity of peptides derived from granulysin.

Table II. *Summary of peptide lysis*

Peptide	Percent Lysis of		
	Salmonella[a]	Jurkat[b]	Liposomes[c]
G8	100	90	100
G9	100	0	100
G10	0	0	10
G11	80	0	40
G12	100	20	100
G13	100	0	10
G14	100	50	100
G15	80	0	50

[a] Percent lysis at 10 μM peptide.
[b] Percent lysis at 20 μM peptide.
[c] Percent lysis at 0.5 μM peptide.

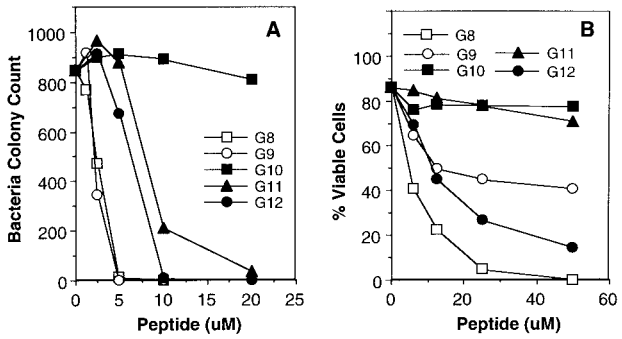

FIGURE 2. Synthetic peptides containing helix 2 or 3 lyse *Salmonella*, but only peptides containing helix 3 lyse Jurkat.

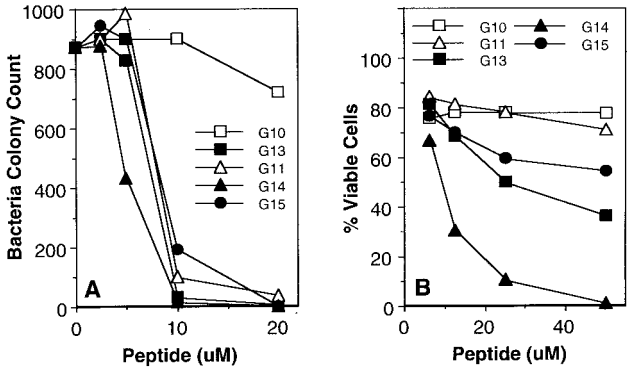

FIGURE 3. Arginine residues in loop 2 contribute to lysis of Jurkat but not *Salmonella*.

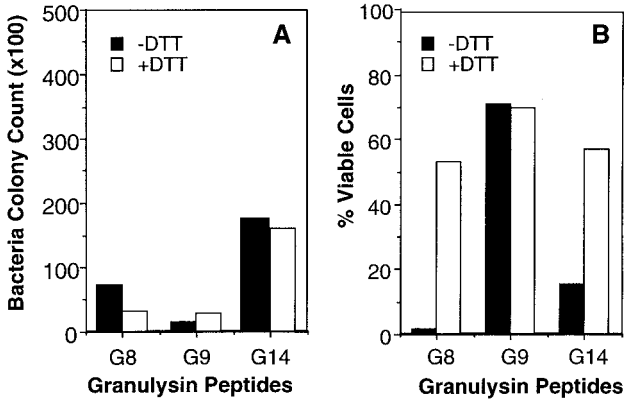

FIGURE 4. Both intra- and intermolecular disulfide bonds in granulysin peptides affect lysis of Jurkat but not *Salmonella*. Peptides G8 (cysteines at residues 34 and 45), G9 (no cysteine residues), or G14 (cysteine at residue 45) were treated with or without DTT as described in *Materials and Methods* and incubated at 10 μM with Jurkat or *Salmonella*.

Granulysin-induced apoptosis is blocked in cells expressing anti-apoptotic genes

Previously, we found that granulysin-induced apoptosis of Jurkat cells is inhibited by Bcl-2 and CrmA.[3] CrmA protection implicates caspases in the death pathway (14), while Bcl-2 protection suggests that the apoptotic pathway induced by granulysin involves mitochondrial damage (15, 16). To determine whether apoptosis induced by granulysin peptides was similar to that induced by recombinant granulysin, we assessed PI uptake in Jurkat cells transfected with Bcl-2, CrmA, or vector control (Fig. 5). Treatment of vector control cells with anti-Fas mAb or recombinant granulysin caused PI uptake in >80% of cells, while both Bcl-2 and CrmA reduced granulysin and anti-Fas mAb-induced death. PI uptake mediated by G8 and G14 was significantly reduced in the Bcl-2 and CrmA transfectants, suggesting that these peptides induce apoptosis by a similar mechanism to intact 9-kDa granulysin.

Discussion

Granulysin is a novel effector molecule present in human CTL and NK cells (2). Granulysin kills bacteria (5), disrupts synthetic liposomes (3), and induces apoptosis of mammalian cells (4). CTL are capable of killing intracellular bacteria via a granule-mediated mechanism (17), and granulysin is the only bactericidal molecule yet identified in CTL granules. In this report, synthetic peptides were tested for lytic activity. Peptides from the central region of granulysin recapitulated the lytic activity, and substitution analyses demonstrate that the mechanism of lysis of bacteria and tumor cells are distinct. Recently, Andreu et al. reported on two synthetic peptides derived from granulysin (18). The first peptide consists of 30 aa, corresponding to most of helix 2 through the beginning of helix 4; the second is a 23-aa peptide that encompasses the end of helix 2 through the beginning of helix 4. Both peptides lysed a variety of bacteria as well as the human NK cell line, K562.

In addition to the highly specific, cell-mediated immune system, vertebrates and other organisms fight infections by releasing broad-spectrum anti-microbial peptides (19–21). Currently, several hundred structures with varying degrees of anti-microbial activity have been described, but very few are of human origin. These "peptide antibiotics" can be divided into three major groups based on gross motifs and three-dimensional structure; sequence similarities within each group are almost negligible. Group I consists of linear peptides that lack cysteine residues, including cecropins, magainins, bombinins, and temporins. Group II consists of peptides with an even number of cysteines intralinked by disulfide bridges, including insect and mammalian defensins. Group III consists of peptides containing a very high content of unusual amino acids, most often proline and arginine together, including porcine PR-39 and bovine Bac5. Peptide antibiotics are usually synthesized as inactive precursors, two to five times the size of active effector molecules.

Granulysin is a member of group II, as it contains four cysteine residues that can form disulfide bonds. NK-lysin, the nearest homologue of granulysin, loses all activity when its disulfide bonds are reduced (9). In contrast, reduction of recombinant granulysin increases lysis of tumors while not changing activity against bacteria. Surprisingly, DTT treatment of peptide G8, which comprises the central helices of granulysin, decreases lysis of Jurkat targets

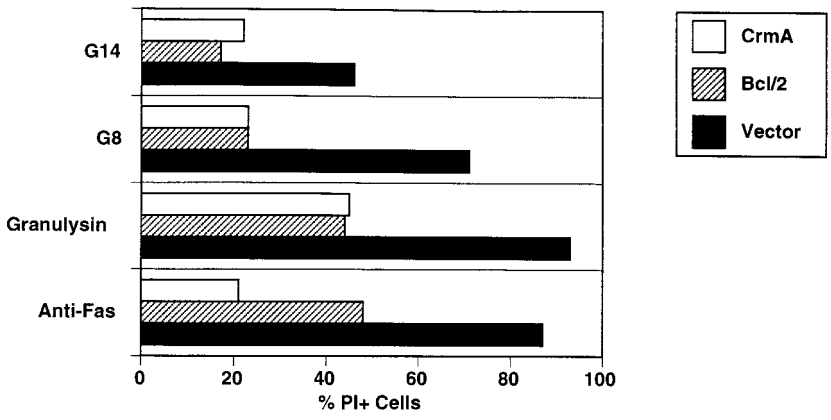

FIGURE 5. CrmA and Bcl/2 protect against lysis by anti-Fas mAb, granulysin, and peptides G8 and G14. Aniti-Fas mAb (5 μg/ml), recombinant granulysin (50 μM), or peptides G8 or G14 (10 μM each) were incubated with Jurkat cells transfected with empty vector (■), Bcl/2 (▨), or CrmA (□). PI uptake was measured after incubation overnight.

but does not affect lysis of *Salmonella*. The cytolytic activity of G14, which contains only one cysteine and therefore cannot form intramolecular disulfide bridges, is also sensitive to DTT, indicating that both inter- and intramolecular bonds affect the activity of granulysin and peptides derived from it.

A high content of positively charged amino acids spread throughout the peptide is another characteristic of many antimicrobial peptides (19, 20). In granulysin, nine of the 29 residues that comprise helix 2 through helix 3 are arginines. However, a high number of positively charged amino acids is not sufficient for lytic activity: seven of the 24 residues in peptide G6 are positive, but it does not exhibit activity against either bacteria or tumor targets. Substitution of two arginine residues with glutamine in G15 abrogates lytic activity against Jurkat and somewhat reduces activity against *Salmonella*.

The mechanism by which peptides G8 and G14 cause apoptosis shares critical features with that of recombinant granulysin. Transfectants expressing Bcl-2 or CrmA are protected from lysis by all three molecules. The anti-apoptotic effect of Bcl-2 on granulysin-induced apoptosis indicates that mitochondrial damage is a critical step in granulysin-induced apoptosis (15, 16). Using the caspase inhibitor z-VAD-fmk, we previously showed that caspases are involved in granulysin-mediated apoptosis (4). This observation is extended here, as overexpression of CrmA, which binds and inactivates certain processed caspases, including activated caspase 8 (22), protects from granulysin- and peptide-mediated apoptosis.

Our findings suggest that the mechanism of lysis of bacteria and tumors by granulysin peptides are different. Peptides containing either helix 2 or helix 3 lyse bacteria, while only peptides containing helix 3 lyse tumor targets. Both intra- and intermolecular disulfide bonds are necessary for peptide lysis of tumor targets, whereas lysis of bacteria is unaffected by reduction of disulfide bonds. In contrast, peptide lysis of both tumor targets and bacteria is sensitive to substitution of arginine residues in loop 2. Collectively, these differences may provide the basis for development of novel selective antibiotics.

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