# Granulysin, a T Cell Product, Kills Bacteria by Altering Membrane Permeability[1]

**William A. Ernst,*‡§ Sybille Thoma-Uszynski,*‡§ Rachel Teitelbaum,¶ Christine Ko,*‡§ Dennis A. Hanson, Carol Clayberger, Alan M. Krensky, Matthias Leippe,# Barry R. Bloom,¶ Tomas Ganz,† Robert L. Modlin[2]*‡§**

**Granulysin, a protein located in the acidic granules of human NK cells and cytotoxic T cells, has antimicrobial activity against a broad spectrum of microbial pathogens. A predicted model generated from the nuclear magnetic resonance structure of a related protein, NK lysin, suggested that granulysin contains a four $\alpha$ helical bundle motif, with the $\alpha$ helices enriched for positively charged amino acids, including arginine and lysine residues. Denaturation of the polypeptide reduced the $\alpha$ helical content from 49 to 18% resulted in complete inhibition of antimicrobial activity. Chemical modification of the arginine, but not the lysine, residues also blocked the antimicrobial activity and interfered with the ability of granulysin to adhere to *Escherichia coli* and *Mycobacterium tuberculosis*. Granulysin increased the permeability of bacterial membranes, as judged by its ability to allow access of cytosolic $\beta$-galactosidase to its impermeant substrate. By electron microscopy, granulysin triggered fluid accumulation in the periplasm of *M. tuberculosis*, consistent with osmotic perturbation. These data suggest that the ability of granulysin to kill microbial pathogens is dependent on direct interaction with the microbial cell wall and/or membrane, leading to increased permeability and lysis.** *The Journal of Immunology*, **2000, 165: 7102–7108.**

It is generally recognized that T cells contribute to host defense against microbial pathogens by secreting cytokines that can activate antimicrobial effector pathways and directly lyse infected targets. A third mechanism involving direct T cell antimicrobial activity was suggested from studies identifying granulysin (1) in cytoplasmic granules of cytolytic T cells (2, 3). Granulysin is a member of the saposin-like protein (SAPLIP)[3] family, including amoebapores, antimicrobial proteins that amoebas use to prevent growth of phagocytosed bacteria (4). Granulysin itself has a broad spectrum of antimicrobial activity, killing bacteria, fungi, and parasites, but is poorly lytic against cells of the monocyte/macrophage lineage (5). Granulysin, in combination with perforin, a lytic molecule that colocalizes with granulysin in cytotoxic granules, can kill the intracellular pathogen *M. tuberculosis* in macrophages (5). These data provide a novel pathway by which cytolytic T cells can directly kill microbial pathogens.

Antimicrobial proteins and peptides are diverse in their structure and mechanism by which they kill infectious agents. Many antimicrobial peptides, however, have a secondary structure based on either $\alpha$ helices or $\beta$ sheets. The antimicrobial activity of these peptides is dependent on their ability to form multimers that facilitate pore formation leading to cell death (6). In addition, most antimicrobial peptides are cationic, although amino acid usage varies, including arginine, histidine, and lysine (7, 8). The putative structure of granulysin is a four $\alpha$ helical bundle similar to the amoebapore family members (3). Because granulysin is the only antimicrobial peptide to have been identified in T cells, we thought it important to clarify its mechanism of action.

Experiments were performed to correlate the structure and function of granulysin using biophysical approaches. Synthetic peptides of granulysin conforming to a putative helix-loop-helix motif (aa 1–35, 36–70, and 31–50) retained 50–80% of anti-bacterial activity, whereas those peptides without this predicted structure (aa 1–20, 16–35, 46–65, 61–80) had $<$20% activity. The structural model also predicts that the $\alpha$ helices are amphipathic, including 15 positively charged amino acids: 12 arginine (16%) and three lysine residues. Chemical modification of the arginine residues caused complete inhibition of the antimicrobial effects of granulysin; however, modification of the lysine residues did not inhibit the antimicrobial activity. Granulysin altered bacterial membranes by increasing their permeability, inducing lesions on the surface of bacteria and separation of the cell wall and membranes from the cytoplasm. These data suggest that the ability of granulysin to kill microbial pathogens is dependent on interactions with the microbial cell wall or membrane leading to increased permeability and osmotic lysis. Differences in structure and function of granulysin compared with amoebapores suggest that granulysin has evolved as a potent weapon of T cells to combat microbial pathogens.

*Division of Dermatology, †Division of Pulmonary Medicine, ‡Department of Microbiology and Immunology, and §Molecular Biology Institute, University of California School of Medicine, Los Angeles, CA 90095; ¶Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY 10461; ‖Office of the Dean, Harvard School of Public Health, Boston, MA 02115; ‖Division of Immunology and Transplantation Biology, Department of Pediatrics, Stanford University, Stanford, CA, 94305; and #Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

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[2] Address correspondence and reprint requests to Dr. Robert L. Modlin, Division of Dermatology, University of California, 52-121 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail address: rmodlin@mednet.ucla.edu

[3] Abbreviations used in this paper: SAPLIP, saposin-like protein; TSB, trypticase soy broth; BAD, butanedione; CAH, citraconic anhydride; ONPG, *o*-nitrophenyl-$\beta$-D-galactoside; NMR, nuclear magnetic resonance.



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## Materials and Methods

### *Production and purification of recombinant granulysin*

Granulysin was produced in *Escherichia coli* BL21 (DE3) transformed with the kanamycin-selective vector, pET28, containing a hexahistidine fusion tag (Novagen, Madison, WI) as previously described (2). Briefly, the transformed *E. coli* were grown in 2× YT and induced with 1 mM isopropyl-β-D-thiogalactoside (Fisher Scientific, Pittsburgh, PA). The bacteria were harvested and denatured in 6 M guanidine HCl/0.05 mM Tris-HCl (pH 7.4). Granulysin was purified via nickel affinity chromatography according to the manufacturer's recommendation (Qiagen, Valencia, CA) and eluted with 0.2 M imidazole, then reduced with 10 mM DTT. The denatured granulysin was renatured in 0.75 M arginine, 0.05 M Tris-HCl (pH 8), 0.05 M KCl, 0.1 mM EDTA, and 10 mM oxidized DTT at a 1/5 dilution with constant stirring for 48 h at 4°C. The renaturing buffer was exchanged by dialysis with 2 mM sodium phosphate and 13 mM NaCl (pH 7.2), then lyophilized. The granulysin pellet was rehydrated and treated with thrombin for 16 h to cleave the hexahistidine tag. Following thrombin cleavage, the protein was loaded onto a Rainin $C_{18}$ reverse phase chromatography column (Braintree, MA) and eluted by a linear gradient of 10–60% aqueous acetonitrile in 0.1% trifluoroacetic acid. The fractions containing granulysin, as determined by Coomassie staining of a 15% SDS-PAGE gel, were lyophilized and hydrated in 10 mM sodium phosphate (pH 7.2) unless otherwise noted. The final protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as a standard. Protein purity was assessed by Coomassie staining of 15% SDS-PAGE gels and was >95%. Additionally, purified granulysin was analyzed by matrix-assisted laser desorption ionization mass spectrometry and shown to contain one species at 9081 Da that corresponded to the calculated m.w., which is 9070.4 assuming that four of the five cysteines are involved in disulfide bridges.

### *Peptide synthesis*

Peptides were synthesized using F-moc chemistry on an Applied Biosystems (Foster City, CA) automatic peptide synthesizer and were purified to >95% homogeneity by reverse phase HPLC, and peptide composition was confirmed by mass spectrometry and amino acid analysis. Stock peptide solutions were prepared at 20 mg/ml in DMSO. The sequences for the peptides used are as follows: 1–35, GRDYRTSLTIVQKLKKMVDKPT QRSVSNAATRVSR; 36–70, TGRSRWRDVSRNFMRRYQSRVIQGLV AGETAQQIS; 1–20, GRDYRTSLTIVQKLKKMVDK; 31–50, TRVSRT GRSRWRDVSRNFMR; 16–35, KMVDKPTQRSVSNAATRVSR; 46–65, RNFMRRYQSRVIQGLVAGET; and 61–80, VAGETAQQISEDLR.

Instead of constructing the 62–74 peptide, additional sequence from the 15-kDa form of granulysin was used to construct the 61–80 peptide and maintain this peptide at a similar length as the others. Amoebapore A was purified from trophozoites of *E. histolytica* using reverse phase HPLC as the final purification step (9). Melittin was obtained from Sigma (St. Louis, MO).

### *CFU assay*

The CFU assay was performed as described previously (5, 10). Briefly, *E. coli* (strain ML-35p) was grown in trypticase soy broth (TSB) collected in mid-log phase and washed three times with 10 mM sodium phosphate, pH 7.4, supplemented with 0.03% TSB. Various concentrations of granulysin and granulysin peptides with 60 μl of $2.5 \times 10^5$ bacteria/ml were incubated at 37°C for 3 h. After incubation, the bacteria were placed on ice, and 10-fold dilutions were prepared and plated on TSB agar plates. The plates were incubated for 2 h at 37°C, then overnight at room temperature. For kinetic studies, bacteria were incubated with 0.5 and 2.5 μM granulysin and then diluted and plated at the indicated times. To evaluate the effects of pH, granulysin was hydrated in 10 mM sodium phosphate supplemented with 0.03% TSB at various pH units. The samples were incubated at 37°C for 3 h and processed as described above. To evaluate the effects of ionic concentration on granulysin, *E. coli* were incubated with various concentrations of granulysin in 10 mM sodium phosphate, pH 7.4, supplemented with 0.03% TSB and 40, 90, 140, and 290 mM NaCl. After the incubation period, the samples were diluted and plated as described above. *M. tuberculosis* was recovered from frozen aliquots and washed twice in Middlebrook 7H9 medium. To prevent clumping, mild sonication was performed before and after washing. Bacteria ($1 \times 10^4$) were added in 90 μl/well of a 96-well flat-bottom tissue culture plate. Recombinant granulysin and synthetic peptides were dissolved and diluted in 10 mM sodium phosphate buffer, pH 7.2, supplemented with 0.03% TSB, and incubated with bacteria at 37°C for 72 h. After incubation, serial dilutions were plated on Middlebrook 7H11 agar plates, and the colonies were counted after 2 wk.

### *Radial diffusion assay*

The radial diffusion assay was used to determine the relative bactericidal activity of chemically modified granulysin and granulysin peptides. A modified assay (11) was performed. Briefly, ML-35p *E. coli* was collected at mid-log phase, and $4 \times 10^6$ bacteria were pour-plated in 10 ml of 1% agarose dissolved in 10 mM sodium phosphate, pH 7.4, supplemented with 0.3% TSB. After the agarose solidified, 3-mm holes were punched and removed from the agarose. Then, 5 μl of the samples were added to the wells and incubated at 37°C for 3 h. After the incubation, 10 ml of 1% agarose and 6% TSB were poured as an overlay. The plates were incubated for an additional 16 h at 37°C. The antimicrobial effects were evaluated by measuring the amount of clearing in millimeters and multiplying by a factor of 10 to yield radial diffusion units.

### *Chemical modification for radial diffusion assay*

To chemically modify granulysin, granulysin peptides and amoebapore A, we incubated 100 μM protein, 40 mM butanedione (BAD) or 20 mM citraconic anhydride (CAH; Sigma), and 10 mM sodium borate, pH 8.0, for 2 h. The samples were placed in the wells of the radial diffusion plate and incubated as described above.

### *Chemical modification for Western blot analysis*

To 1 μg of granulysin, varying doses of BAD and CAH were used for chemical modification. The modified protein was incubated with $1.5 \times 10^4$ *E. coli* or $2.5 \times 10^5$ *M. tuberculosis* in 10 mM sodium phosphate, pH 7.4, supplemented with 0.03% TSB for 1 h, then washed three times with 10 mM sodium phosphate, pH 7.4, to remove unbound granulysin. The bacteria were suspended in 20% Tween 20 and subjected to two rounds of freezing and boiling. The level of granulysin retained with the *E. coli* was determined by Western blot analysis. Samples were run on a 15% SDS-PAGE and blotted to nitrocellulose. Granulysin was detected using the anti-granulysin mAb DH4 (12).

### *Membrane permeability of granulysin and granulysin peptides*

The membrane permeability effects of granulysin and amoebapore A were assayed by adding protein to stationary phase *E. coli* ML-35p. Bacteria were grown for 16 h at 37°C in TSB broth, then washed three times in 10 mM sodium phosphate, pH 7.4. *O*-nitrophenyl-β-D-galactoside (ONPG; Sigma; 2.5 mM), $1 \times 10^7$ bacteria, and 2.5 μM of protein were added to a well of a microtiter plate (Costar, Cambridge, MA). As a control, 1 μM melittin was incubated with *E. coli* as described above. The plate was read in a microtiter plate reader at 410 nm. To assess the effect of chemically modified granulysin on membrane permeability, 2.5 μM granulysin was preincubated with 40 mM BAD and then added to the assay system described above. BAD (40 mM) had no effect on the assay system (data not shown).

### *Circular dichroic analysis of granulysin*

Circular dichroic measurements were performed on an Aviv 62A DS circular dichroic spectrapolarimeter (Aviv, Lakewood, NJ) using a quartz cuvette with a pathlength of 10 mm at 25°C as described previously (13). The data are reported as the average of 10 scans at 50 nm/min with a 0.1-nm step resolution. Spectra were recorded in the far UV at a protein concentration of 1 mg/ml in 10 mM sodium phosphate buffer, pH 7.2. For the evaluation at various pH units, granulysin was diluted in 10 mM sodium phosphate at the appropriate pH. Data are reported as the mean residue ellipticity, $[\theta]$, in units of degrees $cm^2$/decimole.

A model of granulysin was constructed based on the nuclear magnetic resonance (NMR) structure of NK-lysin (PDB accession code 1NKL) (14). Briefly, using the fold recognition server (http://fold.doe-mbi.ucla.edu/), the sequence of granulysin was threaded onto the NMR backbone of NK-lysin (15). Rasmol freeware (http://www.ncbi.nlm.nih.gov/Structure/CN3D/rasmol.html) was used to visual the three-dimensional structure of granulysin. Estimation of the α helical concentration of granulysin was performed using Prosec secondary structural analysis software (Aviv).

### *Transmission electron microscopy*

For transmission electron microscopy $5 \times 10^6$ tubercle bacilli (Erdman strain) were incubated with either 30 μM purified granulysin or control protein for 80 h. Bacilli were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, buffered solution for 6 h. Bacteria were post-fixed in 1% osmium tetroxide, en bloc stained in uranyl acetate, dehydrated in a graded ethanol series, then embedded in LX112 (Ladd Research, Burlington, VT) under vacuum in beem capsules and polymerized at 60°C.

Thin sections were stained with uranyl acid and lead citrate before examination on a JEOL 1200EX or JEOL 100CX transmission electron microscope (Peabody, MA).

## Results

*Antimicrobial effects of granulysin peptides on* E. coli

The ability of peptide constituents of the full-length 9-kDa human granulysin were tested for antimicrobial activity against *E. coli* using a CFU assay. Three peptides (peptides 1–35, 36–70, and 31–50) had significant antimicrobial activity, reducing by >4 logs the number of viable *E. coli* (Fig. 1*A*). Four peptides had no or only weak antimicrobial activity (peptides 1–20, 16–35, 46–65, and 61–80), reducing by <2 logs the number of viable bacteria (Fig. 1*B*). These results were confirmed using a radial diffusion assay (data not shown). The predicted structure of the peptides with antimicrobial activity corresponded to protein segments of granulysin with a helix-loop-helix motif according to a model of granulysin based on the NMR structure of NK-lysin (Fig. 1*D*). In addition, these peptides were rich in positively charged amino acids, arginine, and lysine residues. In contrast, the peptides that had weaker antimicrobial activity either corresponded to the protein segment of granulysin with a single $\alpha$ helix or helical-loop structure or had fewer positively charged residues. These data correlate a helix-loop-helix motif containing positively charged residues with the antimicrobial activity of granulysin.

*Antimicrobial effects of granulysin peptides on* M. tuberculosis

Because we have previously shown that granulysin killed *M. tuberculosis*, it was reasonable to determine the antimicrobial effect of the granulysin peptides on this pathogen. Similar to the results observed when granulysin was incubated with *E. coli*, full-length granulysin had the strongest antimicrobial effect against *M. tuberculosis*, with 65% killing at 25 $\mu$M. When *M. tuberculosis* was treated with peptides 1–35, 36–70, and 31–50, killing was observed in a dose-dependent fashion (Fig. 1*C*). However, compared with granulysin only 25–40% of the bacteria were killed when treated with 25 $\mu$M peptide, with as much as 45–60% killing at a peptide concentration of 100 $\mu$M. Similar to the experiments with *E. coli*, peptide 1–20 had no effect on *M. tuberculosis*. However, peptide 31–50, which showed at least 2-fold less activity than peptides 1–35 and 36–70 against *E. coli*, showed equal activity to these two peptides against *M. tuberculosis*. These differences may be due to differences in the cell wall and membrane structure between the two organisms and their ability to interact with the granulysin peptides.

*Secondary structural analysis of recombinant granulysin*

The putative three-dimensional structure of granulysin modeled on the backbone of the NK lysin NMR data indicated that granulysin conforms to a four $\alpha$ helical bundle, with a predicted $\alpha$ helical content of ~74%. The $\alpha$ helical content of recombinant granulysin was measured by circular dichroism according to its molecular ellipticity. We observed spectral minimum at both 222 and 208 nm, indicating a highly $\alpha$ helical structure, and based on secondary structural analysis, this was determined to be 49%. The difference between the measured and calculated $\alpha$ helical content may reflect inaccuracies in the estimation or indicate that part of the granulysin did not refold during purification. We found no difference in the spectra of granulysin from pH 8.2 to pH 3.0, indicating that granulysin is an acid-stable protein (Fig. 2*A*). To denature granulysin, granulysin was boiled, but this did not alter its circular dichroic spectra. However, boiling in the presence of 2-ME, to destroy the disulfide linkages, altered the circular dichroic spectra with a calculated reduction in $\alpha$ helical content to 18% (Fig. 2*B*). The loss of granulysin $\alpha$ helices correlated directly with decreased antimicrobial activity, as measured by the radial diffusion assay (Fig. 2*C*). These data further implicate the three-dimensional structure of granulysin in its antimicrobial activity.

*Functional analysis of the antimicrobial activity of granulysin*

To further characterize the antimicrobial activity of granulysin according to pH and ionic strength, a series of experiments was performed. The kinetic activity of granulysin was analyzed to determine the rate at which granulysin kills *E. coli*. By incubating *E. coli* with either 0.5 or 2.5 $\mu$M granulysin at pH 7.2 and measuring CFU vs time, we observed a 5-fold decrease in CFU after 2 h (Fig.

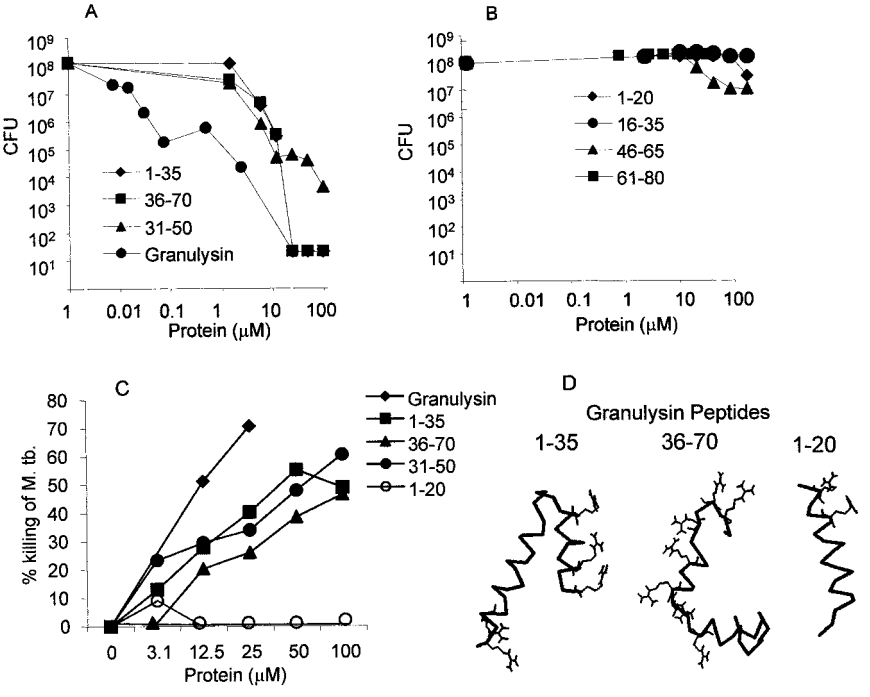


**FIGURE 1.** Antimicrobial activity of granulysin and granulysin peptides. *A*, Synthetic peptides of granulysin predicted to conform to a helix-loop-helix motif (residues 1–35, 36–70, and 31–50) and the 9-kDa granulysin were incubated with *E. coli* at various concentrations for 3 h, diluted in 10 mM $NaPO_4$ with 0.03% TSB, and plated on TSB agar plates to determine antimicrobial activity by CFU assay. *B*, Additional synthetic granulysin peptides (residues 1–20, 16–35, 46–65, and 61–80) were compared with the 9-kDa granulysin for antimicrobial activity by CFU assay. *C*, Various concentrations of synthetic peptides of granulysin and granulysin were incubated with *M. tuberculosis* and tested for antimicrobial activity by the CFU assay. *D*, Putative structures of three granulysin peptides modeled after the NK-lysin molecule. The 1–35 and 36–70 peptides show a distinct helix-loop-helix motif, whereas the 1–20 peptide shows a single helix.

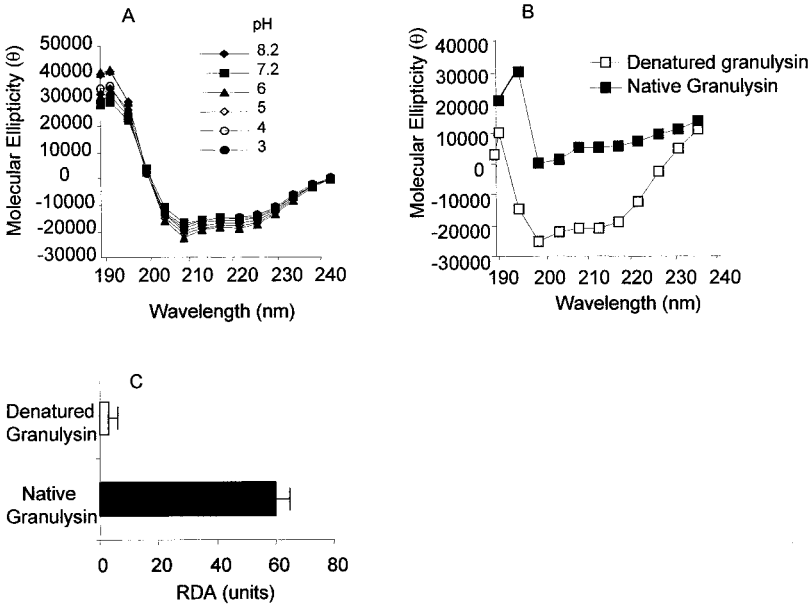


**FIGURE 2.** Secondary structural analysis of granulysin and denatured granulysin. *A*, The secondary structure of granulysin at various pHs was determined by measuring the circular dichroic spectra of granulysin at 208 and 222 nm. The data collected were used to determine the relative amount of $\alpha$ helices using Prosec secondary structural analysis software. *B*, The secondary structure was measured for denatured granulysin. Denatured granulysin (boiled for 5 min in the presence of 140 mM 2-ME) was compared with native granulysin. *C*, The antimicrobial activity of denatured granulysin was compared with native granulysin using the radial diffusion method.

3*A*). The effect of pH on granulysin antimicrobial activity was examined from a range of pH 8 to pH 4.5 (Fig. 3*B*). Granulysin at pH 8 had the highest specific activity against *E. coli*, with nearly a 7-log reduction in CFU with 0.1 $\mu$M. Granulysin at pH 7.2 showed a 3-log reduction in CFU at 0.1 $\mu$M. There was very little difference in the antimicrobial effects of granulysin from pH 6 to pH 4.5. The ability of granulysin to lyse liposomes was similarly pH dependent (12).

Because other cationic antimicrobial proteins exhibit their effects at least partially through ionic interaction, we tested the ability of granulysin to kill at varying ionic concentrations (Fig. 3*C*). At 10 mM salt, granulysin exhibited the highest level of killing, with a 6-log decrease in CFU with 2.5 $\mu$M granulysin. At 50 mM salt the level of killing decreased by a factor of 10. By 100 mM salt there was only a 1.5 log decrease at the highest concentration tested, 25 $\mu$M granulysin. In summary, granulysin effectively killed *E. coli* at 120 min and optimally did so at neutral pH and low ionic strength. These data are reminiscent of the effects of pH and ionic strength on defensins and suggest that electrostatic interactions are the predominant determinants of granulysin activity.

### *Evaluation of chemically modified granulysin*

We therefore reasoned that granulysin was acting at least in part through electrostatic interaction of its cationic amino acid side chains with the negatively charged bacterial cell membrane. Because 17.5% of the protein is composed of arginine residues (13 of 74 residues), and 5.5% are lysine residues (4 of 74 residues), granulysin is a highly basic protein with a calculated pI of 10.8. To establish the role the basic amino acids in the antimicrobial activity, we altered both arginine and lysine residues with specific chemical modifiers. To verify the specificity of the modification, amoebapore A was also studied as a control. Amoebapore A has

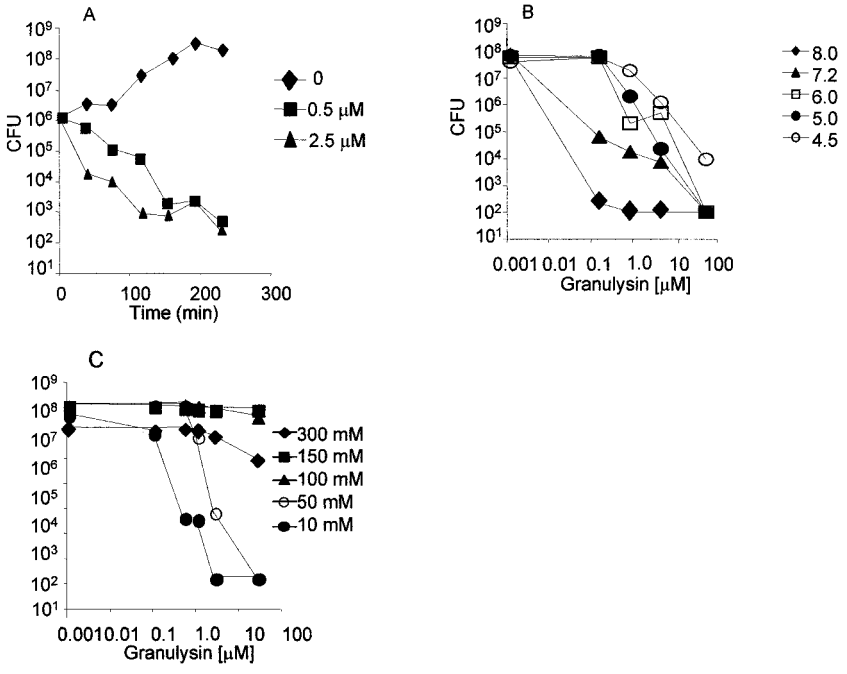


**FIGURE 3.** An analysis of the rate of killing and effect of ionic interaction between granulysin and *E. coli*. *A*, The rate at which granulysin kills *E. coli* was analyzed by incubating either 0.5 or 2.5 $\mu$M granulysin for up to 4 h. Samples were taken at various time points, diluted, and plated onto TSB plates to determine the number of CFU. *B*, The effect of pH on granulysin activity was examined. Various concentrations of granulysin were incubated with *E. coli* with varying concentrations of salt for 3 h. The bacteria were diluted and plated to determine the number of CFU. *C*, The effect of ionic strength on granulysin activity was examined. Various concentrations of granulysin were incubated with *E. coli* with varying concentrations of salt for 3 h. The bacteria were diluted and plated to determine the number of CFU.

been shown previously to have antimicrobial activity against a wide range of microbes, and modification of its lysines inhibited binding to synthetic phospholipid vesicles. Unlike granulysin, the amoebapore protein does not contain any arginines.

Modification of the arginine residues with BAD significantly decreased the antimicrobial activity of granulysin and the three active peptides against *E. coli*, but had no effect on the activity of amoebapore A (Fig. 4*A*). In contrast, modification of the lysine residues with CAH had no effect on the antimicrobial activity of granulysin, but significantly inhibited the antimicrobial activity of amoebapore A (Fig. 4*B*). To verify that granulysin was modified, MALDI-TOF mass spectroscopy was performed and showed an increase in the mass of the modified granulysin with BAD, but not CAH, indicating that the lysines were not available for modification (data not shown) and therefore may not have a role in the antimicrobial activity. Together, these data point to a differential role of arginine and lysine residues in the antimicrobial activity of granulysin and amoebapore A.

We hypothesized that the positively charged arginine residues mediate the binding of granulysin to the negatively charged bacterial surface. The interaction of granulysin with the bacteria was measured incubating the granulysin with *E. coli* and assaying for the amount of granulysin associated with *E. coli* by Western blot. Using this approach, it was possible to demonstrate the direct interaction of granulysin with *E. coli* (Fig. 4*C*). Treatment of granulysin with increasing levels of BAD completely inhibited the ability of granulysin to bind to *E. coli* (Fig. 4*C*). This decrease in Ab recognition as observed by Western blot analysis was not due to modification of the Ab epitope, because modified granulysin could be detected independently of the concentration of the BAD used (data not shown). BAD did not alter the secondary structure of granulysin as judged by circular dichroism studies (data not shown). Additionally, modification of the granulysin with CAH did not inhibit the interaction of granulysin with *E. coli* (Fig. 4*D*). Thus, the ability of granulysin to directly interact with bacteria is dependent on its positively charged arginine residues. We also tested whether BAD-treated granulysin would bind to *M. tuberculosis* (Fig. 4*E*). We found that BAD pretreatment of granulysin inhibited granulysin binding to *M. tuberculosis* in a dose-dependent manner. These data provide evidence the positively charged arginine residues of granulysin are required for binding to bacterial membranes.

### *Granulysin increases membrane permeability of* E. coli

We examined whether the binding of granulysin to the surface of bacteria leads to an increase in membrane permeability. Membrane permeability was measured in a permease-deficient strain of *E. coli* by the accessibility of cytosolic β-galactosidase to its membrane-impermeant substrate, ONPG. Granulysin increased membrane permeability in a dose- and time-dependent manner (Fig. 5*A*). In contrast, amoebapore A had no effect on membrane permeability. The known membranolytic peptide, melittin, is shown as a positive control.

To further evaluate the involvement of the arginine residues in the protein-membrane interaction, chemically modified granulysin was subjected to the same assay conditions to evaluate the effect on membrane permeability. Granulysin and granulysin modified with

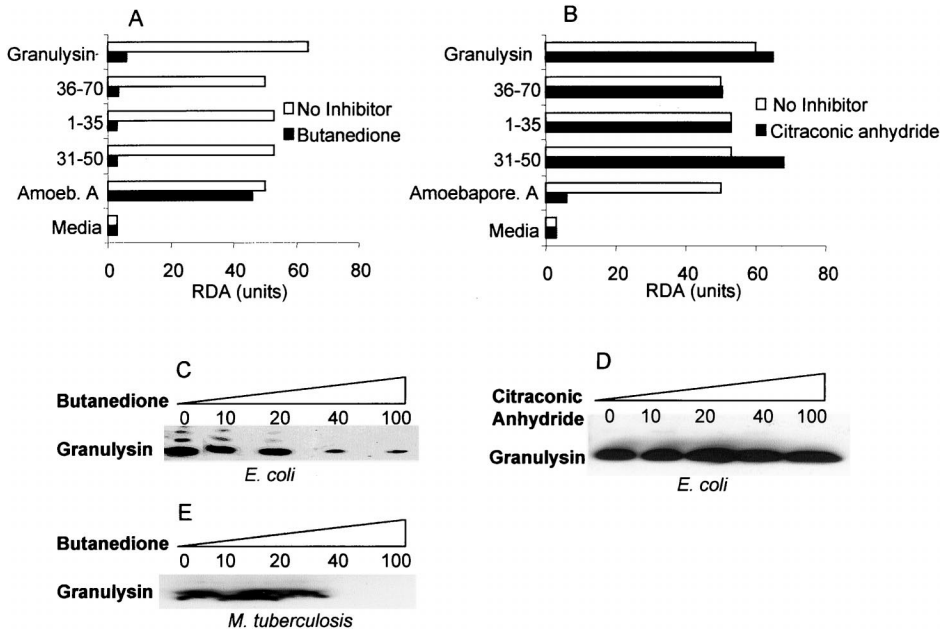


**FIGURE 4.** Inhibition of the antimicrobial activity of granulysin by specific chemical modification. *A*, Ten micrograms of granulysin, synthetic granulysin peptides, and amoebapore A were incubated with 40 mM BAD (pH 8), a chemical modifier of arginine residues, for 2 h, then added to a radial diffusion plate to determine the antimicrobial activity. Zones of clearing were measured after 16 h, and the level of activity was analyzed. B, Ten micrograms of granulysin, synthetic granulysin peptides, and amoebapore A were incubated with 20 mM CAH (pH 8), a chemical modifier of lysine residues, for 2 h, then added to a radial diffusion plate to determine the antimicrobial activity. Zones of clearing were measured after 16 h, and the level of activity was analyzed. C, Two micrograms of granulysin was incubated with various concentrations of BAD (2 h), then incubated with *E. coli* (1 h). The bacteria were washed three times with 10 mM $NaPO_4$ to remove unassociated granulysin, then resuspended in 2% Tween 20, and the amount of associated granulysin was evaluated by Western blot analysis. *D*, Two micrograms of granulysin was incubated with various concentrations of CAH (2 h), then incubated with *E. coli* (1 h). The bacteria were washed three times with 10 mM $NaPO_4$ to remove unassociated granulysin, then resuspended in 2% Tween-20, and amount of associated granulysin was evaluated by Western blot analysis. E, Two micrograms of granulysin was incubated with various concentrations of BAD (2 h), then incubated with *M. tuberculosis* (1 h). The bacteria were washed three times with 10 mM $NaPO_4$ to remove unassociated granulysin, then resuspended in 2% Tween 20, and amount of associated granulysin was evaluated by Western blot analysis.

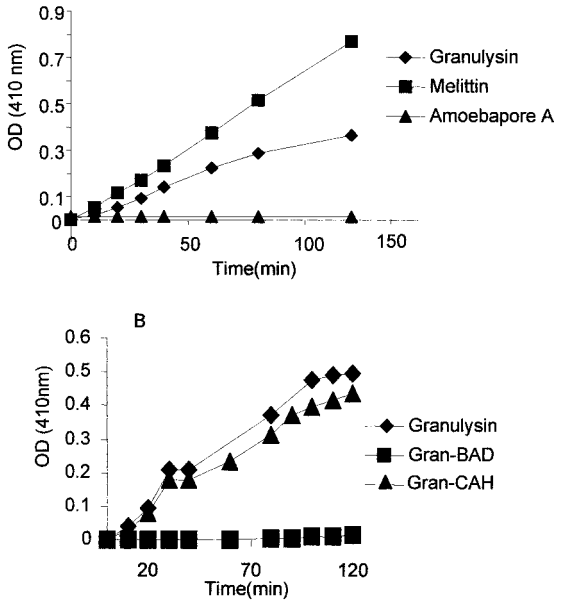


**FIGURE 5.** *E. coli* membrane permeability of granulysin. *A*, Granulysin (2.5 $\mu$M), 2.5 $\mu$M amoebapore A, and 1 $\mu$M melittin were incubated with *E. coli* in the presence of ONPG and incubated at 37°C. *E. coli* membrane permeabilization was evaluated by monitoring the absorbance at 410 nm for 2 h. *B*, Chemically modified granulysin with either BAD or CAH was incubated with *E. coli* in the presence of ONPG and incubated at 37°C. *E. coli* membrane permeabilization was evaluated by monitoring the absorbance at 410 nm for 2 h.

CAH showed similar permeabilizing effects; however, BAD-modified granulysin was completely inactive (Fig. 5*B*). The addition of modifiers alone to this assay system did not elicit a response (data not shown). We also tested the control protein amoebapore A, which killed *E. coli* in a manner dependent on its lysine residues. However, amoebapore A did not permeabilize the bacterial membrane (Fig. 6*A*), indicating that while these two proteins both interact by electrostatic mechanisms with bacteria, they eventually cause cell death through different mechanisms.

### *Morphologic effect of granulysin on* M. tuberculosis *as determined by transmission electron microscopy*

To directly visualize the effect of granulysin on *M. tuberculosis*, granulysin was incubated with the bacteria for varying incubation times and viewed by transmission electron microscopy. Fig. 6 shows an untreated bacterium as well as that treated with granulysin for 24 h (*middle panel*) and 48 h (*bottom panel*). Compared with the untreated control, the granulysin-treated mycobacterium exhibited cell membrane and cell wall separation similar to that observed in osmotic lysis. We speculate that these data are consistent with the possibility that granulysin is creating pores in the bacterial membrane or alternatively perturbing the membrane releasing the cytoplasm. A rapid release of the cytoplasm may lead to the separation of the membranes and eventually cell death.

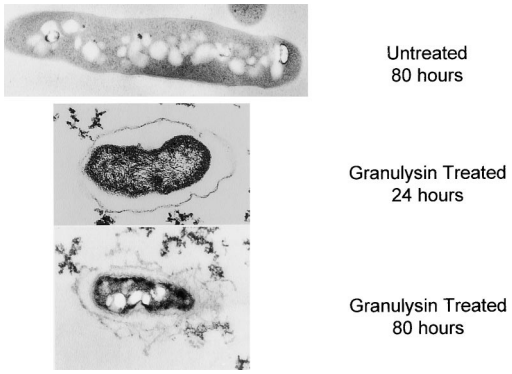


**FIGURE 6.** Effect of granulysin on *M. tuberculosis.* Transmission electron micrographs were performed of *M. tuberculosis* untreated for 80 h (*top panel*), incubated with granulysin (2.5 $\mu$M) for 24 h (*middle panel*), or incubated with granulysin for 80 h (*bottom panel*). After 24 h, the outer cell membrane had detached from the inner cell membrane, but not until 80 h of incubation did the cell membrane rupture, releasing the cytosol.

## Discussion

The ability of T cells to secrete the antimicrobial peptide granulysin is the only defined pathway by which T cells directly kill a variety of bacterial, fungi, and parasites (5). We therefore investigated the mechanism by which granulysin kills microbial pathogens. Our data suggest a model in which granulysin is attracted to the bacterial cell membrane and/or cell wall by ionic interaction mediated by the positively charged arginine residues of granulysin that probably interact with the negatively charged phospholipids present on the surface of the pathogen. Following insertion of granulysin into the microbial cell membrane, granulysin increases the permeability of bacterial membranes, leading to separation of the outer membrane from the cytoplasm with the accumulation of fluid in the cytoplasm. Together, these data indicate that the T cell product granulysin kills microbial targets by altering membrane permeability, thereby leading to osmotic lysis.

Amino acid sequence comparison indicates that granulysin is a member of the SAPLIP family of lipid binding proteins. Within this family, granulysin is most similar to NK lysin (43% identify and 67% similarity), a porcine granule protein with antibacterial activity (16). Other SAPLIP family members include the amoebapores, antibacterial peptides that amoebas use to kill bacterial prey (9). To determine the minimum active portion of the granulysin molecule retaining antimicrobial activity, peptides were synthesized to encompass the domains of structural homology with other members of this antimicrobial protein family. Although we cannot be certain that these peptides assume the same structure and function as their corresponding segments do in the intact protein, three of the peptides tested retained significant antimicrobial activity and were found to have a highly cationic charge, with contributions from both arginine and lysine residues. Similar to other antimicrobial peptides, granulysin and these constituent peptides had optimal activity at neutral to slightly basic pH and low ionic strength buffers, suggesting an essential role for an ionic interaction between the positively charged arginine residues of granulysin and the negatively charged bacterial membrane (17, 18). Chemical modification of the arginine residues with BAD not only inhibited the antimicrobial activity of granulysin, but also interfered with the protein interaction with the bacterial cell wall or membrane. Previous studies indicate the role of the negatively charged bacterial cell membrane with cationic antimicrobial peptides (19). Taken together, these data indicate that ionic interactions between granulysin and bacterial membranes are required for optimal antimicrobial activity.

When its primary structure is modeled on the related protein porcine NK-lysin (20), granulysin is predicted to form a four $\alpha$ helical bundle. Based on computer modeling and secondary structural analysis, the putative $\alpha$ helical conformation of granulysin displays hydrophobic residues on one surface and hydrophilic residues on the opposite surface (data not shown). Such a structure is common among proteins and peptides that interact with the phospholipid bilayers of cell membranes. Circular dichroic analysis

from three separate preparations of recombinant granulysin indicates a high $\alpha$ helical content of 49%. Additionally, we have shown that the specific three-dimensional structure of granulysin is required for its antimicrobial activity, because denaturation completely abolished its ability to kill *E. coli*. Studies of synthetic peptides corresponding to selected regions of granulysin suggest that one or more $\alpha$ helical segments may be required for activity. The three peptides of granulysin that retained antimicrobial activity were predicted to conform to the helix-loop-helix motif. Similarly, a previous study found that synthetic peptides of granulysin conforming to a helix-loop-helix region spanning the second and third helices were anti-mycobacterial (21). The helix-loop-helix structure of the antimicrobial peptides of granulysin is similar to the structure of cecropins, antimicrobial peptides present in insects (22, 23).

Our data indicate that granulysin kills bacteria by perturbing the cell membrane. When granulysin was incubated in the presence of *E. coli* and a substrate for the cytoplasmic $\beta$-galactosidase, ONPG, granulysin permeabilized the membrane and allowed the cytoplasmic enzyme to hydrolyze the normally excluded substrate. Transmission electron microscopy of granulysin-treated *M. tuberculosis* demonstrated the separation of the outer cell wall and membrane from the cytoplasm and the accumulation of electron-lucent material, presumably water, in the cytoplasm. Previous analysis of granulysin-treated bacteria by scanning electron microscopy indicated distortions in the outer cell wall and membrane of the pathogen (5). Together, these data are consistent with incorporation of granulysin into the membrane, perhaps forming pores in the bacterial membrane and disrupting the osmotic gradient between the cytoplasm and the external environment. Alternatively, the incorporation of granulysin into the bacterial membrane could lead to outer membrane expansion and disruption of the barrier to the extracellular environment.

Two types of membrane-active proteins have been shown to kill or lyse a broad range of targets. These two species of proteins include 1) small peptides (typically 16–40 aa), e.g., magainins, cecropins, defensins, protegrins, and tachyplesins (8, 17, 24, 25); and 2) larger proteins with multiple $\alpha$ helical domains, e.g., amoebapores, porcine NK-lysin, and human granulysin (16, 26). All these proteins are cationic amphipathic species whose basic residues are thought to interact with the negatively charged membrane. Yet the mechanisms by which two of these proteins, granulysin and amoebapores, kill pathogens appear different. Although both are positively charged molecules, the antimicrobial activity of granulysin was dependent on arginine residues, while that of amoebapores was dependent on lysine residues. The permeabilization experiments indicate that granulysin can perturb both the inner and outer bacterial membranes, but amoebapore A had no measurable effect on the *E. coli* inner membrane.

Over a billion years of evolution, the conformation of SAPLIP family members has been preserved, from the amoebapores of amoebas to granulysin of humans (2, 3). This evolutionary change coincides with the development of the immune system; amoebapores represent a basic form of innate immunity that amoebas use to prevent growth of bacterial prey, whereas granulysin is secreted by human T cells, thus integrating this form of antimicrobial defense into the adaptive immune response. Amoebapores kill bacteria and are cytotoxic to human cells, whereas granulysin kills tumor cells, but is relatively inactive against normal human cells, requiring perforin as a cofactor to enter cells and to kill intracellular pathogens. Because of its low toxicity to normal host cells, granulysin is an attractive template for the development of novel antimicrobial therapeutics to combat the emergence of drug-resistant pathogens.

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