The Journal *of* Immunology

A Distinct Pathway of Cell-Mediated Apoptosis Initiated by Granulysin

Allan A. Kaspar, Satoshi Okada, Jayant Kumar, Francis R. Poulain, Katerina A. Drouvalakis, Ameeta Kelekar, Dennis A. Hanson, Ruth M. Kluck, Yasumichi Hitoshi, Daniel E. Johnson, Christopher J. Froelich, Craig B. Thompson, Donald D. Newmeyer, Alberto Anel, Carol Clayberger and Alan M. Krensky

---

References This article **cites 46 articles**, 21 of which you can access for free at:
http://www.jimmunol.org/content/167/1/350.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

A Distinct Pathway of Cell-Mediated Apoptosis Initiated by Granulysin[1]

Allan A. Kaspar,[2]* Satoshi Okada,[2]* Jayant Kumar,* Francis R. Poulain,[§]
Katerina A. Drouvalakis,* Ameeta Kelekar,[¶] Dennis A. Hanson,* Ruth M. Kluck,[∥]
Yasumichi Hitoshi,[‡] Daniel E. Johnson,[#] Christopher J. Froelich,** Craig B. Thompson,[††]
Donald D. Newmeyer,[∥] Alberto Anel,[‡‡] Carol Clayberger,*[†] and Alan M. Krensky[3]*

Granulysin is an antimicrobial and tumoricidal molecule expressed in granules of CTL and NK cells. In this study, we show that granulysin damages cell membranes based upon negative charge, disrupts the transmembrane potential ($\Delta\psi$) in mitochondria, and causes release of cytochrome *c*. Granulysin-induced apoptosis is blocked in cells overexpressing Bcl-2. Despite the release of cytochrome *c*, procaspase 9 is not processed. Nevertheless, activation of caspase 3 is observed in granulysin-treated cells, suggesting that granulysin activates a novel pathway of CTL- and NK cell-mediated death distinct from granzyme- and death receptor-induced apoptosis. *The Journal of Immunology,* 2001, 167: 350–356.

G ranulysin is a cytolytic molecule present in human CTL and NK cell granules (1). It was first identified by subtractive hybridization of CTL minus B cell mRNA, and is specifically expressed by CTL 3–5 days after activation (2). Granulysin is released from killer cells by granule exocytosis and is lytic against a variety of tumor cell targets (1) and microbes (3), including Gram-positive and -negative bacteria, fungi, and parasites. In combination with purified perforin, recombinant granulysin lyses 90% of intracellular *Mycobacterium tuberculosis*, inducing lesions on the mycobacterial cell surface (3).

CTL kill targets in part by granule exocytosis (4). Granule-mediated cytotoxicity involves the action of the pore-forming protein perforin and a family of serine proteases, the granzymes. Although granzyme B can cleave many caspase substrates in vitro (5, 6), it was recently demonstrated that granzyme B-induced apoptosis results from its ability to directly cleave BH3-interacting domain death agonist (Bid)[4] (7), a Bcl-2 family member, which then transmits the death signal through the mitochondrion. Granzyme A causes apoptosis through a caspase-independent pathway (8). Cell-mediated cytotoxicity can also involve ligation of death receptors on the target cell using a second signaling pathway for initiating caspase-dependent apoptosis (9, 10). Granulysin is an additional secreted protein present in cytolytic granules (1).

We previously showed that recombinant granulysin, independent of other granule constituents, induces apoptotic cell death of Jurkat tumor cells (11). Target cells show the physical hallmarks of apoptosis, including nuclear condensation, membrane ruffling, and translocation of phosphatidylserine to the outer leaflet of the plasma membrane, as detected by annexin V-FITC staining. Granulysin activates a sphingomyelinase in target cells, increasing the cellular ceramide/sphingomyelin ratio. We also showed that a general inhibitor of caspases, the peptide benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, partially protects against granulysin-induced cell death, whereas a more specific inhibitor of caspase 3-like enzymes, the peptide *N*-acetyl-Asp-Glu-Val-Asp aldehyde, does not protect.

Granulysin is homologous to the saposin-like protein (SAPLIP) family of lipid-binding proteins (1). The homology of granulysin to saposins suggests that it may exert its cytolytic function and antibacterial activity via interaction with lipids. In this study, we demonstrate that granulysin disrupts negatively charged synthetic liposomes and cell membranes. Cells expressing the antiapoptotic gene *Bcl-2* are protected against granulysin-induced cell death. The interaction of granulysin with cells causes a change in mitochondrial potential ($\Delta\psi$) and release of cytochrome *c*. Surprisingly, however, procaspase 9 is not processed to its active form, although an early caspase 3 activation is observed. Thus, granulysin is a CTL- and NK cell-specific product that activates a death pathway distinct from those induced by Fas or granzymes.

*Division of Immunology and Transplantation Biology, Department of Pediatrics, and Departments of [†]Cardiothoracic Surgery and [‡]Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305; [§]Department of Pediatrics, University of California, San Francisco, CA 94143; [¶]Gwen Knapp Center for Lupus and Immunology Research, Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637; [∥]Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121; [#]Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15213; **Evanston Northwestern Research Institute, Northwestern University, Evanston, IL 60201; [††]Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA 19104; and [‡‡]Department of Bioquimica, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain

[4] Abbreviations used in this paper: Bid, BH3-interacting domain death agonist; SAPLIP, saposin-like protein; AIF, apoptosis-inducing factor; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CL, cardiolipin; CrmA, cytokine response modifier; POPC, palmitoyloleoylphosphatidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; Rho-123, Rhodamine-123; tBid, truncated human Bid.

Materials and Methods

Materials

Reagents used include chemicals (Sigma, St. Louis, MO), propidium iodide (Molecular Probes, Eugene, OR), lipids (Avanti Polar Lipids, Alabaster, AL), fluorescent probes 1-aminonaphtlene-3,6,8-trisulfonic acid, and N,N'-(p-phenylenedimethylene)bis(pridinium bromide), and fura 2 (Molecular Probes), HRP-conjugated secondary Abs (Cappell, Durham, NC), and ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Cells

The Jurkat T cell leukemia (American Type Culture Collection, Manassas, VA) was cultured as described (11). Jurkat cells transfected with Bcl-2 or cytokine response modifier (CrmA) were maintained in medium supplemented with 0.5 mg/ml G418, as described (12). Jurkat cells overexpressing Toso were sorted by flow cytometry selecting for green-fluorescent protein expression from the viral construct used for infection (13). FL5.12 cells were grown as described (14).

Expression and purification of recombinant granulysin and purification of perforin

Recombinant granulysin was expressed and purified as described (1). Briefly, recombinant 9-kDa granulysin was expressed using the vector pET28a (Novagen, Madison, WI). 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 PBS, and concentration was determined using a protein assay (Bio-Rad, Hercules, CA) with lysozyme as a standard. Human perforin was purified by metal-affinity chromatography from the human NK cell line YT2C2, as described (6).

Liposome lysis assays

Disruption of liposomes was measured as described (15). Briefly, the fluorescent probe 1-aminonaphtlene-3,6,8-trisulfonic acid and its collisional quencher N,N'-(p-phenylenedimethylene)bis(pridinium bromide) were incorporated into palmitoyloleoylphosphatidylglycerol (POPG) and palmitoyloleoylphosphatidylcholine (POPC) large unilamellar vesicles. POPC and cardiolipin (CL) were mixed in a 1:1 ratio to form large unilamellar vesicles. Release of the fluorescent probe was measured on a spectrophotometer. Liposomes (25 μM phospholipids) were treated with 550 nM granulysin, and the percentage of specific lysis was calculated.

Cytotoxicity assays

Jurkat cells were seeded in 96-well plates at a density of 2×10^5/ml (100 μl/well) in medium containing 0.1% FCS. Cells were cultured for 16 h in the presence of medium, granulysin (11), or anti-Fas mAb (CH-11, 100 ng/ml; Beckman-Coulter, Fullerton, CA). Cell death was measured by propidium iodide uptake and by gating of live cells based on forward vs side scatter. Similar results with Jurkat cells were obtained using additional cytotoxicity assays, including ^{51}Cr release (1), MTT assay (16), and annexin V-FITC staining (11). Plasma membrane integrity was measured by propidium iodide uptake after 15 min of culture in the presence of granulysin.

Measurement of mitochondrial membrane potential with Rho-123

Jurkat cells were washed, resuspended at 5×10^5/ml in RPMI 1640 supplemented with 0.1% FCS, and incubated for 1 h at 37°C. A total of 10^5 cells were treated with recombinant granulysin for 2 h at 37°C, washed, resuspended in PBS, and placed on an inverted coverslip chamber (pretreated with polylysine). After 5 min, 200 μl of 20 μM Rho-123 (Molecular Probes) was added, and the excess was washed away after 2 min. Confocal images were collected using a Molecular Dynamics (Sunnyvale, CA) MultiProbe 2010 Confocal Laser Scanning Microscope, with a Nikon Diaphot 200 inverted with epi-fluorescence attachment. A krypton/argon laser with 488-nm excitation and a 590-nm long pass emission filter was used to collect images through a ×60 (oil) objective. After images had been collected, 200 μl of 2 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma) was added to the cells in the chamber, and, after 30 s, additional images were collected. Results are representative of 10 fields for each sample.

Ca²⁺ flux measurements

Jurkat cells were loaded with fura 2 in a 1:1 vol of cell suspension with dye by incubating at 37°C for 60 min. The cells were then washed twice with buffer and incubated for a further 30 min for complete deesterification. The induction of a calcium flux was observed using an Attofluor digital fluorescence microscope (Atto Instruments, Rockville, MD). Cell suspension was loaded into chambers and, after ~20 s, these cells were treated with ionomycin or granulysin. The Ca^{2+}-dependent fluorescence signal was obtained using excitation at 340 and 380 nm and ratioing the fluorescence intensities detected at 510 nm.

Subcellular fractionation and Western blot analysis

Mitochondrial and cytosolic (S100) fractions were prepared by resuspending FL5.12 cells in 0.8 ml ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml PMSF, 8 μg/ml aprotinin, 2 μg/ml leupeptin (pH 7.4)). Cells were homogenized using a prechilled cylinder cell homogenizer (H&Y Enterprise, Redwood City, CA). Unlysed cells and nuclei were pelleted at 750 $\times$ g for 10 min, and the supernatant was centrifuged at 10,000 $\times$ g for 25 min. The pellet, representing the mitochondrial fraction, was resuspended in buffer A, and the supernatant was subjected to further centrifugation at 100,000 $\times$ g for 1 h. The supernatant from the final centrifugation represents the S100 fraction. Equivalent amounts of mitochondrial and S100 fractions were subjected to Western blot analysis, as previously described (14). The primary Abs were either a 1/1000 dilution of the 7H8.2C12 cytochrome c mAb (a gift from R. Jemmerson, Department of Microbiology and Center for Immunology, University of Minnesota, Minneapolis, MN) in TBS, 0.2% Tween 20; or 10 ng/ml 20E8-C12 cytochrome oxidase subunit IV mAb (Molecular Probes) as a control for mitochondrial inner membrane integrity.

For caspases 3 and 9, Jurkat cells were treated with 50 μM granulysin for 3 h and washed, and whole-cell lysates were prepared by lysing cells in 2% SDS, 137 mM NaCl, 2.7 mM KCl, and 8 mM $NaHPO_4$ (pH 7.4) (17, 18). Samples were separated on 15% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat milk, 0.1% Tween 20 in TBS and then incubated with specific Abs to caspase 9 (Cell Signaling Technologies, Beverly, MA) or caspase 3 (BD PharMingen, San Diego, CA) and α-actin (Calbiochem, La Jolla, CA). Primary Abs were detected by peroxidase-labeled secondary Ab and developed using ECL (Amersham Pharmacia Biotech) and exposure to film.

Xenopus egg extracts and incubations

Recombinant truncated human Bid (tBid) was produced as previously described (19). *Xenopus* egg mitochondria were isolated as previously described (20). Mitochondrial incubations were reconstituted from mitochondria (1 mg/ml; biuret method) in buffer A (250 mM sucrose, 20 mM HEPES/KOH (pH 7.5), 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 5 μg/ml cytochalasin B, and 50 μg cycloheximide) and an ATP-regenerating system of 10 mM phosphocreatine, 2 mM ATP, and 150 μg/ml creatine phosphokinase. To examine mitochondrial cytochrome c content, extract aliquots (10 μl) were removed at 3 h and mixed with 0.5 ml buffer A before centrifugation (12,000 $\times$ g, 5 min) to pellet mitochondria. Western blot of mitochondrial cytochrome c was performed as described previously (19).

Results

Granulysin induces membrane disruption by interaction with lipids

Granulysin, a lytic molecule of human CTL and NK cell granules, is homologous to the SAPLIP family of lipid-binding proteins (1). The homology of granulysin to saposins suggests that it may exert its lytic activity via interaction with membrane lipids. To assess granulysin interaction with lipids, the lytic effect of granulysin on synthetic liposomes of defined composition was examined (Fig. 1A). Negatively charged POPG liposomes were disrupted, whereas neutral POPC liposomes were not. Liposomes consisting of a mixture of POPC and CL, a negatively charged lipid, were disrupted by granulysin, indicating that lysis was associated with negatively charged lipids. Disruption of liposomes by granulysin was dependent on both granulysin (Fig. 1B) and lipid concentration (Fig. 1C). In these experiments, we reproducibly detect significant disruption of liposomes at granulysin concentrations as low as 0.1 μM. Thus,
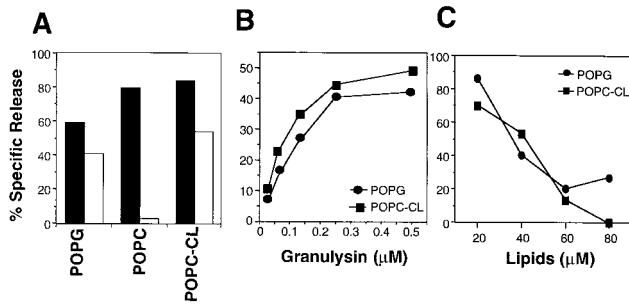
FIGURE 1. Granulysin disrupts negatively charged synthetic liposomes. *A*, Effect of granulysin (□) or surfactant protein B (■) on liposomes of different compositions. Granulysin (0.5 μM) or surfactant protein B was added to 25 μM liposomes, and the percentage of specific release was measured. *B*, Dose-dependent disruption of liposomes by granulysin. A total of 25 μM POPG or POPC-CL liposomes were treated with increasing amounts of granulysin. *C*, Lipid concentration dependence of granulysin-induced disruption of liposomes. Granulysin (0.5 μM) was added to cuvettes containing increasing amounts of POPG or POPC-CL liposomes, and specific release was measured. Data in each panel are from independent experiments using separate liposome preparations. Data represent duplicate experiments.

granulysin, like other SAPLIP family members, interacts with lipid membranes.

The ability of recombinant granulysin to disrupt membranes of nucleated mammalian cells was assessed by treating the human T cell tumor Jurkat with granulysin and monitoring uptake of propidium iodide. Within 30 min, 49.5% of granulysin-treated Jurkat cells were propidium iodide positive, as compared with 21.6% of control-treated cells, indicative of granulysin-induced membrane disruption (Fig. 2). Perforin is shown as a positive control for membrane disruption. The combination of granulysin and perforin was additive rather than synergistic, indicating that these two molecules act independently to induce membrane damage (data not shown). Thus, granulysin perturbs the plasma membrane by a direct interaction with lipids. This activity is accompanied by nuclear condensation, membrane ruffling, and annexin V-FITC staining, as previously described (11).

Granulysin-induced apoptosis involves mitochondrial release of cytochrome c

Bcl-2 protects cells from the mitochondrial apoptotic pathway (21), whereas CrmA protects cells from death receptor-induced apoptosis by inhibiting several caspases (22). Jurkat cells transfected with the genes for Bcl-2 or CrmA (12) were protected to some extent against granulysin-induced death relative to vector controls (Fig. 3). Bcl-2 protection indicates that the apoptotic pathway induced
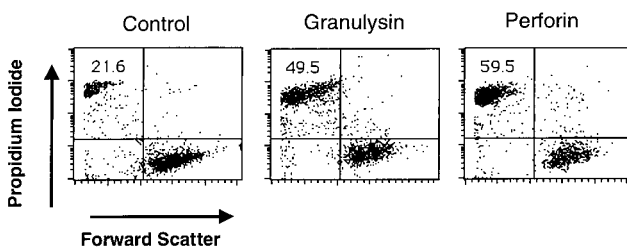
by granulysin involves mitochondrial damage (21), whereas CrmA protection further implicates caspases in the death pathway (22).

The ability of granulysin to induce mitochondrial damage was assessed by measuring mitochondrial transmembrane potential ($\Delta\psi$) (Fig. 4). Rho-123 is a cationic fluorophore that is taken up and concentrated in electronegative mitochondria in response to the $\Delta\psi$. CCCP is a protonophore that disrupts the $\Delta\psi$, leading to the dissipation of the dye into the cytoplasm (23–25). Jurkat cells incubated in medium plus Rho-123 show a punctate fluorescence (Fig. 4*A*) that is abolished in cells after the addition of CCCP (Fig. 4*B*). Granulysin-treated cells do not stain with Rho-123 (Fig. 4*C*), and addition of CCCP does not cause any change in the staining (Fig. 4*D*). Similar results were obtained with DiOC6 (3) and JC-1, two other potential sensitive dyes (not shown).
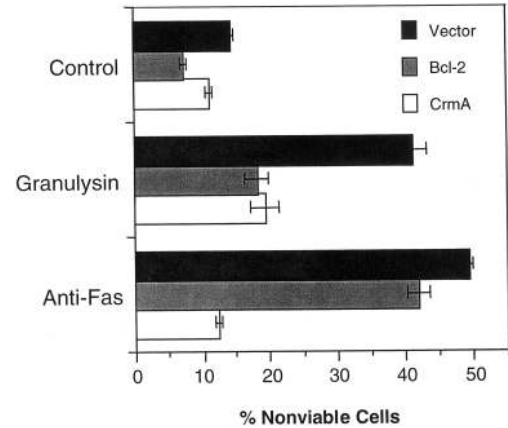

FIGURE 3. Jurkat cells expressing Bcl-2 or CrmA are protected from granulysin-induced cell death. Jurkat cells transfected with Bcl-2 or CrmA were cultured for 16 h in the absence or presence of 50 μM granulysin, and cell death was analyzed by flow cytometry. The data (mean ± SD) shown are the percentage of cells determined nonviable by forward/side scatter analysis and propidium iodide staining.

FIGURE 2. Granulysin disrupts Jurkat cell plasma membranes. Jurkat cells were cultured with 50 μM granulysin in 0.1% FCS medium containing propidium iodide for 15 min before analysis by flow cytometry. A concentration of perforin was chosen to effect ~50% lysis of the cells. Data are representative of cells treated and analyzed in triplicate.
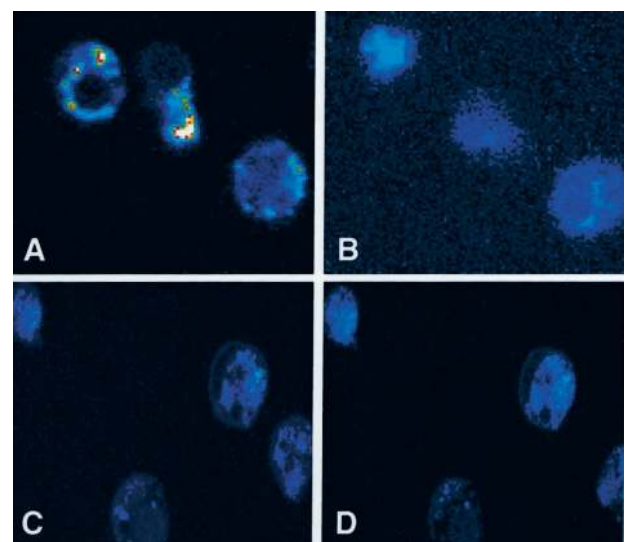

FIGURE 4. Granulysin disrupts $\Delta\psi$ in mitochondria. *A*, Mitochondria of Jurkat cells incubated in medium and then stained with Rho-123 show a punctate yellow-orange staining. *B*, Addition of CCCP abolished the punctate fluorescence in the cells. Granulysin-treated cells (*C*) do not stain with Rho-123, and addition of CCCP did not cause any change in the staining (*D*).

Bcl-2 protection from granulysin-induced death suggests that cytochrome c release from mitochondria may initiate caspase activation, because one effect of Bcl-2 is to prevent the release of cytochrome c (26). Therefore, we next assayed for the release of cytochrome c from the mitochondria of granulysin-treated cells. Within 1 h of granulysin treatment, cytochrome c was detectable in cytosolic extracts of granulysin-treated target cells, whereas the inner membrane protein cytochrome oxidase remained in the mitochondrial fraction (Fig. 5A), implicating granulysin-induced cytochrome c release as an initiating signal for caspase activation.

Because granulysin disrupts the cell plasma membrane, internalized granulysin may directly damage mitochondria, effecting cytochrome c release. We tested the ability of granulysin to directly damage mitochondria in vitro. Isolated mitochondria were treated with either granulysin or tBid and analyzed for loss of cytochrome c in the mitochondrial pellet. In contrast to tBid, which caused release of cytochrome c from mitochondria, 50 μM granulysin did not disrupt isolated mitochondria (Fig. 5B), indicating that the mitochondrial disruption observed in cells is not a direct effect of pure granulysin.

One indirect mechanism by which granulysin-induced plasma membrane damage could affect mitochondria is through an influx of ions across the plasma membrane. Granulysin could induce an influx of Ca^{2+} across the plasma membrane, which would induce permeability transition, secondary swelling of mitochondria, rupture, and release of cytochrome c. Chelating divalent cations with EDTA partially inhibits granulysin-induced death in chromium release assays of labeled target cells (Fig. 6A), but does not affect the ability of granulysin to disrupt cell membranes (Fig. 6B) or liposomes (data not shown). The calcium flux induced by granulysin was directly detected by the use of the dye fura 2. Jurkat cells loaded with fura 2 and treated with either ionomycin or granulysin show a rapid increase in fluorescence, indicative of a calcium flux into the cytosol (Fig. 6C). Thus, there is a requirement for extracellular ions in granulysin-induced apoptosis, and an ion flux induced by disruption of the plasma membrane may initiate cell death. Additionally, these data show that unlike perforin, there is not a requirement for calcium in granulysin-induced membrane damage, indicating a distinct mechanism for granulysin-induced plasma membrane disruption.
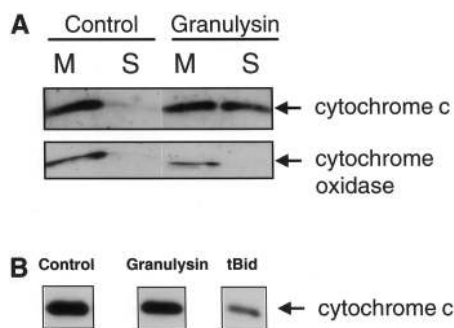

FIGURE 5. Granulysin damages mitochondria in intact cells. *A*, FL5.12 cells were cultured in the absence or presence of 50 μM granulysin and fractionated. Mitochondrial fractions (M) and cytosolic S-100 fractions (S) were analyzed on a single Western blot probed sequentially with mAb to cytochrome c (*upper panel*) or cytochrome oxidase (*lower panel*). *B*, Granulysin does not directly disrupt isolated mitochondria. Isolated *Xenopus* mitochondria were incubated in the absence or presence of 50 μM granulysin or tBid for 3 h, and mitochondrial pellets were analyzed by immunoblot with anti-cytochrome c Ab. Bands represent the cytochrome c present in the mitochondrial pellet. No effect was seen at 100 μM granulysin, the highest granulysin concentration tested (data not shown). Data are representative of two independent experiments.

Caspases in granulysin-induced death

Granulysin-induced apoptosis involves caspases, as both CrmA and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone partially protect target cells. To identify specific caspases involved in granulysin-induced death, we examined three initiator caspases involved in Fas-, granzyme B-, and genotoxic-mediated apoptosis.

The cowpox serpin CrmA inhibits death pathways mediated by Fas and chemotherapeutic agents (27). We observed that CrmA, an inhibitor of serine and cysteine proteases, including caspase 8 (28), protects from granulysin-induced death (Fig. 3). Because the Jurkat-Bcl-2 transfectant is resistant to granulysin but is susceptible to Fas-induced death, it is unlikely that the defined death receptor pathway is involved in granulysin-induced apoptosis. To further evaluate the role of caspase 8 in the granulysin-mediated pathway, we tested the ability of another inhibitor to protect Jurkat cells from granulysin-induced death. Nolan and coworkers (13) identified a molecule designated Toso that inhibits Fas- and Fas-associated death domain protein-induced apoptosis. Toso prevents cell death by inducing the expression of cellular Fas-associated death domain-like IL-1-converting enzyme inhibitory protein (29) and blocking adapter-induced caspase 8 activation (30). Jurkat cells expressing Toso were protected from anti-Fas-induced death, but were not protected from granulysin-induced death (data not shown), again indicating that granulysin-induced death is different from the death receptor signaling pathway mediated by Fas.

Caspase 3 is a substrate for granzyme B (5, 6). Previously, we reported minimal processing of procaspase 3 in granulysin-treated cells and that *N*-acetyl-Asp-Glu-Val-Asp aldehyde, a peptide inhibitor of caspase 3-like enzymes, only weakly protected against granulysin-induced apoptosis of Jurkat cells (11). In those experiments, cells were incubated with granulysin for 16 h before measuring apoptosis or procaspase 3 cleavage. Because granulysin-mediated damage is detectable much earlier, we decided to assay procaspase 3 activation at earlier times. As shown in Fig. 7, procaspase 3 is processed to some extent in cells treated for 3 h with 50 μM granulysin or 2 μM staurosporin.

Caspase 3 is an effector caspase that can be activated downstream of caspase 8 or caspase 9. Experiments using cells overexpressing Toso showed that caspase 8 is not activated. Therefore, we turned our attention to activation of caspase 9. When cytochrome c is released from mitochondria, it binds Apaf-1, which recruits procaspase 9, leading to caspase 9 activation (31) and initiation of the caspase cascade induced by genotoxic agents. Immunoblots using a caspase 9-specific antiserum were conducted on the same lysates that showed processing of procaspase 3 after a 3-h incubation with either granulysin- or staurosporine-treated cells (Fig. 7). Processing of caspase 9 in staurosporine-treated cells is apparent. However, processed forms of caspase 9 were not detected in granulysin-treated cells. Thus, granulysin induces the activation of caspase 3 by a mechanism that is independent of processing of caspase 9.

Discussion

Granulysin, a novel effector molecule of human CTL and NK cells, is bactericidal (3) and induces apoptosis in mammalian cells (11). CTL are capable of killing intracellular bacteria via a granule-mediated mechanism (32), and granulysin is a bactericidal molecule present in CTL granules (3). We previously suggested that granulysin interacts directly with lipids in cell membranes, and showed that granulysin induces lesions on the surface of *M. tuberculosis* consistent with a direct action on the mycobacterial glycolipid envelope (3). The current work provides further insights into how granulysin kills target cells.

FIGURE 6. Granulysin induces an ion flux in target cells. *A*, EDTA inhibits granulysin-induced chromium release. ^{51}Cr-labeled JY target cells were incubated in the presence of 50 μM granulysin for 4 h in the presence or absence of EDTA. NaCl was used as a control buffer. Radiolabel released into the supernatant was measured to determine specific cytotoxicity induced by granulysin. *B*, EDTA does not inhibit granulysin-induced membrane damage. Jurkat cells were treated with granulysin or perforin in the presence or absence of EDTA and analyzed 15 min later for propidium iodide uptake. Data shown (mean ± SD) are representative of three independent experiments. *C*, Granulysin induces an ion flux in Jurkat cells. Jurkat cells were loaded with fura 2 and, after 20 s, were treated with either ionomycin or granulysin. Fluorescence was observed using an Attofluor digital fluorescence microscope. Data shown are representative of three independent experiments.
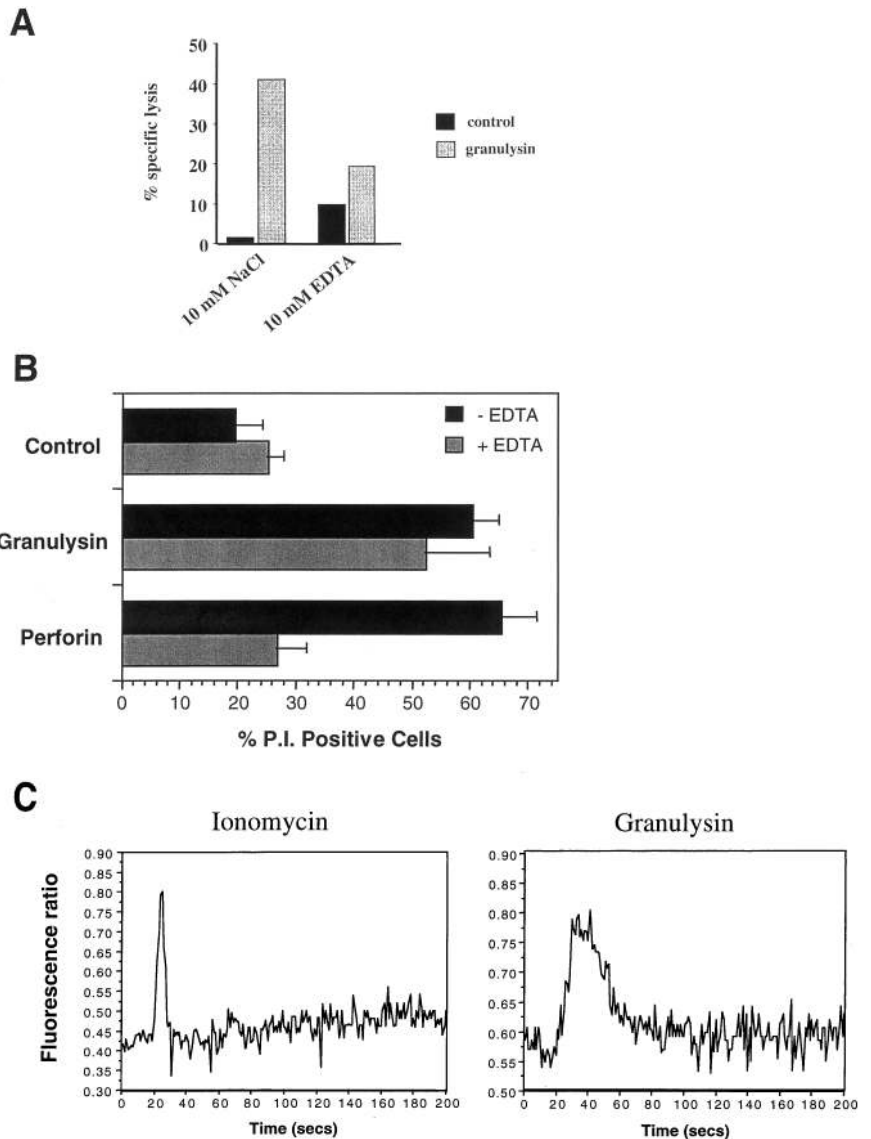
In vitro, we find that granulysin strongly induces apoptosis of tumor target cells at a concentration of 50 μM. This relatively high concentration is consistent with granulysin-inducing apoptosis in a receptor-independent manner (33). However, it is unlikely that such high concentrations of granulysin are necessary to effect target cell lysis in vivo. In vivo, granulysin would be delivered in a relatively concentrated form at the junction of the CTL/target (4). Furthermore, granulysin most likely works in concert with other constituents of cytolytic granules, as previously shown for both granzymes and perforin. Finally, conditions in the local environment will also affect granulysin-mediated lysis, because protein, salt, and H$^+$ can alter the lytic activity of recombinant granulysin (34).

An immediate effect of granulysin on target cells is disruption of plasma membrane integrity. Positively charged granulysin lyses negatively charged, but not neutral, liposomes. NK-lysin, the SAP-LIP family member with the greatest homology to granulysin (35), also affects lipid membranes (36). NK-lysin disrupts lipid vesicles and induces random current fluctuations through a planar lipid membrane when voltage is applied across the membrane, indicating that the lipid membrane-disrupting activity of NK-lysin does not involve formation of a stable pore (36). Our findings suggest a similar mechanism of action for granulysin. Unlike the pore-forming molecule perforin (37), granulysin-induced membrane damage

leads to activation of the apoptotic machinery. Granulysin disruption of the plasma membrane leads to a calcium flux that may in turn damage mitochondria. Mitochondrial damage is apparent within minutes of cell surface exposure to granulysin, but this effect is indirect in that recombinant granulysin is incapable of directly damaging isolated mitochondria in vitro. The inability of granulysin to directly disrupt the mitochondrial membrane may be either due to differences between the lipid compositions of the plasma membrane and mitochondrial membrane or that an intermediate is involved in transmitting the granulysin signal. One candidate for such an intermediate is an ion flux. We show that EDTA partially inhibits granulysin-induced death in chromium release assays, but it has no effect on the membranolytic properties of granulysin. Furthermore, there is a rapid rise in intracellular Ca^{2+} associated with granulysin treatment of cells, suggesting that plasma membrane damage may provoke mitochondrial damage and cytochrome *c* release.

Previous studies with caspase inhibitors indicated that caspase activation is involved in granulysin-mediated cell death (11). In the present study, we further evaluated the role of caspases in the granulysin pathway. A transfectant overexpressing the serpin CrmA protected against granulysin-mediated cell death. CrmA is reported to preferentially inhibit caspase 8 and caspase 1 at the
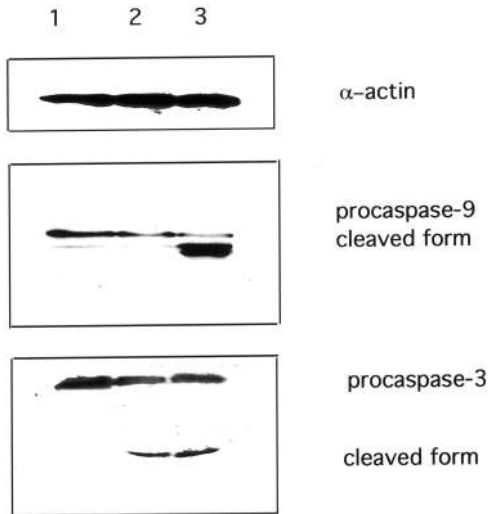
FIGURE 7. Caspase 3, but not caspase 9, is processed in granulysin-treated cells. Cells were treated with medium (*lane 1*), 50 μM granulysin (*lane 2*), or 2 μm staurosporine (*lane 3*), as described in *Materials and Methods*, and immunoblots were probed as indicated.

concentrations generally present in transfections (28), but our experiments using Toso transfectants indicate that death receptors are not involved in granulysin-mediated cell death (13). Toso expression induces Fas-associated death domain-like IL-1-converting enzyme inhibitory protein expression, which acts to protect cells from Fas- and Fas-associated death domain protein-induced death (29). We find that, although Toso-expressing Jurkat are protected from anti-Fas, they are not protected from granulysin. Thus, granulysin-induced death is not dependent on the death receptor pathway mediated by Fas.

Consistent with this conclusion, Jurkat cells overexpressing Bcl-2 showed only weak protection from anti-Fas-induced death (Fig. 3), indicating that our Jurkat cell line is not a type II cell in which the death receptor and mitochondrial death pathways intersect (38). As these cells are protected from granulysin-induced death, the granulysin pathway is clearly distinct from the death receptor pathway mediated by Fas.

The antiapoptotic effect of Bcl-2 on granulysin-induced apoptosis suggests that mitochondrial damage is a critical step in granulysin-induced apoptosis (21). Apaf-1, the mammalian *ced*-4 homologue, binds cytosolic cytochrome *c* and recruits procaspase 9, leading to activation. Although we are unable to detect processing of procaspase 9 in granulysin-treated cells, it has been reported that unprocessed procaspase 9, when complexed with cytochrome *c* and dATP, can activate downstream caspases (39). It is also possible that low levels of processed caspase 9, sufficient to cause apoptosis but undetectable by immunoblot, may be present in granulysin-treated cells. Alternatively, activation of caspase 3 may occur by a caspase 9-independent mechanism. Disruption of $\Delta\psi$ in mitochondria releases not only cytochrome *c*, but also a number of other molecules, including apoptosis-inducing factor (AIF). AIF is sufficient to activate endonucleases in isolated nuclei (40–42). Anel and coworkers have recently demonstrated that AIF is released from mitochondria in cells treated with granulysin.[5]

A large number of loss of function studies performed by many laboratories over the last 5 years have clearly delineated the roles of perforin/granzymes and Fas systems for CTL-mediated cytotoxicity (43, 44). It is clear from these papers that most of the measurable in vitro cytotoxicity delivered by CTL in the reported systems can be attributed to these two pathways. This calls into question the biologic function of granulysin in other than an accessory role. We have been unable to identify a murine homologue for granulysin using a variety of approaches. Granulysin-specific antisera fail to Western blot a protein from mouse CTL extracts; no signal is obtained using several murine CTL/NK cell cDNA or genomic libraries probed with radiolabeled granulysin cDNA; and no murine homologue is present in databases. This observation is reminiscent of other antimicrobial peptides such as human defensins for which there are no structural murine homologues (45). Thus, granulysin appears not to have a mouse homologue that could conceivably complement the deficiency of perforin or granzyme B in knockout mice. Murine systems, including knockouts, cannot conclusively determine the functional role for granulysin- or other species-specific proteins in humans. It should also be noted that the various reported systems only address limited biologic functions. For example, granulysin may be important in local environments such as the gut or CNS. Although no granulysin knockout is possible in mice because there is no homologue, experiments with human granulysin transgenic mice are planned.

CTL evolved to induce target cell death via several pathways (4). Apoptosis is initiated by the delivery of granzymes through granule exocytosis and by stimulation of cell surface death receptors (46). Granzyme B activates several substrates, including caspase 3 (5, 6), whereas death receptor pathways of the TNF family, like Fas, use caspase 8 as the initiator (9). In this study, using a variety of approaches, we show that granulysin disrupts cell membranes, damages mitochondria, and causes release of cytochrome *c* and other factors (21). Thus, granulysin, a member of the SAPLIP family, acts via a distinct molecular mechanism for induction of target cell death by CTL and NK cells.

Acknowledgments

References

1. Peña, S. V., D. A. Hanson, B. A. Carr, T. J. Goralski, and A. M. Krensky. 1997. Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. *J. Immunol. 158:2680.*

2. Jongstra, J., T. J. Schall, B. J. Dyer, C. Clayberger, J. Jorgensen, M. M. Davis, and A. M. Krensky. 1987. The isolation and sequence of a novel gene from a human functional T cell line. *J. Exp. Med. 165:601.*

3. Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melián, C. Bogdan, et al. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science 282:121.*

4. Henkart, P. A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity 1:343.*

5. Darmon, A. J., D. W. Nicholson, and R. C. Bleackley. 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature 377: 446.*

6. Yang, X., H. R. Stennicke, B. Wang, D. R. Green, R. U. Janicke, A. Srinivasan, P. Seth, G. S. Salvesen, and C. J. Froelich. 1998. Granzyme B mimics apical caspases: description of a unified pathway for *trans*-activation of executioner caspase-3 and -7. *J. Biol. Chem. 273:34278.*

7. Heibein, J. A., I. S. Goping, M. Barry, M. J. Pinkoski, G. C. Shore, D. R. Green, and R. C. Bleackley. 2000. Granzyme B-mediated cytochrome *c* release is regulated by the Bcl-2 family members Bid and Bax. *J. Exp. Med. 192:1391.*

8. Beresford, P. J., Z. Xia, A. H. Greenberg, and J. Lieberman. 1999. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity 10:585.*

9. Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signaling and modulation. *Science 281:1305.*

10. Green, D. R. 1998. Apoptotic pathways: the roads to ruin. *Cell 94:695.*

11. Gamen, S., D. A. Hanson, A. Kaspar, J. Naval, A. M. Krensky, and A. Anel. 1998. Granulysin-induced apoptosis. I. Involvement of at least two distinct pathways. *J. Immunol. 161:1758.*

12. Dou, Q. P., B. An, K. Antoku, and D. E. Johnson. 1997. Fas stimulation induces RB dephosphorylation and proteolysis that is blocked by inhibitors of the ICE protease family. *J. Cell. Biochem. 64:586.*

13. Hitoshi, Y., J. Lorens, S. I. Kitada, J. Fisher, M. LaBarge, H. Z. Ring, U. Francke, J. C. Reed, S. Kinoshita, and G. P. Nolan. 1998. Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. *Immunity 8:461.*

14. Kelekar, A., B. S. Chang, J. E. Harlan, S. W. Fesik, and C. B. Thompson. 1997. Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol. Cell. Biol. 17:7040.*

15. Chang, R., S. Nir, and F. R. Poulain. 1998. Analysis of binding and membrane destabilization of phospholipid membranes by surfactant apoprotein B. *Biochim. Biophys. Acta 1371:254.*

16. Alley, M. C., D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker, and M. R. Boyd. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res. 48:589.*

17. Granville, D. J., C. M. Carthy, H. Jiang, G. C. Shore, B. M. McManus, and D. W. Hunt. 1998. Rapid cytochrome *c* release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. *FEBS Lett. 437:5.*

18. Kingham, P. J., and J. M. Pocock. 2000. Microglial apoptosis induced by chromogranin A is mediated by mitochondrial depolarization and the permeability transition but not by cytochrome *c* release. *J. Neurochem. 74:1452.*

19. Kluck, R. M., M. D. Esposti, G. Perkins, C. Renken, T. Kuwana, E. Bossy-Wetzel, M. Goldberg, T. Allen, M. J. Barber, D. R. Green, and D. D. Newmeyer. 1999. The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J. Cell Biol. 147:809.*

20. Newmeyer, D. D., D. M. Farschon, and J. C. Reed. 1994. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell 79:353.*

21. Green, D. R., and J. C. Reed. 1998. Mitochondria and apoptosis. *Science 281: 1309.*

22. Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and D. J. Pickup. 1992. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell 69:597.*

23. Kim, M., D. D. Cooper, S. F. Hayes, and G. J. Spangrude. 1998. Rhodamine-123 staining in hematopoietic stem cells of young mice indicates mitochondrial activation rather than dye efflux. *Blood 91:4106.*

24. Zahrebelski, G., A. L. Nieminen, K. al-Ghoul, T. Qian, B. Herman, and J. J. Lemasters. 1995. Progression of subcellular changes during chemical hypoxia to cultured rat hepatocytes: a laser scanning confocal microscopic study. *Hepatology 21:1361.*

25. Mathur, A., Y. Hong, B. K. Kemp, A. A. Barrientos, and J. D. Erusalimsky. 2000. Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. *Cardiovasc. Res. 46:126.*

26. Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science 275:1132.*

27. Antoku, K., Z. Liu, and D. E. Johnson. 1997. Inhibition of caspase proteases by CrmA enhances the resistance of human leukemic cells to multiple chemotherapeutic agents. *Leukemia 11:1665.*

28. Zhou, Q., S. Snipas, K. Orth, M. Muzio, V. M. Dixit, and G. S. Salvesen. 1997. Target protease specificity of the viral serpin CrmA: analysis of five caspases. *J. Biol. Chem. 272:7797.*

29. Irmler, M., M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J. L. Bodmer, M. Schroter, K. Burns, C. Mattmann, et al. 1997. Inhibition of death receptor signals by cellular FLIP. *Nature 388:190.*

30. Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell 81:505.*

31. Srinivasula, S. M., M. Ahmad, T. Fernandes-Alnemri, and E. S. Alnemri. 1998. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol. Cell 1:949.*

32. Stenger, S., R. J. Mazzaccaro, K. Uyemura, S. Cho, P. F. Barnes, J. P. Rosat, A. Sette, M. B. Brenner, S. A. Porcelli, B. R. Bloom, and R. L. Modlin. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science 276:1684.*

33. Leippe, M. 1995. Ancient weapons: NK-lysin is a mammalian homologue to pore-forming peptides of a protozoan parasite. *Cell 83:17.*

34. Hanson, D. A., A. A. Kaspar, F. R. Poulain, and A. M. Krensky. 1999. Biosynthesis of granulysin, a novel cytolytic molecule. *Mol. Immunol. 36:413.*

35. Andersson, M., H. Gunne, B. Agerberth, A. Boman, T. Bergman, R. Sillard, H. Jörnvall, V. Mutt, B. Olsson, H. Wigzell, et al. 1995. NK-lysin, a novel effector peptide of cytotoxic T and NK cells: structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumor activity. *EMBO J. 14:1615.*

36. Ruysschaert, J. M., E. Goormaghtigh, F. Homblé, M. Andersson, E. Liepinsh, and G. Otting. 1998. Lipid membrane binding of NK-lysin. *FEBS Lett. 425:341.*

37. Shiver, J. W., and P. A. Henkart. 1991. A noncytotoxic mast cell tumor line exhibits potent IgE-dependent cytotoxicity after transfection with the cytolysin/perforin gene. *Cell 64:1175.*

38. Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer, and M. E. Peter. 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J. 17:1675.*

39. Stennicke, H. R., Q. L. Deveraux, E. W. Humke, J. C. Reed, V. M. Dixit, and G. S. Salvesen. 1999. Caspase-9 can be activated without proteolytic processing. *J. Biol. Chem. 274:8359.*

40. Zamzami, N., S. A. Susin, P. Marchetti, T. Hirsch, I. Gomez-Monterrey, M. Castedo, and G. Kroemer. 1996. Mitochondrial control of nuclear apoptosis. *J. Exp. Med. 183:1533.*

41. Susin, S. A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp. Med. 184:1331.*

42. Susin, S. A., N. Zamzami, M. Castedo, E. Daugas, H. G. Wang, S. Geley, F. Fassy, J. C. Reed, and G. Kroemer. 1997. The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. *J. Exp. Med. 186:25.*

43. Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell, and T. J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell 76:977.*

44. Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science 265:528.*

45. Ganz, T., and J. Weiss. 1997. Antimicrobial peptides of phagocytes and epithelia. *Semin. Hematol. 34:343.*

46. Berke, G. 1995. The CTL's kiss of death. *Cell 81:9.*