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Granulysin-Induced Apoptosis. I. Involvement of at Least Two Distinct Pathways¹

Susana Gamen,* Dennis A. Hanson,[†] Allan Kaspar,[†] Javier Naval,* Alan M. Krensky,^{2†} and Alberto Anel*

Granulysin is a newly described cytolytic molecule released by CTL and NK cells via granule-mediated exocytosis. It shares homology with saposin-like proteins, including NK-lysin and amoebapores, and has been implicated in the lysis of tumor cells and microbes. In the present study we show that recombinant granulysin alone induces apoptosis of Jurkat cells. This apoptosis is associated with a sixfold increase in the ceramide/sphingomyelin ratio, implicating the activation of sphingomyelinases. Granulysin- and ceramide-induced apoptosis are similar in that they both are only minimally inhibited by the more selective cysteine protease p32 (caspase 3)-like caspase inhibitor *N*-acetyl-Asp-Glu-Val-Asp aldehyde, while they are significantly inhibited by the more general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). Nevertheless, while Z-VAD-fmk almost completely inhibits ceramide-induced apoptosis, a Z-VAD-fmk-resistant component was observed using granulysin. Granulysin also causes apoptosis in cells depleted of sphingomyelin by prolonged treatment with the ceramide synthase inhibitor fumonisins B₁. These data indicate that granulysin induces target cell death by both ceramide- and caspase-dependent and -independent pathways. *The Journal of Immunology*, 1998, 161: 1758–1764.

Cell-mediated cytotoxicity plays an essential role in defense against viral infection and malignancy. CTL and NK cells induce target cell death by two major mechanisms (1–3). The first mechanism involves Ca²⁺-dependent secretion of preformed cytoplasmic granules that contain cytotoxic molecules, the pore-forming protein perforin and several serine proteases, most importantly granzyme A and granzyme B in humans (4–6). Perforin pores allow entry of granzymes into the cytoplasm of target cells (6), where they induce apoptotic cell death. Although exogenous granzymes can also enter target cells by themselves, via a classical endocytic pathway, they are not cytotoxic unless liberated from endosomes into the cytosol by perforin or another endosomolytic agent, such as adenovirus (7). The second mechanism involves the interaction of Fas (CD95), a TNF receptor-like molecule, with its ligand (1, 8).

Cytolysis induced by both granule-mediated and Fas-based mechanisms exhibits the typical features of apoptosis (9). The biochemical mechanism for Fas-induced apoptosis involves the recruitment and activation of intracellular cysteine proteases of the caspase family (10). In addition, granzyme B can cleave and ac-

tivate several caspases (11, 12). Although nuclear manifestations of granzyme B-induced apoptosis are dependent on caspase activation (7, 13–15), cytolysis appears to be caspase independent (13, 15). In contrast, the manifestations of granzyme A-induced apoptosis appear independent of caspase activation (14).

Granulysin is a protein localized inside CTL granules with an expression pattern similar to those of perforin and granzymes, being selectively up-regulated during the stage at which these cells are able to exert cytolytic function (16, 17). This protein is exocytosed after CTL stimulation through the TCR and exerts lytic activity as measured by ⁵¹Cr release assays (17). These data suggest that granulysin-induced cytolysis could constitute another granule-associated lytic pathway in addition to the perforin/granzyme pathway (17). Granulysin is highly homologous to another lytic molecule localized inside porcine NK cell granules, termed NK-lysin (18), and to *Entamoeba histolytica* amoebapores (19), proteins capable of forming pores into lipid membranes. These homologies suggest that granulysin might also form pores. Granulysin also shares sequence homology with saposins (17). Saposins are not pore-forming proteins, but are proteins that interact with lipid membranes and activate lipid-degrading enzymes (specifically, glucosylceramidases and sphingomyelinases) (20, 21). The immediate consequence of activation of these enzymes is the increase in cellular ceramide content (22). Ceramide, of note, has been proposed as a mediator in some apoptotic processes (23, 24), and when added exogenously, ceramide can induce apoptosis (25, 26).

The partial homology of granulysin with saposins suggested that it might induce cytotoxicity through activation of sphingomyelinases and an increase in cellular ceramide content. Here we demonstrate that granulysin causes apoptotic cell death. Jurkat tumor cells treated with recombinant granulysin exhibit a significant increase in ceramide content and a decrease in sphingomyelin. However, granulysin also caused apoptosis in cells depleted of sphingomyelin by prolonged treatment with the ceramide synthase inhibitor fumonisins B₁. This finding suggests that granulysin may induce apoptosis via more than one pathway.

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Materials and Methods

Cells

The Jurkat T cell leukemia (American Type Culture Collection, Rockville, MD; clone E6.1) was used as the target in the granulysin- and ceramide-induced cytotoxicity assays performed in this study. Jurkat cells were cultured in RPMI 1640 medium (BioWhittaker, Barcelona, Spain) supplemented with 5% FCS, L-glutamine, and penicillin/streptomycin (hereafter, complete medium) using standard cell culture procedures.

Materials

Fumonisin B₁ from *Fusarium moniliforme*, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)³, ceramide type III from bovine brain, and dodecane were purchased from Sigma (Madrid, Spain). *N*-acetyl-Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were obtained from Bachem (Bubendorf, Switzerland). [¹⁴C]palmitic acid was purchased from Amersham (Madrid, Spain). Mouse IgG2a anti-human CPP32 (caspase 3, clone 19) was obtained from Transduction Laboratories (Affinity, Mamhead, U.K.).

Expression and purification of recombinant functional granulysin

Recombinant granulysin was expressed and purified essentially as previously described (17). Briefly, a portion of the 519 cDNA coding for glycine residue 63 through the arginine residue 136 of P520 (27) followed by a stop codon was cloned into the pET28a vector (Novagen, Madison, WI) to express polyhistidine-tagged recombinant 9-kDa granulysin in *Escherichia coli* BL21 (DE3). A control construct of similar size, coding for residues 549 through 646 of human heat shock protein 70 (HSC70) was produced for parallel expression and purification. The recombinant proteins were expressed and purified on a nickel column under denaturing conditions according to Novagen's instructions. After purification, the proteins were reduced by the addition of 1 mM DTT, allowed to refold in the presence of oxidized DTT, and dialyzed against Tris-buffered saline; the histidine tag removed by thrombin treatment and purified by reverse phase HPLC. Quantification of the protein was performed using a standard commercial kit from Bio-Rad (Richmond, CA) with lysozyme as a standard. Protein purity was always >95%, as assessed by Coomassie staining of 15% SDS-PAGE gels.

Cytotoxicity assays

Jurkat cells were seeded in flat-bottom, 96-well plates at an initial density of 2×10^5 cells/ml (100 μ l/well) and cultured for 16 h in complete medium in the presence or the absence of 25 ng/ml of the cytotoxic anti-Fas mAb CH-11, as described previously (26, 28). For granulysin-induced lysis, cells were similarly resuspended in RPMI 1640 medium supplemented with 1% FCS and incubated for 16 h at 37°C in the presence or the absence of 20 to 50 μ M recombinant granulysin. Natural (C₁₈) ceramide (50–200 nM) was first dissolved in ethanol/dodecane (98/2, v/v) and then added to serum-free culture medium (RPMI 1640/DMEM/Ham's F-12, 2/1/1) as previously described (26, 29). At the doses used, ethanol and dodecane had no effect on cell proliferation. Ac-DEVD-CHO, an inhibitor of CPP32-like caspases (30), or Z-VAD-fmk, a more general caspase inhibitor (31), were used at 600 and 100 μ M, respectively. Peptide inhibitors were first dissolved in DMSO and diluted in culture medium (final DMSO concentration, $\leq 0.4\%$, v/v). The addition of the peptide inhibitors at concentrations up to 1.2 mM did not affect to the growth rate or cell morphology of Jurkat cells. Cells were preincubated for 1 h with the protease inhibitors before adding the toxic stimuli to assure a sufficient incorporation by cells (26, 28, 32). Jurkat cells were depleted of sphingomyelin and ceramide by culture for 3 days in complete medium supplemented with 50 μ M fumonisin B₁, a potent inhibitor of ceramide synthase (33). Cell viability was determined by a modification of the MTT reduction method of Mosmann (34) and expressed as a percentage of that in control cultures. Cell death was determined by the trypan blue exclusion test and microscopy of stained cells. Phosphatidylserine (PS) exposure during apoptosis was evaluated by annexin V-FITC staining (35). Briefly, cells were washed with PBS and incubated in a solution of 0.5 μ g/ml FITC-labeled annexin V (Bender, Barcelona, Spain) in binding buffer (140 mM NaCl, 2.5 mM CaCl₂, and 10

mM HEPES/NaOH, pH 7.4) at 4°C for 30 min. Cells were then centrifuged, washed, resuspended in 1 ml of binding buffer, and analyzed by flow cytometry.

Ceramide and sphingomyelin determinations

Ceramide and sphingomyelin levels were assayed essentially as previously described (26). Jurkat cells (1.8×10^6 in 10 ml) were labeled for 48 h with 5 μ Ci of [¹⁴C]palmitic acid bound to fatty acid-free serum albumin (1/1, molar ratio) in complete medium. Cells were resuspended in RPMI 1640 medium supplemented with 1% FCS at 4×10^5 cells/ml, and granulysin (50 μ M) or the control recombinant protein HSC70 was added. After incubation for 16 h at 37°C, the cells were harvested, washed with cold RPMI medium, and counted. Cell viability was determined in an aliquot of the corresponding cell suspensions by the MTT assay. Total cell lipids were extracted at 4°C with chloroform/methanol (2/1, v/v) (36). Radioactivity in aliquots from chloroformic phases was determined by liquid scintillation counting, and equal amounts of radioactivity for each sample were applied to TLC Silica gel G plates (Scharlau, Barcelona, Spain). Plates were prewashed with chloroform/methanol (1/1, v/v) and heat-activated at 110°C for 1 h. A first development of TLC-loaded samples was performed with chloroform/methanol/water (60/30/5, v/v) up to 10 cm from the bottom of the plate, and a second development was performed to its full length with hexane/diethyl ether/acetic acid (80/20/2, v/v/v). Plates were air-dried, and radiolabeled bands were located by film autoradiography (Hyperfilm β -max, Amersham) at room temperature for 2 to 3 days. Then, sample lanes were covered with glass, allowing the marker lanes to be iodine stained. Standards of ceramide, sphingomyelin, cholesterol, phosphatidylcholine, and PS (Sigma) were used as markers. The corresponding radiolabeled lipids were scraped and transferred to vials, to which 4 ml of scintillation mixture (Normascint 11, Scharlau) containing 10% (v/v) methanol were added. Radioactivity in samples was determined by liquid scintillation counting, and results were expressed as ceramide/sphingomyelin (Cer/SM) ratios.

Fluorescence microscopy

Morphologic evaluation of apoptosis was monitored by cell labeling with the nuclear stain *p*-phenylenediamine (PPDA) and then visualized by fluorescence microscopy (37). Briefly, cells were washed with PBS, pH 7.4, and fixed at room temperature with 1% paraformaldehyde in PBS for 15 min. Fixed cells were washed with PBS, centrifuged onto glass coverslips in wells of a 24-well plate, mounted on a glass slide over a drop of PPDA stain (10 mg of PPDA in 1 ml of PBS mixed with 9 ml of oxidized glycerol) (28), and photographed.

Analysis of CPP32 activation by immunoblotting

CPP32 activation was evaluated by Western blot analysis of cell homogenates with a specific anti-human CPP32 Ab (38). Jurkat cells (5×10^6 in 1 ml) were treated with 100 nM ceramide, as indicated above, for 5 h at 37°C. At the end of the incubations, cells were recovered by centrifugation at 4°C, washed twice with cold PBS, and lysed in 1 ml of lysis buffer (50 mM Tris/HCl, pH 7.6, containing 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 30 mM NaF, 10 μ g/ml leupeptin, and 1 mM PMSF), as previously described (39). Cell lysates were centrifuged (4°C, 12,000 \times g, 15 min), and solubilized proteins were separated by SDS-PAGE in an SDS-12%-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham). Membranes were then sequentially incubated with 50 ng/ml anti-CPP32 Ab in PBS containing 5% BSA and with 0.2 μ g/ml goat anti-mouse IgG coupled to alkaline phosphatase (Sigma) for 1 h and revealed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (40).

Results

Granulysin induces apoptotic cell death

Recombinant granulysin is cytotoxic by itself in a ⁵¹Cr release assay using B lymphoblastoid and other target cells (17). If this lysis were due to pore formation in the plasma membrane and disruption of osmotic equilibrium, as has been described for NK-lysin and amoebapores (18, 19), necrotic, rather than apoptotic, cell death should be observed. Jurkat cells were treated with various doses of recombinant granulysin, and toxicity was tested by the MTT assay. The IC₅₀ dose was 40 μ M, with no toxicity at 10 μ M and almost 100% cell death at 100 μ M. This dose response is similar to that previously obtained for granulysin-induced ⁵¹Cr release from YAC cells (17). To test the type of cell death induced

³ Abbreviations used in this paper: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp aldehyde; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; CPP32, cysteine protease p32 (caspase 3); PS, phosphatidylserine; Cer/SM, ceramide/sphingomyelin ratio; PPDA, *p*-phenylenediamine.

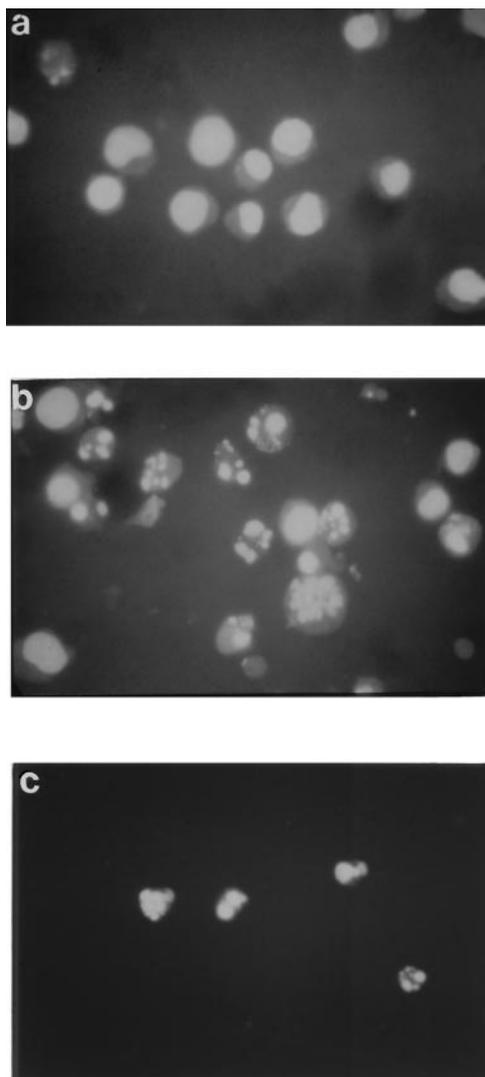


FIGURE 1. Granulysin induces apoptotic morphology. Jurkat cells were cultured for 16 h in medium supplemented with 1% FCS in the absence (A) or the presence of either 20 ng/ml of anti-Fas mAb (B) or 50 μ M granulysin (C). Cell nuclei were stained with PPDA and photographed under epifluorescence illumination. Original magnification, $\times 400$.

by granulysin, Jurkat cells were treated with 50 μ M recombinant granulysin or anti-Fas mAb, and nuclei were stained with the fluorescent probe PPDA. Both granulysin and anti-Fas mAb induced nuclear features of typical apoptosis: cell shrinkage, blebbing, chromatin condensation, and nuclear fragmentation (Fig. 1). Another early event that takes place during apoptosis is the redistribution of PS from the inner to the outer leaflet of the plasma membrane. Annexin V has a great affinity for PS and annexin V FITC staining is a good measure of PS translocation (35). As shown in Figure 2, granulysin induces increased annexin V staining. We previously showed that granulysin also induces cell death of JY (a B lymphoblastoid cell line), K562, and YAC (17). Treatment of these cell lines with granulysin similarly increases annexin V staining (data not shown). Hence, granulysin treatment of cells causes cell death with the characteristics of apoptosis.

Granulysin-induced apoptosis is associated with an increase in the Cer/SM ratio

The effect of granulysin on ceramide and sphingomyelin levels in target cells was examined. Jurkat cells treated with 50 μ M granu-

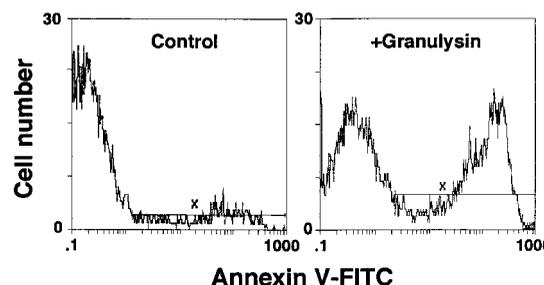


FIGURE 2. Granulysin induces PS translocation. Jurkat cells were cultured for 16 h in medium supplemented with 1% FCS in the absence (Control) or the presence of 50 μ M granulysin (+ Granulysin). PS exposure was analyzed by measuring annexin V-FITC binding by flow cytometry.

lysin for 16 h were killed, as determined by the MTT assay and trypan blue staining, while cells treated with recombinant HSC-70 protein were not (Fig. 3c). Granulysin induced a significant increase in cellular ceramide content and a decrease in that of sphingomyelin (Fig. 3a, lane 3), while no changes in sphingomyelin and ceramide levels were observed in HSC-70-treated cells (Fig. 3a, lane 2). The Cer/SM ratio was increased 6-fold in granulysin-treated cells (Fig. 3b). By comparison, anti-Fas mAb-induced apoptosis, which is associated with sphingomyelinase activation with a similar time course, increased the Cer/SM ratio by 2.6-fold (26) (data not shown). Thus, granulysin-induced apoptosis is associated with sphingomyelinase activation and ceramide generation.

Granulysin induces apoptosis in sphingomyelin and ceramide-depleted cells

Ceramide has been proposed as a mediator of some apoptotic processes (23, 24), and it induces apoptosis when added exogenously to cells (25, 26). To study the involvement of ceramide in granulysin-induced apoptosis, Jurkat cells were cultured for 72 h in the presence of 50 μ M fumonisins B₁, a potent inhibitor of ceramide synthase. This treatment resulted in almost complete depletion of sphingomyelin and ceramide (Fig. 4a). Ceramide levels did not increase in fumonisins B₁-treated cells incubated with either anti-Fas mAb (40) or granulysin (data not shown). However, granulysin-induced Jurkat cell death was similar in cells treated or not with the ceramide synthase inhibitor (Fig. 4b). Granulysin induced chromatin condensation and nuclear fragmentation in fumonisins B₁-treated cells (Fig. 4c) similarly to that observed in cells not treated with the ceramide synthase inhibitor (see Fig. 1C).

Ceramide-induced apoptosis in Jurkat cells is inhibited by Z-VAD-fmk, but not by Ac-DEVD-CHO

Intracellular cysteine proteases with Asp specificity (caspases) are the main executioners of apoptosis (41, 42). We previously reported that Ac-DEVD-CHO, a tetrapeptide inhibitor of the CPP32-like subfamily of caspases, completely inhibits Fas-induced apoptosis in Jurkat cells, but does not affect ceramide-induced cell death (26). CPP32, like other members of the caspase family, is activated by proteolytic processing (38). Activation of CPP32 can be analyzed by Western blotting with a specific mAb by monitoring the disappearance of the 32-kDa proenzyme. Treatment of Jurkat cells with anti-Fas mAb resulted in strong activation of CPP32 (Fig. 5a, lane 2), while treatment with exogenous ceramide caused only minor reduction in the levels of the proenzyme (Fig. 5a, lane 3). While Fas-induced apoptosis was completely blocked by the peptide inhibitor Ac-DEVD-CHO, apoptosis of ceramide-treated Jurkat cells was unaffected by the same concentration of inhibitor, indicating that neither CPP32 nor other CPP32-like

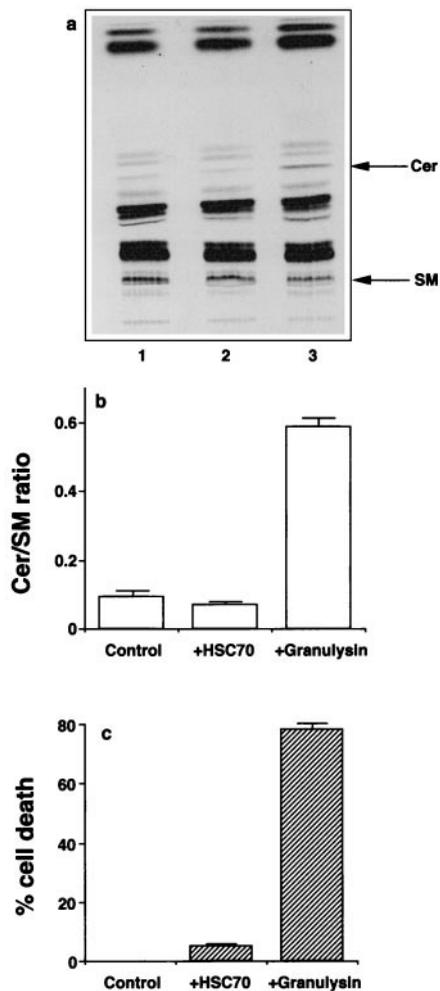


FIGURE 3. Granulysin toxicity is associated with an increase in cellular ceramide and a decrease in sphingomyelin content. *a*, TLC separation of cellular lipids. Jurkat cells previously labeled with [^{14}C]palmitic acid were incubated in medium supplemented with 1% FCS for 16 h in the absence (*lane 1*) or the presence of 50 μM recombinant HSC-70 (*lane 2*) or 50 μM granulysin (*lane 3*). Cell lipids were then extracted, separated by TLC, and revealed by autoradiogram. The positions of ceramide (Cer) and sphingomyelin (SM) are indicated on the *right* of the autoradiography. *b*, Radioactivity associated with the ceramide and sphingomyelin fractions was determined by liquid scintillation counting. Results are expressed as the ratio of radioactivity in the ceramide to sphingomyelin fractions (Cer/SM ratio). Counts per minute recovered were ≤ 800 and 5000 for the Cer and SM fractions, respectively. *c*, Toxicity induced by the indicated treatments was determined by the MTT method. Data in *b* and *c* are the mean \pm SD of duplicate determinations in two different experiments.

caspases are implicated in ceramide-induced apoptosis (Fig. 5, *b* and *c*).

Z-VAD-fmk is a more general caspase inhibitor, which has been demonstrated to inhibit CPP32 processing by inhibition of an upstream caspase (43), presumably FLICE (caspase-8) (44). Z-VAD-fmk almost completely blocked apoptosis induced by either anti-Fas mAb or exogenous ceramide in Jurkat cells (Fig. 5, *b* and *c*). Thus, in Jurkat cells, ceramide-induced apoptosis is mediated by the activation of one or several Z-VAD-sensitive, DEVD-insensitive caspases.

Effect of peptide caspase inhibitors on granulysin-induced apoptosis

The effect of granulysin treatment on CPP32 activation was tested by immunoblot, as indicated above for ceramide and anti-Fas

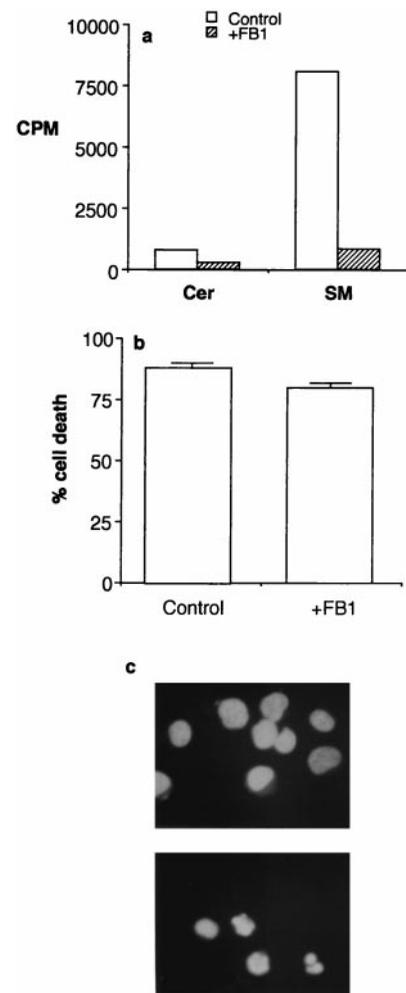


FIGURE 4. Chronic treatment with fumonisin B₁ greatly reduces cellular ceramide and sphingomyelin content but does not affect granulysin-induced apoptosis. *a*, Jurkat cells were pretreated with 50 μM fumonisin B₁ (FB₁) or medium (control) for 24 h. [^{14}C]palmitic acid (0.5 $\mu\text{Ci/ml}$) was then added, and cells were cultured for an additional 48 h. Cellular lipids were extracted and analyzed by TLC. Radioactivity in the ceramide (Cer) and sphingomyelin (SM) fractions was determined by liquid scintillation counting. *b*, Control or FB₁-treated cells were washed and resuspended in medium supplemented with 1% FCS in the absence (control) or the presence of the same FB₁ concentration. Granulysin (50 μM) was then added, and the cells were incubated for 16 h. Toxicity was determined by the MTT method. *c*, FB₁-treated cells were treated with granulysin (*lower photomicrograph*) or not (*upper photomicrograph*), as indicated in *b*. Cell nuclei were stained with PPDA and photographed under epifluorescence illumination. Original magnification, $\times 400$.

mAb. As shown in Figure 6*a*, CPP32 processing as a consequence of granulysin treatment was very limited, similar to that observed in ceramide-treated cells (see Fig. 5*a*, *lane 3*). The effect of peptide caspase inhibitors on granulysin-induced apoptosis was also tested. Apoptosis of Jurkat cells treated with granulysin was only slightly decreased by Ac-DEVD-CHO, whereas Z-VAD-fmk significantly protected Jurkat cells from granulysin-induced cell death (60% of protection, Fig. 6, *b* and *c*). This pattern was similar to that observed for ceramide-induced apoptosis (see Fig. 5) and could reflect the contribution of ceramide generation induced by granulysin (Fig. 3) to cell death. However, there is a significant (40%) Z-VAD-resistant component in granulysin-induced apoptosis. This component appears to be ceramide independent and may correspond to the mechanism involved in cytolysis of fumonisin B₁-treated cells (Fig. 4). In fact, while Z-VAD-fmk partially inhibited

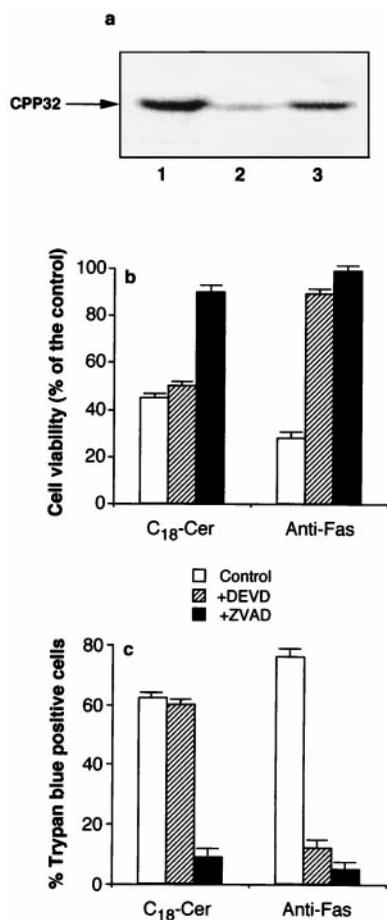


FIGURE 5. Ceramide induces limited CPP32 activation. Effect of peptide caspase inhibitors on ceramide- and Fas-induced apoptosis. *a*, Jurkat cells (5×10^5 cells/ml) were incubated for 3 h in serum-free medium supplemented with 0.4% ethanol/dodecane (98/2, v/v; lane 1), 20 ng/ml of anti-Fas mAb (lane 2), or 200 nM of C₁₈-ceramide dissolved in ethanol/dodecane (98/2, v/v; lane 3). Cell proteins were separated by SDS-12% PAGE, and CPP32 activation was analyzed by immunoblotting using an anti-CPP32 Ab. *b* and *c*, Cells were preincubated for 1 h in the absence (□) or the presence of 600 μ M DEVD-CHO (▨) or 100 μ M Z-VAD-fmk (▩). C₁₈-ceramide (200 nM) dissolved in ethanol/dodecane (98/2, v/v) or 20 ng/ml of anti-Fas mAb was then added, as indicated, and cells were incubated for another 16 h. The final concentration of ethanol/dodecane was 0.4% (v/v). Cell viability was determined by the MTT assay (*b*), and cell death was quantitated by trypan blue exclusion method (*c*). Results are the mean \pm SD of quadruplicate determinations in two different experiments.

granulysin toxicity in Jurkat cells (Fig. 6), it had no effect on granulysin-induced apoptosis in ceramide- and SM-depleted Jurkat cells (Fig. 7). The granulysin toxicity on fumonisins B₁-treated cells was similar to that in normal Jurkat cells, in agreement with the results shown in Figure 4.

Discussion

Granulysin is expressed in CTL and NK granules when these cells become cytolytic against targets, paralleling the time course of expression of other granule-specific cytotoxic molecules, perforin and granzymes (16, 17). Granulysin is cytotoxic by itself and can thus constitute an additional granule-associated lytic mechanism. By homology with amoebapores (19), which form pores in lipid membranes, granulysin may be a pore-forming protein, but no experimental data are currently available. In the present work, we initiated characterization of the cellular biochemical pathways in-

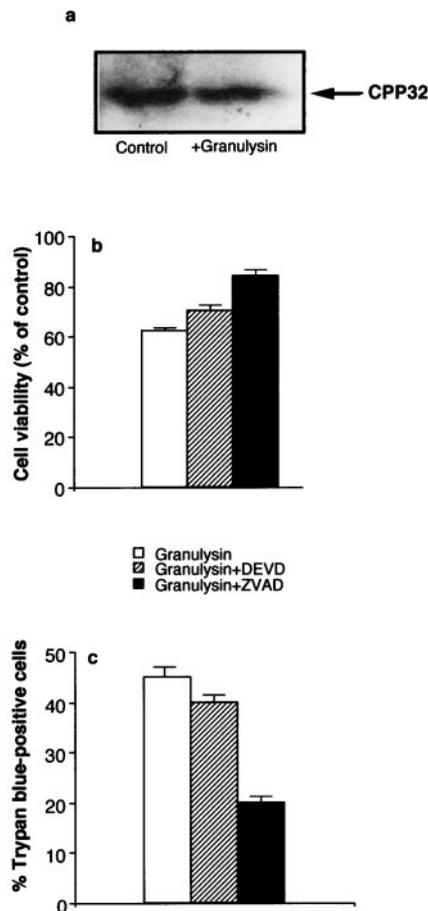


FIGURE 6. Granulysin does not activate CPP32. Effect of peptide caspase inhibitors on granulysin-induced apoptosis. *a*, Jurkat cells (5×10^5 cells/ml) were incubated for 16 h in medium supplemented with 1% FCS in the absence (Control) or the presence of 50 μ M granulysin (+ Granulysin). Cell proteins were separated by SDS-12% PAGE, and CPP32 activation was analyzed by immunoblotting using an anti-CPP32 Ab. *b* and *c*, Jurkat cells were preincubated for 1 h in medium supplemented with 1% FCS in the absence (□) or the presence of 600 μ M DEVD-CHO (▨) or 100 μ M Z-VAD-fmk (▩). Granulysin (50 μ M) was then added, and cells were incubated for an additional 16 h. Cell viability was determined by the MTT assay (*b*), and cell death was quantitated by trypan blue exclusion (*c*). Results are the mean \pm SD of quadruplicate determinations in two different experiments.

duced by granulysin treatment. We show that granulysin induces the typical features of apoptosis, including cell shrinkage, chromatin condensation, nuclear fragmentation, and PS translocation. Granulysin-induced apoptosis is associated with an increase in ceramide content and a concomitant decrease in sphingomyelin, suggesting the activation of sphingomyelinases. Perforin, another granule-specific, pore-forming protein, does not appear to induce apoptotic cell death by itself (45, 46), although this independent induction is controversial (47). In any case, perforin is not a saposin-like protein and is unable to activate sphingomyelinases.

By homology with pore-forming proteins, such as amoebapores and perforin, granulysin could interact with lipids in the plasma membrane, forming pores. Two hypothetical models could then explain granulysin-induced sphingomyelinase activation: 1) in a manner similar to that proposed for saposin A in the activation of acidic sphingomyelinase (21), granulysin could activate plasma membrane neutral sphingomyelinase at the same time as granulysin forms pores in the membrane; or 2) as described for all saposins, granulysin could activate acidic sphingomyelinase (20, 21,

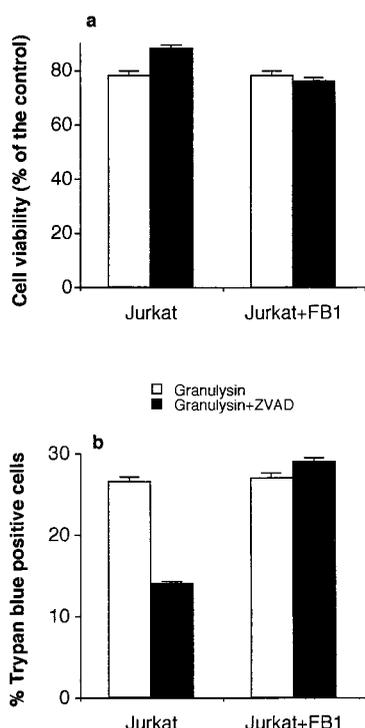


FIGURE 7. Granulysin toxicity is not inhibited by Z-VAD-fmk in SM-depleted Jurkat cells. Jurkat cells were pretreated (Jurkat-fumonisin B₁) or not (Jurkat) with 50 μ M fumonisin B₁ (FB₁) for 72 h. Then, they were preincubated for 1 h in serum-free medium in either the absence (\square) or the presence (\blacksquare) of 100 μ M Z-VAD-fmk before the addition of 50 μ M granulysin. The cells were incubated with granulysin for an additional 16 h. Cell viability was determined by MTT assay (a), and cell death was quantitated by trypan blue exclusion (b). Results shown are the mean \pm SD of quadruplicate determinations in one experiment.

48). Since acid sphingomyelinase is restricted to the lysosomal compartment, the second possibility would implicate granulysin internalization and localization into this compartment, a more complex process than mere insertion into the plasma membrane. Additional studies are needed to characterize the mechanism of action of granulysin, especially regarding its subcellular localization while inducing its toxic effect and its potential for pore formation.

The marked (sixfold) increase in the Cer/SM ratio associated with granulysin toxicity appears to implicate ceramide generation as a mediator of granulysin-induced apoptosis. Nevertheless, granulysin was able to exert a toxic effect in cells depleted of sphingomyelin and ceramide by prolonged culture in the presence of the ceramide synthase inhibitor fumonisin B₁. Apoptosis induced by exogenous ceramide was inhibited by Z-VAD-fmk (this work and Ref. 49), but not by Ac-DEVD-CHO (this work and Ref. 26). Z-VAD-fmk inhibits the proteolytic activity of several caspases (41, 43), while Ac-DEVD-CHO preferentially inhibits CPP32 and related caspases (41). Z-VAD-fmk inhibits the processing and activation of CPP32, at least during Fas-induced apoptosis (43), presumably by inhibiting FLICE (caspase-8) (44). Together these data suggest that ceramide-induced apoptosis is dependent on a Z-VAD-sensitive, DEVD-insensitive caspase. Caspases 1, 4, 5, and 6 exhibit similar sensitivities to these peptides (41, 50), although only activated CPP32 (caspase-3) and Mch2 (caspase-6) have been detected in apoptotic Jurkat cells (51).

Granulysin-induced cell death had an inhibitory profile similar to that induced by exogenous ceramide: weak protection by Ac-

DEVD-CHO and strong protection by Z-VAD-fmk. These data suggest that the observed increase in cellular ceramide content as a consequence of granulysin treatment is indeed an important part of granulysin toxicity. However, there is also a Z-VAD-fmk-resistant component (>40%) in granulysin-induced apoptosis that is not observed when cell death is induced by exogenous ceramide. This component should be ceramide independent and could correspond to the lytic mechanism that mediates the entire granulysin toxic effect in SM-depleted cells. In fact, the observation that Z-VAD-fmk had no effect on granulysin toxicity in SM-depleted cells indicates that the ceramide-dependent and the Z-VAD-sensitive components are equivalent in granulysin-induced apoptosis.

The ceramide-independent granulysin-induced lytic pathway may be associated with the formation of membrane pores, although further studies are needed to demonstrate this possibility. In this respect, the dose-response toxicity obtained with Jurkat cells is similar to that previously obtained for granulysin-induced ⁵¹Cr release in YAC cells, suggesting that apoptosis, pore formation, and ⁵¹Cr release occur at the same doses of granulysin. The ceramide-independent component of granulysin-induced lysis is also apoptotic, associated with chromatin condensation and nuclear fragmentation, as demonstrated in SM-depleted cells. This effect could be associated with the increase in intracellular Ca⁺² concentration induced by pores in the plasma membrane, since apoptotic cell death can be induced by calcium ionophores alone (52, 53). However, perforin-induced lysis has not been associated with nuclear fragmentation in some studies despite the formation of pores in the plasma membrane (45, 46), and ceramide-independent apoptotic cell death induced by granulysin could be dependent on some uncharacterized particular property of this protein.

In summary, we provide the first evidence for the biochemical mechanism of granulysin-induced lysis. Granulysin treatment of cells induces sphingomyelin-derived ceramide generation and the activation of Z-VAD-sensitive, DEVD-insensitive caspases, leading to apoptosis. This may constitute an additional lytic mechanism used by CTL and NK cells to eliminate virus-infected and tumor cells. However, granulysin-induced apoptosis also takes place through ceramide- and caspase-independent mechanisms that remain to be elucidated.

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