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Cytotoxic Cell Granule-Mediated Apoptosis: Perforin Delivers Granzyme B-Serglycin Complexes into Target Cells without Plasma Membrane Pore Formation

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Summary

The mechanism underlying perforin (PFN)-dependent delivery of apoptotic granzymes during cytotoxic cell granule-mediated death remains speculative. Granzyme B (GrB) and perforin were found to coexist as multimeric complexes with the proteoglycan serglycin (SG) in cytotoxic granules, and cytotoxic cells were observed to secrete exclusively macromolecular GrB-SG. Contrary to the view that PFN acts as a gateway for granzymes through the plasma membrane, monomeric PFN and, strikingly, PFN-SG complexes were shown to mediate cytosolic delivery of macromolecular GrB-SG without producing detectable plasma membrane pores. These results indicate that granule-mediated apoptosis represents a phenomenon whereby the target cell perceives granule contents as a multimeric complex consisting of SG, PFN, and granzymes, which are, respectively, the scaffold, translocator, and targeting/informational components of this modular delivery system.

Introduction

The ability of cytotoxic lymphocytes to initiate apoptosis in pathogen-infected and transformed cells depends on two fundamentally distinct signal transduction strategies. Through a series of complex molecular interactions, the Fas pathway involves receptor-dependent transmission of signals that activates the death caspases and other host responses. The granule secretion pathway, on the other hand, appears to require the direct intracellular delivery of a family of granule-associated-serine proteases, which activate both caspase-independent (granzyme A, GrA) and dependent (granzyme B, GrB) death programs to ensure that the targeted cell dies. Perforin (PFN), well-known for its pore-forming capacity, has long been considered the vehicle that provides the gateway for entry of the granzymes through the plasma membrane (Young et al., 1986). Although appealing, experimental evidence for this model is marginal, leaving the mechanism underlying PFN-dependent delivery of the granzymes unexplained. We have previously demonstrated that GrB binds to target cells in a specific, saturable manner but after internalization remains innocuously confined to endocytic vesicles (Froelich et al., 1996a). Effecting release of the granzyme to the cytosol requires the presence of PFN or a demonstrated endosomolytic agent (e.g., adenovirus [AD], listeriolysin) (Froelich et al., 1996a; Browne et al., 1999). Furthermore, disruption of vesicular trafficking impairs this process (Browne et al., 1999), providing indirect evidence that PFN may act at the endosomal level to deliver the granzymes. Elaborating on the model originally proposed by the Podack laboratory (Podack et al., 1988), we predicted that PFN pores in the plasma membrane would undergo internalization with bound granzyme to an endocytic vesicle where the established channels then facilitate escape of the protease (Froelich et al., 1998). Contrary to this prediction, we show here that cells that are destined to become apoptotic after exposure to PFN and GrB do not possess detectable plasma membrane pores.

Complementing our observations of PFN-mediated delivery, evidence is also presented that serglycin (SG), the primary proteoglycan of cytotoxic granules, plays a central role in granule-mediated apoptosis. SG, distinguished by linkage of chondroitin sulfate (CS) glycosaminoglycans (GAG) to Ser-Gly repeats in the central portion of the core protein (Stevens et al., 1988), reportedly facilitates packaging of proteases and cytokines and is postulated to participate in their transport to extracellular sites (Stevens et al., 1988; Matsumoto et al., 1995). We show here that GrB is secreted solely in a macromolecular form bound to SG and preformed GrB-SG complexes are delivered by PFN to induce apoptosis through a process that does not involve detectable plasma membrane permeabilization. Furthermore, PFN retains the capacity to deliver the macromolecular granzyme complex when ionically linked to SG. The results suggest that PFN, either in monomeric form or complexed to SG, enters the target cell delivering internalized macromolecular granzyme complex from a vesicular compartment in a manner similar to viral-mediated cellular penetration.

Results

Transmembrane Pores Formed by Sublytic PFN Is a Constrained Outcome

Experimental models of GrB-induced cell death involve the addition of isolated PFN to target cells in "sublytic" quantities that irreversibly damage a minority of the target cells (5%–20%) when evaluated microscopically. It has been assumed that the remaining viable targets also undergo membrane damage but the pores are eliminated by exocytic or endocytic processes. To document the dynamics of membrane permeabilization produced

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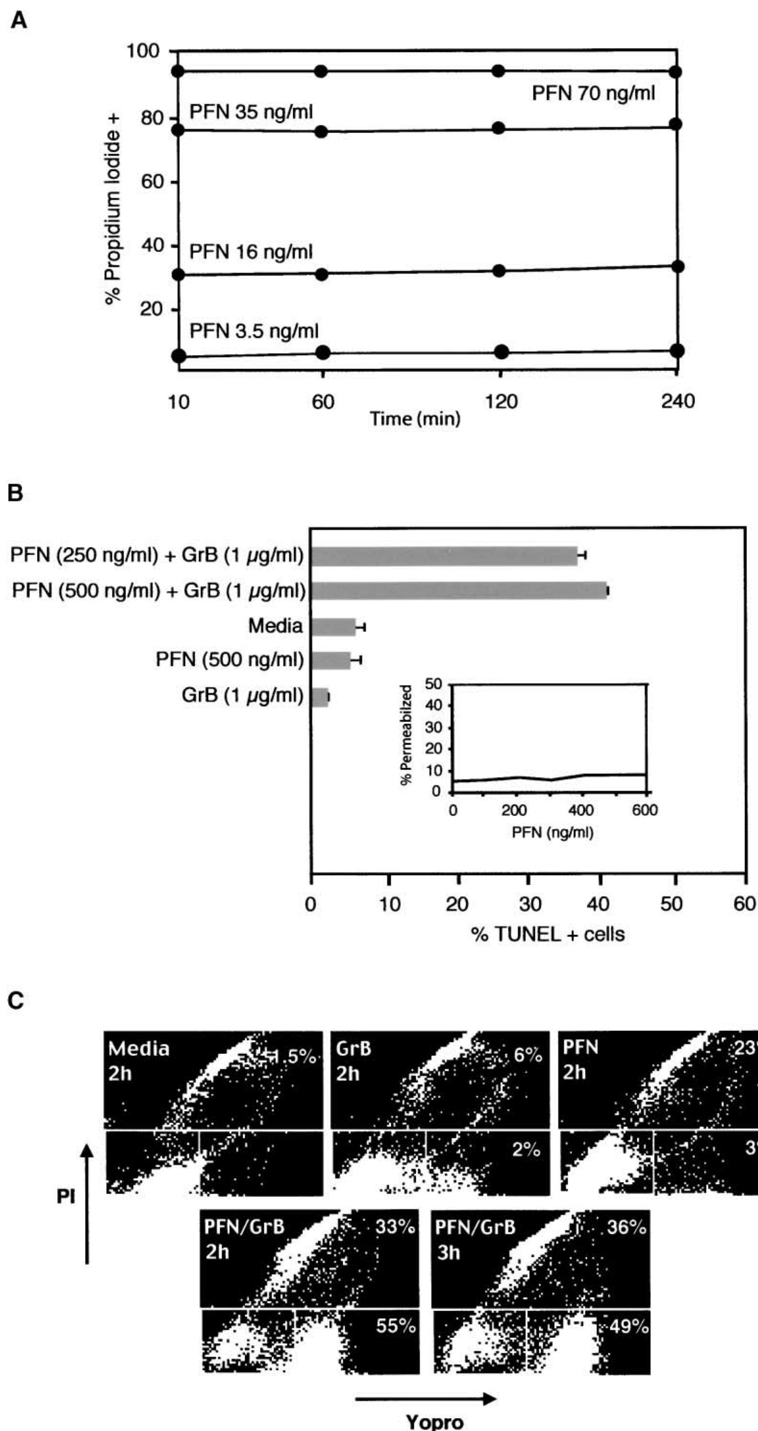

Figure 1. Inability to Detect PFN-Mediated Plasma Membrane Perforations in Cells Undergoing Apoptosis

(A) Isolated PFN leads to rapid appearance of a stable subset of permeabilized cells. Jurkat cells were preincubated with PI for 10 min (37°C), followed by treatment with PFN at indicated concentrations. Aliquots were analyzed at indicated times with a FACScalibur instrument, and the number of PI positive cells (permeabilized) was enumerated. Results are representative of three separate experiments.

(B) PFN delivers GrB in 0.2 mM Ca buffer without plasma membrane permeabilization. Jurkat cells were treated with PFN at indicated concentrations for 30 min (37°C), and the percent of permeabilized cells was determined by trypan blue stain (inset). For the apoptosis assay, cells were treated with a combination of indicated PFN and GrB (1ug/ml) for 4 hr under identical conditions, fixed, and evaluated microscopically (Hoescht) and flow cytometrically (TUNEL). Data for TUNEL is shown (mean $\pm$ SD, n $=$ 2) where Hoescht stain produced similar results (data not shown).

(C) Absence of membrane permeabilization in cells undergoing apoptosis. Dot-plots of Yopro-1 (FL-1, *x* axis) versus PI (FL-3, *y* axis) fluorescence are shown. Cells were preincubated with PI and Yopro-1 for 10 min (37°C), followed by PFN (30 ng/ml) and/or GrB (2 μg/ml) in high Ca buffer, and analyzed by flow cytometry. Note: apoptotic cells that are Yopro-1^{+} exclude PI (lower right quadrant). Results are representative of three experiments.

by sublytic PFN, the generation and potential clearance of membrane pores were evaluated in Jurkat cells treated sequentially with propidium iodide (PI) and PFN in the presence of extracellular calcium (1.5 mM). Isolated PFN produced the rapid appearance of a stable subset of PI^{+} cells (Figure 1A). Measurement of light scatter characteristics showed that PI^{+} cells possessed reduced cell volume (data not shown). The shrunken cells were viewed as dying, being unable to repair membrane damage (i.e., eliminate pores) and sustain normal volume. Viable cells that transiently expressed pores should, on the other hand, be PI^{+} but maintain normal volume. A population of cells with these characteristics could not be identified. Furthermore, if a subset of targets transiently expressed pores, then the number of PI positive cells should decrease as the time between the addition of PFN and PI was lengthened. We found that the percentage of permeabilized cells was comparable whether the PI was added prior to PFN or 5–10 min afterward (data not shown). Together the data indicate

that a sublytic dose of PFN rapidly permeabilizes a limited number of targets, leaving the majority unaffected. GrB would, therefore, interact with two subsets: a permeabilized population plus another which might undergo apoptosis despite the absence of membrane alterations. This distinction is crucial because both subsets could show signs of nuclear apoptosis due to PFN-dependent delivery of granzyme B regardless of the response to PFN exposure.

To investigate whether GrB induced apoptosis in both permeabilized and intact targets, two approaches were taken. First, cell death was evaluated under conditions that would favor delivery of GrB by PFN yet absolutely minimize plasma membrane permeabilization. Second, membrane and nuclear alterations were evaluated simultaneously in targets undergoing PFN/GrB-induced apoptosis. After identifying the minimum Ca^{2+} concentration that supported delivery of GrB by PFN (0.2 mM), we generated a broad concentration curve to determine whether PFN permeabilized the target cells. For concentrations of PFN ranging from 25 to 500 ng/ml, the number of permeabilized targets was comparable to media (Figure 1B, inset). PFN, nonetheless, produced a robust apoptotic response in the presence of GrB, providing indirect evidence that PFN has the capacity to deliver the granzyme through a process that does not involve plasma membrane pore formation (Figure 1B). Although this correlative study appeared to exclude a role for plasma membrane pore formation in the delivery of the granzyme, definitive proof required the simultaneous evaluation of membrane and nuclear alterations in the same target cell. This was achieved by adding the membrane impermeant nuclear dye, Yopro-1, and the plasma membrane impermeant probe, PI, to the targets prior to incubation with PFN and GrB. The outcome was then monitored by flow cytometry. Besides the predicted permeabilized subset, the appearance of Yopro-1^+, PI^- cells (Figure 1C) indicated that targets succumbing to GrB-induced apoptosis fail to display early evidence of membrane damage. As the period of incubation lengthens, the number of Yopro-1^+, PI^- cells declined and were replaced by apoptotic cells that developed secondary membrane damage (Yopro-1^+, PI^+). PFN appears, therefore, to act through two distinct processes after addition to suspended target cells: (1) very rapid plasma membrane poration and (2) a process which involves intracellular delivery of granule proteins without overt membrane perforation. It is noteworthy that the concentration of PFN necessary for pore formation markedly exceeds values required for granzyme delivery.

Identification of PFN-GrB-SG Complexes in Cytotoxic Granule Extracts

To minimize the disruption of a cytotoxic granule, PFN as well as the granzymes are postulated to be sequestered by the granule-associated proteoglycan, serglycin (MacDermott et al., 1985). To document the association of these constituents, the interaction of PFN and GrB with SG was evaluated by coimmunoprecipitation (IP). Granules from ^{35}S-labeled YT cells were treated with Zwittergent 3–12 (0.5%), the detergent providing optimal solubilization (data not shown), and IP was performed. The anti-GrB mAb precipitated a diffuse band consistent with SG as determined by sensitivity to chondroitinase ABC and resistance to heparinase/heparitinase digestion (Figure 2A) plus comparison to the mobility of isolated SG by SDS-PAGE and silver stain (Figure 2A). Anti-δG9 PFN IP yielded a diffuse band consistent with SG but with lesser intensity, reflecting perhaps the lower endogenous levels of the PFN in granules (Figure 2A). In companion experiments designed to evaluate a trimolecular interaction, IP with anti-GrB and anti-δG9 PFN yielded PFN and GrB, respectively (Figures 2B and 2C). The association of GrB, PFN, and SG was confirmed by subjecting the anti-δG9 PFN immunoprecipitate to GrB ELISA (Figure 2D), and the presence of the highly glycosylated SG in the immunoprecipitate was validated by the Blyscan assay, which quantitatively detects proteoglycans (Figure 2D). Together the results are the first to show a direct multimeric interaction between PFN, GrB, and SG in granules of cytotoxic cells.

GrB Is Exocytosed from Cytotoxic Cells in a Macromolecular Form

Since GrB appears to interact with SG in cytotoxic granules (Figure 2A), the hypothesis was evaluated that the granzyme would be released as a macromolecular complex during stimulated secretion. Degranulate from ^{35}S biosynthetically labeled LAK cells was obtained by stimulation with anti-CD2 mAb as described (Galvin et al., 1999). Complexed GrB was distinguished from the monomeric form by size exclusion membrane filtration, followed by measurement of granzyme content in the two compartments (Figure 3A). SG was subsequently identified indirectly by IP of the retentate fraction with anti-GrB mAb and digestion with chondroitinase ABC (Figure 3B). These results establish that GrB is secreted from cytotoxic cells only as a macromolecular complex with SG and that PFN would then have to deliver not merely the monomeric granzyme but a much larger moiety (>300 kDa) to effect cell death.

GrB-SG Complexes—Stability, Proteolytic Properties, and Capacity to Induce Apoptosis after Intracellular Delivery

To learn whether we could reconstitute proapoptotic granzyme complexes in vitro, the stability of a mixture of GrB and SG was evaluated under extracellular conditions (Figure 4). Complexes were assembled by mixing GrB with SG isolated from the monocytic cell line, THP-1, followed by size exclusion membrane filtration to remove monomeric granzyme. Using agarose gel electrophoresis and combined toluidine blue and Coomassie blue staining to detect the proteoglycan and protease, respectively, we directly evaluated the molecular interaction of the cationic granzyme and anionic SG. Compared to a set of proteoglycan standards (aggrecan, $\sim 2.5 \times 10^6$ kDa; biglycan, ~350 kDa; decorin, ~100 kDa; CS, ~30 kDa), SG, on the basis of mass and charge, migrated with the predicted M_r approximating 250–300 kDa, whereas the free granzyme shifted upward in the gel due to its cationic state. When the mobility of the preformed complexes was examined, we failed to detect free GrB, and the SG band appeared to redistribute into two components where the first was situated slightly

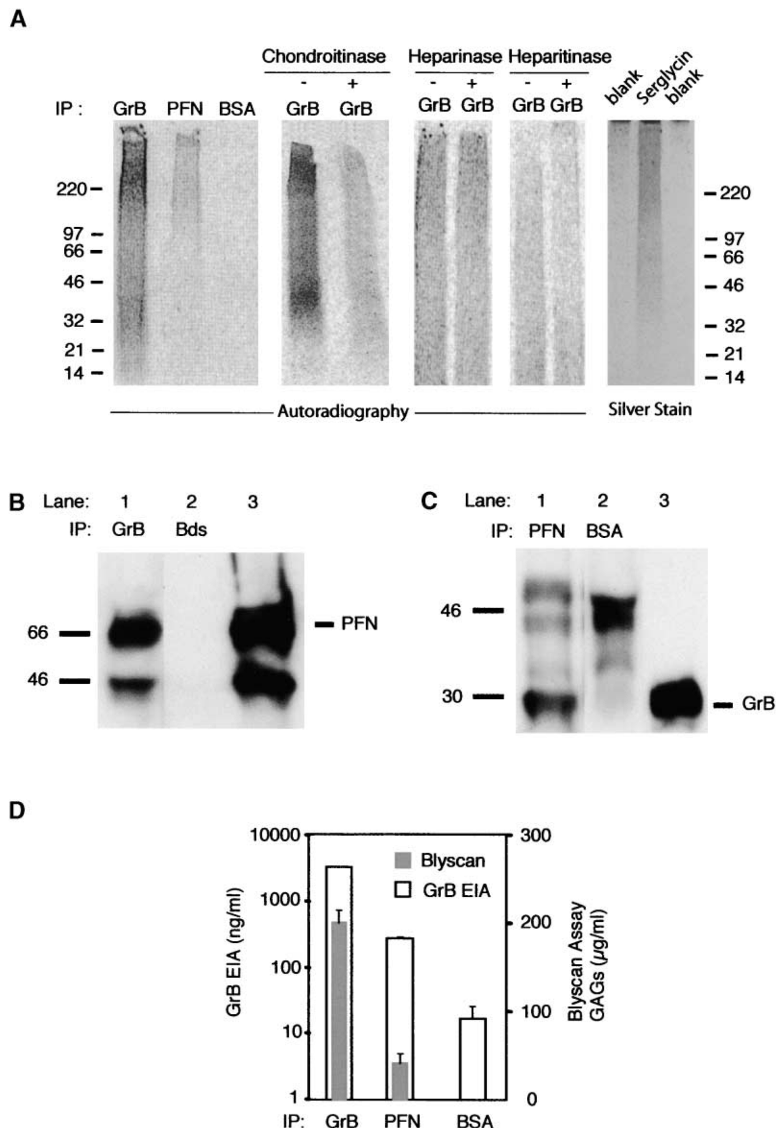

Figure 2. GrB and PFN Are Associated with SG in YT Cytotoxic Granules

(A) GrB and PFN associate with a highly sulfated macromolecule in cytotoxic granules. Zwittergent solubilized, ^{35}S-labeled YT granule extract was subjected to IP with antibodies to GrB, PFN, or BSA as indicated, and the material was run on 4%–15% gradient gels followed by fluorography. Anti-GB10 immunoprecipitated material was subjected to chondroitinase ABC, heparinase, or heparitinase digestion as described. Isolated SG (6.25 μg) was electrophoresed and silver stained for comparison.

(B and C) GrB and PFN form multimeric complexes in cytotoxic granules. Solubilized cytotoxic granules were subjected to IP as described followed by immunoblotting for PFN (B) or GrB (C). Isolated PFN or GrB served as controls in (B) and (C), respectively (lane 3). In (B), Sepharose beads alone incubated with solubilized granules served as control, and an immunoprecipitating antibody for BSA functioned similarly in (C). For (B) (lane 1), bands immediately beneath nominal PFN reactive band represent heavy chain for immunoprecipitating anti-GrB antibody. For (C), doublet near 46 kDa marker is from nonspecific staining of anti-GrB.

(D) Anti-GrB and anti-δG9 PFN immunoprecipitates GAGs. Solubilized cytotoxic granules were subjected to IP with indicated antibodies, and associated GrB and GAG content was quantitated by ELISA and Blyscan assay, respectively. Open bars represent GrB values on a log scale, while gray bars represent total GAG on linear scale (mean + SD, n = 2).

above the band that migrated in a position similar to SG alone (Figure 4A). The presence of the granzyme in the redistributed SG bands was then verified by extracting the protein from the agarose and performing an anti-GrB immunoblot (Figure 4B). In addition to confirming that GrB forms a stable interaction with SG, these results suggest that the granzyme forms multimeric complexes where the stoichiometric ratio of GrB:SG exceeds 1:1. Indeed, titration experiments indicate that as many as 20 GrB molecules may interact with a single SG (data not shown). Finally, complementary experiments were performed to ensure stable complexes after dilution with the targets. Complexes were serially diluted 2-fold and membrane filtered. Using IETD-pNA, GrB activity remained entirely in the retentate (data not shown).

The proteolytic activities of GrB-SG and free GrB were then compared against two physiologically relevant substrates, caspase-3 and -7 (Yang et al., 1998). Although comparable molar quantities of GrB-SG and free GrB (determined by EIA) produced equivalent esterolytic activities (BAADT assay, Figure 4C), GrB-SG complexes processed both caspases with less efficiency than the free granzyme (Figure 4D). The apoptotic activity of GrB-SG was then evaluated using AD, the PFN substitute we have previously described to deliver granzymes intracellularly (Froelich et al., 1996a). Although GrB-SG displayed a reduced capacity to process caspases-3 and -7 in vitro, GrB-SG delivered by AD produced a greater concentration-dependent level of apoptosis (Figure 4E). Finally and most notably, PFN was as effective as AD in delivering the macromolecular GrB-SG complexes into target cells through a process that did not involve plasma membrane permeabilization of the cells undergoing apoptosis (Figure 4F).

PFN-SG Complexes Deliver Macromolecular GrB in the Absence of Demonstrable Plasma Membrane Pores

Since PFN also appeared to interact with SG in secretory granules, we asked whether the pore-forming protein might also remain stably complexed to SG in vitro and deliver the macromolecular granzyme complex. Conse-

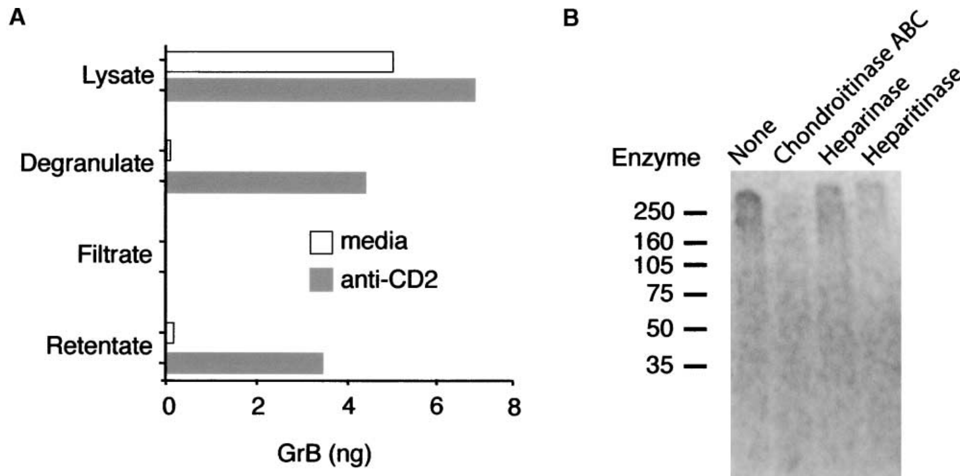

Figure 3. Granzyme B Is Secreted Exclusively in a High M_r Form from LAK Cells Coupled to SG

(A) GrB is exocytosed exclusively in a high M_r form. LAK cells were either untreated or induced to degranulate for 5 hr. Supernatants were collected and subjected to 100 kDa filtration, and the amount of GrB in the various fractions was quantitated by ELISA. GrB levels were normalized for volume and are representative from one of three normal donors. The filtrate fractions lacked detectable GrB.

(B) The high M_r form of secreted GrB is sensitive to chondroitinase ABC digestion. Degranulates from ^{35}S-labeled LAK cells were filtered and immunoprecipitated with anti-GrB. Samples were subjected to overnight endoglycosidase digestions (chondroitinase ABC, lane 2; heparinase, lane 3; heparitinase, lane 4; left untreated, lane 1), followed by electrophoresis and phosphoimaging.

quently, it was crucial to ensure that the PFN-SG interaction was stable during contact with the target cell. PFN-SG was formed by mixing SG and PFN (150 mM NaCl [pH 7.4]) and subjecting the mixture to size exclusion membrane filtration (100 kDa). By semiquantitative silver stain, approximately 10%–25% of the PFN remained in the retentate, suggesting a stable interaction (Figure 5A). Complex formation, however, was then directly verified by agarose gel electrophoresis of putative PFN-SG complexes retrieved after membrane size exclusion, followed by immunoblotting for PFN extracted from bands containing SG (Figures 5B and 5C). When the mobility of the preformed complexes was examined, we failed to detect free PFN by protein stain, and the SG band detected by toluidine blue stain appeared to redistribute more broadly than SG alone (Figure 5B), suggesting that an interaction with PFN reduced the mobility of SG. The presence of PFN in the redistributed SG band was then confirmed by agarose extraction and anti-PFN immunoblotting (Figure 5C). Thereafter, the influence of increasing pH and Ca^{2+} concentration on the interaction of PFN and SG was evaluated using chondroitin sulfate A (CS) coupled to Sepharose as surrogate. These results indicate (Figures 5D and 5E) that substantial amounts of PFN remain complexed to CS-Sepharose as pH and Ca^{2+} are elevated to extracellular values. The combined results indicate that both free and complexed PFN are likely present extracellularly after granule exocytosis and that both forms of PFN could contribute to the delivery of the granzyme.

Since the interaction of PFN with glycosaminoglycans has been reported to inhibit pore formation (Tschopp and Masson, 1987), SG was also predicted to reduce this biologic activity, and the decreased membrane permeabilization mediated by PFN-SG would be associated with a reduction in the ability to deliver the granzyme. We have observed that free PFN, averaging 5–20 ng/ml, rapidly damages $>$50% of Jurkat (1×10^7/ml). PFN-SG, on the other hand, is more than 5- to 10-fold less potent (Figure 6A), indicating that the proteoglycan does indeed suppress membranolytic activity. If plasma membrane pore formation is a prerequisite for delivery of the granzymes, then free PFN should be more efficient than PFN bound to SG. PFN-SG, on a molar basis, was found to more effectively deliver GrB-SG as well as the free granzyme (Figure 6B). Therefore, although PFN-SG was significantly less membranolytic than free PFN, the complexed form displayed a similar or greater ability to deliver the granzyme. Together the results indicate that PFN-SG appears to interact with target cells delivering macromolecular granzyme through a process whereby membrane pore formation is not an obligatory step.

Discussion

Evidence is presented that PFN functions in an unforeseen manner to deliver the physiologically relevant form of secreted GrB, a macromolecular complex of granzyme and SG. We have proposed that granule-mediated apoptosis is fundamentally a variant of virus-dependent endocytosis where PFN facilitates delivery of internalized granzyme (Froelich et al., 1998). We had shown that target cells internalize membrane-bound granzyme and speculated that delivery of the granzyme to the cytosol was dependent on uptake of membrane remnants carrying PFN pores (Froelich et al., 1996a). The granule-mediated cell death pathway appears, however, to share a greater similarity to viral-mediated cellular penetration than initially anticipated. A macromolecular granule complex consisting of granzyme, SG, and PFN analogous to a viral particle may be the central effector. The granule-associated proteoglycan, SG, contributes to granule-mediated apoptosis by acting as a carrier, which facilitates internalization of GrB and/or PFN. Ac-

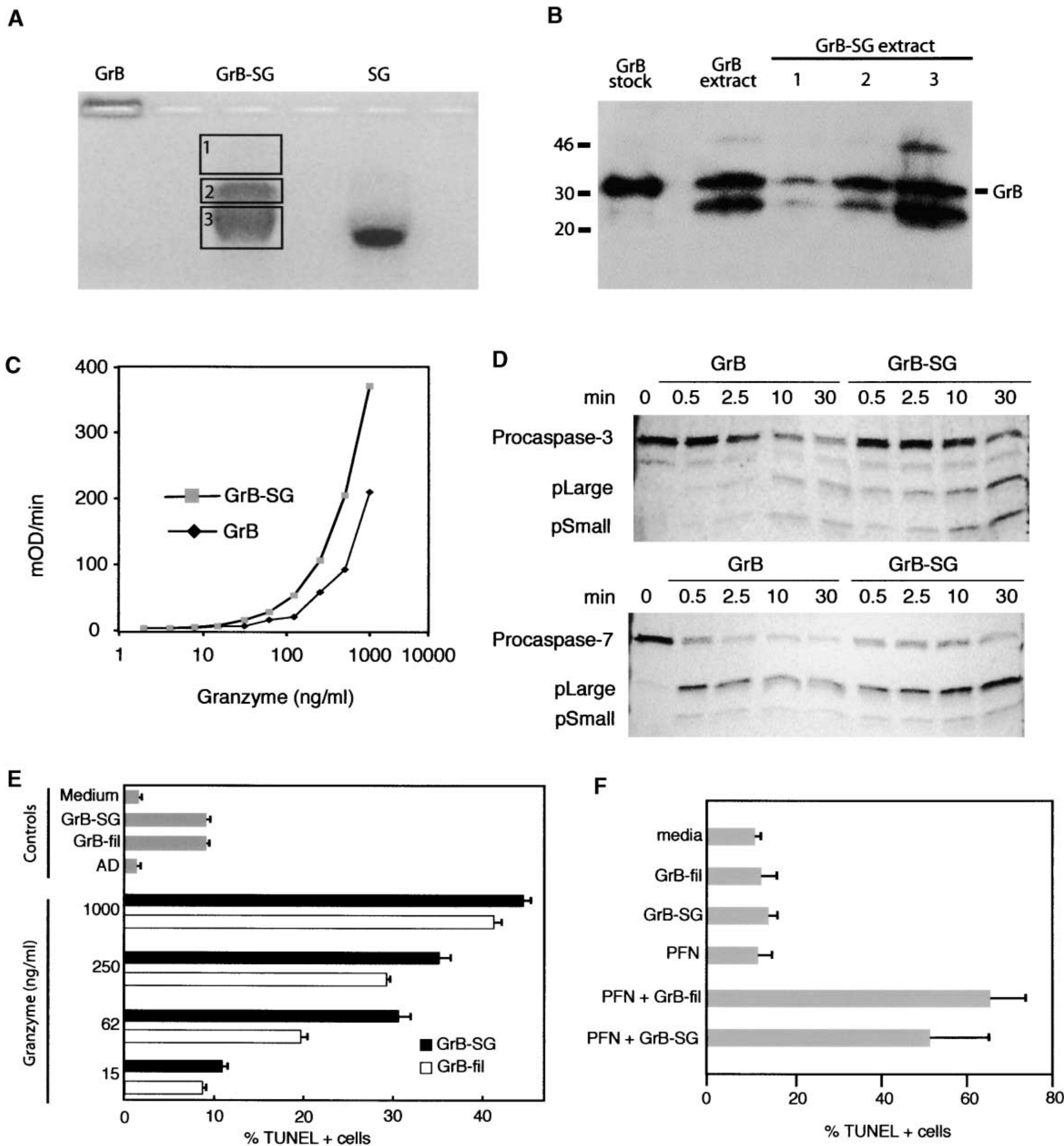

Figure 4. GrB-SG Complexes—Stability, Proteolytic Properties, and Capacity to Induce Apoptosis after Intracellular Delivery

(A) Agarose gel electrophoresis of GrB-SG complexes. GrB (2.4 μg), SG (8.9 μg), and GrB-SG mixture underwent 1% agarose gel electrophoresis followed by sequential toluidine blue and gel code blue staining. GrB alone migrates upward (negative electrode), while GrB in the GrB-SG complex lane migrates along with SG.

(B) Anti-GrB immunoblotting of GrB-SG complexes extracted from agarose gel. Slabs were obtained from GrB and GrB-SG lanes as diagrammed ([A], boxes 1–3), extracted, and immunoblotted for anti-GrB. Stock GrB (125 ng) was positive control for blotting. GrB extracted from agarose (GrB extract) was control for extraction efficiency. Slices corresponding to boxes 1, 2, and 3 from agarose gel contained putative GrB-SG complexes (GrB-SG extract). Maximum signal for GrB colocalized to box 3, which migrated similarly to SG (see [A]). A substantial signal was also associated with box 2. The signal below 32 kDa GrB band represents deglycosylated granzyme generated by alkaline hydrolysis during extraction.

(C) Measurement of esterolytic activity of GrB/GrB-SG complex by BAADT assay. Free GrB and GrB-SG complex were incubated with substrate and hydrolysis reported as mOD/min.

(D) GrB-SG is less efficient than free GrB in processing recombinant caspases-3 and -7 in vitro. Kinetics of proteolysis of caspase-3 (top, 500 nM) and -7 (bottom, 500 nM) were determined with either free GrB or GrB-SG (5 nM) for times indicated (min). At each time point, the reaction was stopped by adding GrB inhibitor IETD-CHO (500 μM), and the sample was electrophoresed and silver stained.

(E) GrB-SG delivered by AD efficiently induces apoptosis. Jurkat cells were treated with increasing concentrations of free or GrB-SG plus AD for 4 hr. The percentage of apoptotic cells determined by TUNEL (mean ± SD, n = 3) was comparable to values observed by Hoescht (data not shown).

(F) PFN-mediated delivery of free and GrB-SG. Jurkat cells were treated with GrB filtrate or GrB-SG (2 μg/ml) and PFN stock (14 ng/ml) for 4 hr at 37°C. The percentage of apoptotic cells by TUNEL (mean ± SD, n = 2) was comparable to values by Hoescht (data not shown).

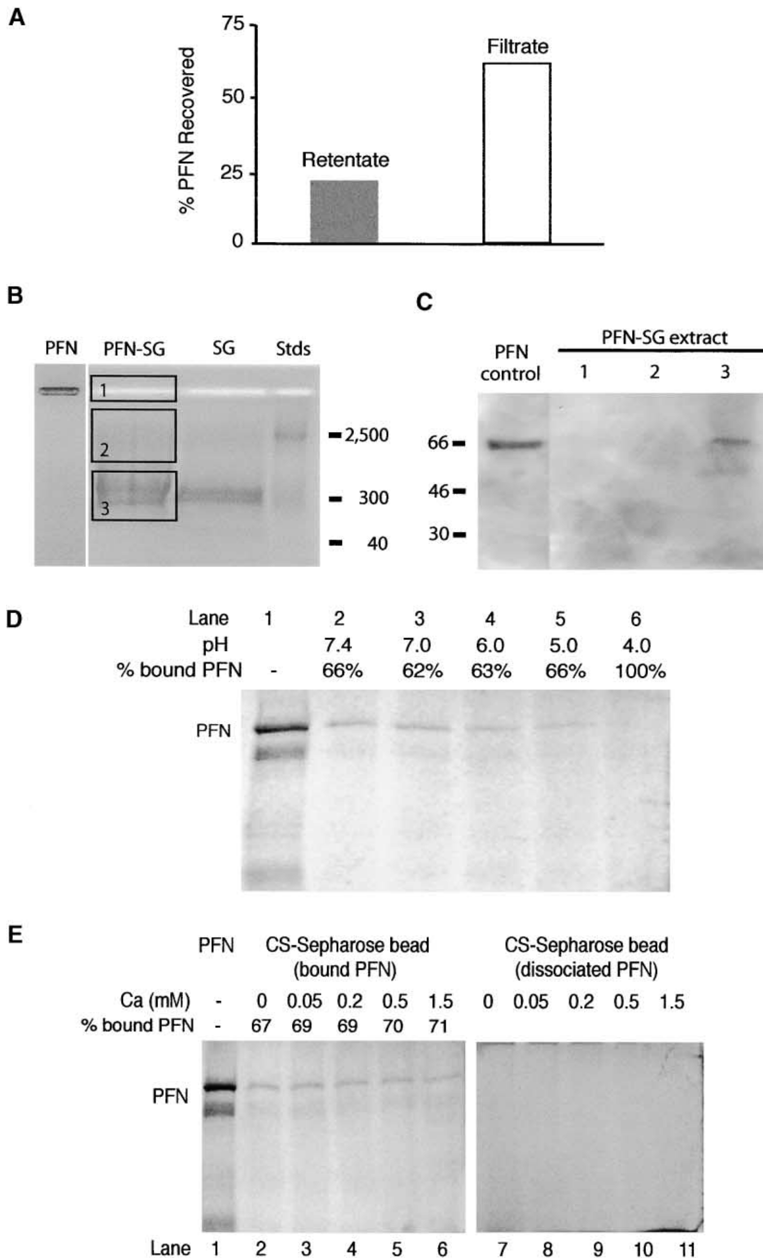

Figure 5. PFN Forms Stable Complexes with SG In Vitro

(A) PFN-SG complexes exist at neutral pH and isotonicity. PFN-SG complexes were generated as described, and PFN complexed to SG determined by subjecting 100 kDa retentate and filtrate fractions to 10% SDS-PAGE, silver stain, and densitometry. Approximately 10%–20% of PFN was retained by 100 kDa membrane.

(B) Agarose gel electrophoresis of PFN-SG complexes. PFN (2 μg), SG (46.1 μg), and PFN-SG mixture underwent 1% agarose gel electrophoresis and was stained to identify PFN and SG. PFN alone migrates slightly upward (negative electrode), while PFN in the PFN-SG complex lane migrates with SG.

(C) Anti-PFN immunoblotting of PFN-SG extracted from agarose gel. Slabs were obtained as diagrammed ([B], boxes 1–3), and protein was extracted and immunoblotted with anti-PFN. PFN (5 ng) served as immunoblot control, and extracts from slabs corresponding to 1, 2, and 3 (PFN-SG extract) were analyzed. Signal for PFN only colocalized to box 3, which migrated similarly to SG (B).

(D) PFN remains associated with chondroitin sulfate (CS) at neutral pH. PFN was mixed with CS-Sepharose beads in buffers with decreasing pH as described ([pH 7.4, 7, 6, 5, and 4], lanes 2–6). Unbound PFN was removed by centrifugation, and beads were washed at room temperature with appropriate buffer. The supernatant was subjected to 10% SDS-PAGE, followed by silver stain and densitometry. PFN alone (lane 1, 500 ng) served as control. Approximately 65% of PFN remained bound to CS-Sepharose at neutral pH, while none was detectable in supernatant at pH 4.0.

(E) Calcium does not dissociate PFN from CS. PFN was mixed with CS-Sepharose, and the beads were washed and resuspended in the same buffer containing increasing concentrations of $CaCl_2$ (0, 0.05, 0.2, 0.5, and 1.5 mM; lanes 2–6, respectively). The beads were incubated at room temperature for 10 min and centrifuged. Both supernatant (free PFN) and bead fractions (bound PFN) were subjected to SDS-PAGE, silver stain, and densitometry. PFN was not detectable in any of the supernatants.

cordingly, the killer cell is predicted to secrete the macromolecular complex into the synapse-like space between effector and target cell. The complex then binds to the target cell and undergoes endocytosis. Within the acidic vesicle, PFN alters the endocytic membrane, facilitating release of the granzyme complex to the cytosol. In support of this unexpected model, our primary observations include: (1) isolated PFN delivers GrB through a process that does not require generation of plasma membrane pores; (2) the macromolecular form of GrB (i.e., GrB-SG) is secreted by cytotoxic cells, is delivered by PFN, and is potently apoptotic; and (3) stable PFN-SG complexes also deliver this physiologic form of the granzyme.

Having predicted that plasma membrane pore formation is a crucial prerequisite for delivery of internalized granzyme, our inability to identify transiently expressed pores on apoptotic target cells was unexpected. The data, nevertheless, provide evidence that monomeric PFN is internalized in a nonpore-forming state to facilitate delivery of GrB. The results in Figure 1C are the first to show that a target cell will ultimately die by apoptosis without demonstrating signs of PFN-mediated membrane damage. Second, using FM1-43 to monitor endocytic activity, the addition of PFN to targets induces uptake of the dye without concomitant pore formation (Kawasaki et al., 2000). Third, targets treated with a combination of PFN and granzyme become apoptotic but continue to exclude low M_r dextran and protein fluoroprobes (Browne et al., 1999). Finally, target cells containing vesicle-associated granzyme (Pinkoski et al., 1998) will undergo apoptosis when subsequently chal-

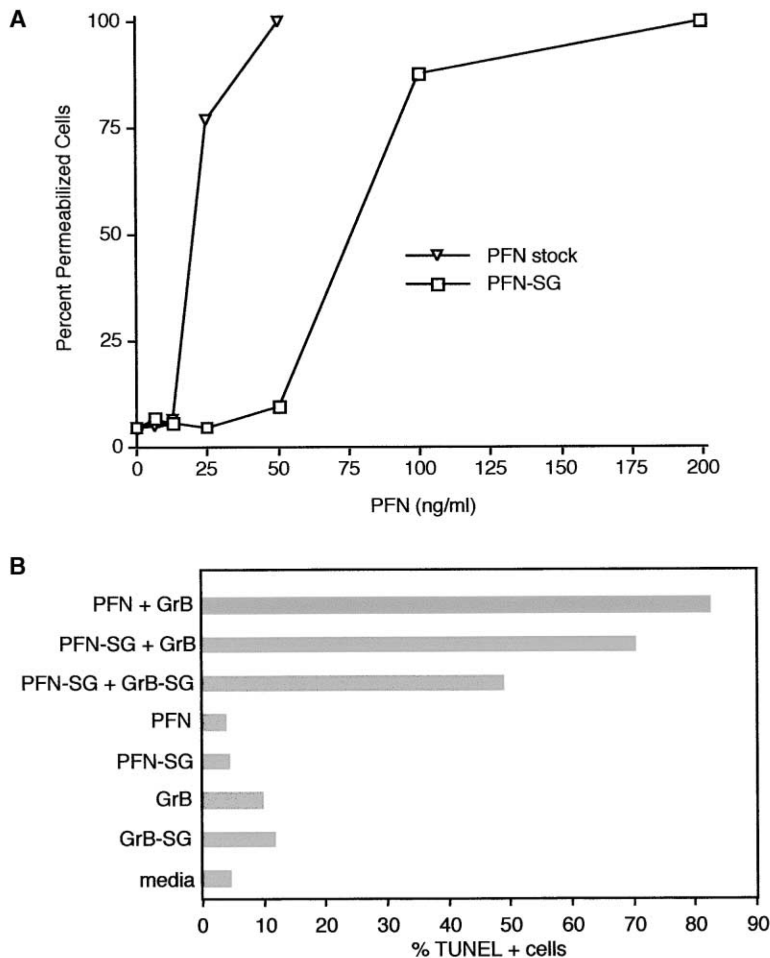

Figure 6. PFN-SG Complexes Deliver GrB-SG without Plasma Membrane Permeabilization

(A) Comparison of permeabilizing activity of PFN and PFN-SG complexes. Jurkat cells were treated with increasing concentrations of stock PFN or PFN-SG for 30 min (37°C), and permeabilized cells were enumerated by trypan blue stain. Results represent one of four experiments.

(B) PFN-SG complexes can deliver GrB-SG to induce apoptosis. Minimally permeabilizing concentrations ($<$20%) of PFN filtrate (66 ng/ml) or PFN-SG (30 ng/ml) were used in combination with GrB-SG or free GrB (2 ug/ml). The number of apoptotic events observed by TUNEL (mean $\pm$ SD, n = 2) were similar to results by Hoechst.

lenged with sublytic amounts of PFN (Froelich et al., 1996a).

PFN has been defined biologically by its ability to induce membrane permeabilization. However, at concentrations whereby permeabilization is virtually nonexistent, targets are rendered susceptible to an apoptotic death if the granzyme is present. The 2-fold effect of PFN reveals an alternate mechanism to explain the action of the porin during granule-mediated apoptosis: PFN monomers (or oligomers) undergo internalization if the target is exposed to a limited amount of the pore-forming protein (Figure 1A). The results are consistent with the collisional model of PFN pore formation (Peters et al., 1990) whereby PFN monomers bind to the plasma membrane with insufficient proximity to form pores and instead undergo clearance from the plasma membrane. Since the interaction of PFN through its C2 domain with membrane-associated phosphorylcholine is considered essential for binding (Uellner et al., 1997), the "phosphorylcholine receptor" remains a plausible candidate to initiate either pore formation or internalization. Similar to certain viruses, PFN could generate channels in vesicular membranes, but the observation that plasma membrane pores do not allow entry of GrB suggests that this mechanism is unlikely. The pore-forming activity of PFN is exquisitely pH dependent with an optimum near pH 7.0. Agents that alkalinize endosomes are reported to reduce PFN-dependent GrB-mediated cell death (Browne et al., 1999), suggesting that PFN may act through a mechanism distinct from membrane pore formation to deliver granzymes from intracellular vesicles. In this regard, the fusogenic properties of PFN could disrupt the endosome in a manner analogous to such pore-forming proteins as listeriolysin. Studies are underway to learn whether the hydrophobic domain of PFN is sufficient to disrupt endosomal membrane and whether PFN acts alone or requires the presence of cofactors such as SG for optimal membranolysis.

PFN was found to interact with SG in granules, suggesting that the pore-forming protein is secreted as a complex and, after granule exocytosis, remains bound and/or separates from the proteoglycan extracellularly. We were unable to detect free or putative PFN-SG complexes after degranulation, in part because the Ca^{2+} required for exocytosis inactivates PFN, making the protein inaccessible to our immunoprecipitating antibody (data not shown), and more likely due to the minuscule amounts of PFN secreted by the effector cell, which are below the detection limits of the antibodies used. Nevertheless, we establish here that isolated PFN does form a stable complex with SG under extracellular conditions, supporting the concept that exocytosed PFN has two molecular forms. Additional studies will be necessary to determine the proportion of free and bound PFN

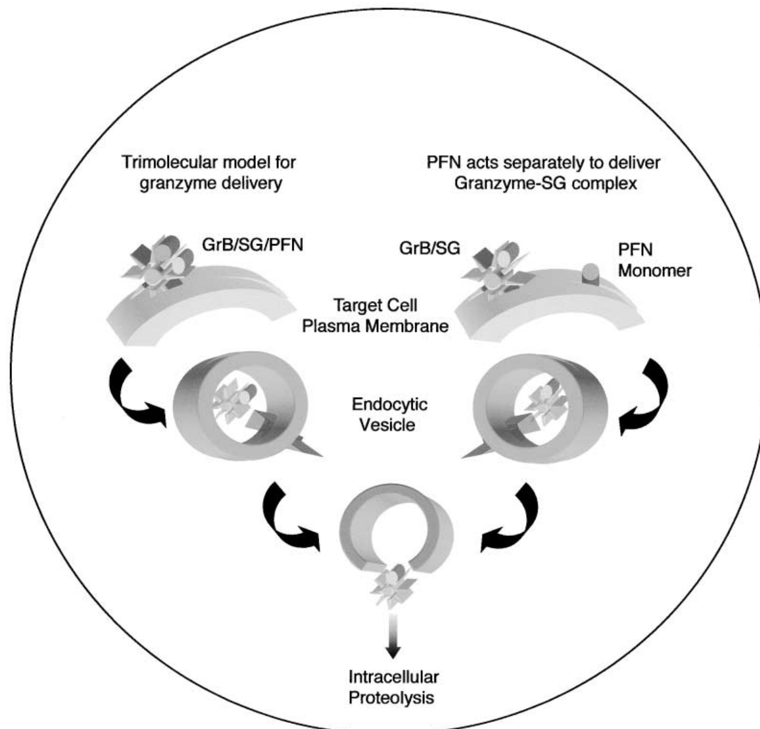

Figure 7. A Macromolecular Proapoptotic Array Secreted by Cytotoxic Cells Induces Death

Cytotoxic cells appear to secrete GrB on a scaffold of SG. This macromolecular complex will also contain PFN monomers and other granzymes. Either GrB-SG-PFN or GrB-SG and dissociated PFN monomer are then internalized. Regardless of the mechanism of PFN uptake, the outcome is endosomolytic delivery of granule proteins.

secreted by cytotoxic cells and their relative contribution to granzyme delivery. Importantly, SG used here was monocytic in origin. We have recently isolated SG from NK cells, and preliminary studies indicate that YT-SG is similar in nature to the THP-1 counterpart, forming stable complexes with GrB/PFN.

Although a caveat for comparing monomeric and complexed PFN is the inability to verify by an independent technique that the two forms contain equivalent active protein, our data suggest that PFN-SG more efficiently delivers the granzyme. Paradoxically, monomeric PFN was substantially more effective than PFN-SG in permeabilizing targets. This dichotomy offers further support that the pore-forming and translocating activities may indeed be dissociated into separate mechanisms. How SG modulates the action of PFN in this delivery process is unclear. Since glycosaminoglycans are reported to block the hemolytic activity of PFN, the prevalent view is that proteoglycans are designed to inhibit PFN extracellularly. In light of the data here, the interaction may serve to protect PFN from Ca-mediated inactivation, allowing either internalization or focusing of PFN monomers to exchange sites of the cell surface. The complexed form of PFN might therefore favor the outcome of endosomolysis by encouraging uptake of the pore-forming protein and reducing membrane permeabilization.

The finding that GrB is secreted by cytotoxic cells and delivered by PFN as a macromolecular complex has enormous implications for the understanding of granule-mediated apoptosis and forces reinterpretation of data from many laboratories. In comparison to the free granzyme, the interaction of GrB with SG will alter cell surface binding characteristics, vesicular trafficking, and cytosolic movement, as well as the efficiency of targeting and processing of putative substrates. To date, studies utilizing the granzyme have employed the free cationic protease, which encourages nonspecific electrostatic interactions with anionic membranes. This was a major concern when we reported that GrB was able to bind to target cell in a receptor-dependent manner. The discovery that GrB uses the mannose-6-phosphate receptor (MPR) for internalization suggested that the elusive "GrB receptor" had been identified (Motyka et al., 2000). However, on the basis of our observation that GrB is secreted wrapped in anionic SG, other proteins are predicted to participate in the internalization of the granzyme complex, particularly the CD44 family of isoforms, which could focus or facilitate internalization of GrB through SG (Toyama-Sorimachi et al., 1995). The likelihood that GrB-SG uses solely the MPR is also diminished by the observation that this trafficking system targets the free enzyme. Detailed studies, therefore, are necessary to examine the contribution of the MPR and CD44 systems for endocytic uptake and trafficking of granzymes. Although a single specific receptor for GrB is attractive, to ensure elimination of target cells, GrB should have the capacity to bind to multiple cell surface structures. Our results are significant because the data provide a level of nonselectivity (i.e., degeneracy) for GrB binding and internalization—a necessary prerequisite for killing. Specificity is ultimately endowed by the effector cell recognition systems and complemented by secretion of PFN, which delivers the internalized granzyme.

Introducing monomeric GrB to the cytosol will result in anomalous targeting of the granzyme to irrelevant cytosolic proteins. Consistent with this possibility, GrB-SG was less efficient in processing caspase-3/-7 in vitro, but the complex more effectively induced cell death.

Although the outcome may be due to enhanced efficiency at multiple steps in the delivery process, we speculate that after release to the cytosol the complex is more appropriately targeted to the caspases, whereas free GrB would nonspecifically bind cytosolic proteins impairing this interaction. Postulated to represent alternate or amplifying apoptotic pathways, GrB has been reported to cleave cytosolic (BID, fibrillin) and, after translocation, nuclear substrates (Andrade et al., 1998; Browne et al., 2000; Froelich et al., 1996b; Sutton et al., 2000). Similarly, Bcl-2 overexpression partially inhibits GrB-mediated death, but cytotoxic cell-induced apoptosis is unaffected (Sutton et al., 1997). Studies will be necessary to delineate the trafficking of GrB-SG after intracellular delivery as well as the protein substrates cleaved by the granzyme complex. The tools are finally available to learn whether GrB induces cell death primarily, if not solely, by activation of the caspase cascade, whether the granzyme retains the capacity to kill cells independent of this death pathway, and how antiapoptotic proteins such as Bcl-2 influence the intracellular behavior of GrB-SG.

Overall, our results indicate that PFN acts through two mechanisms in vitro: the commonly perceived capacity to perforate plasma membrane and the incompletely understood process of endosomolysis. Although the major determinant that predicts the cellular outcome in vitro is the amount of PFN encountered by the target (Figure 1A), it remains to be determined whether PFN lyses cells in vivo. Numerous Cr release studies are interpreted to support the notion that PFN-mediated membrane permeabilization is a common outcome. However, these studies often do not distinguish Cr release due to PFN per se versus the response to the apoptotic process. Isolated reports that examine targets for signs of PFN-mediated membrane damage in effector:target conjugates by CLSM do show that cytotoxic cells have the capacity to secrete PFN in amounts sufficient to cause severe membrane damage, but these studies do not assess the relative frequency of the process (Oshimi et al., 1996). Recent single cell studies that examine the morphological effects of CTL on targets have shown that membrane permeabilization is not a customary early event but rather a late consequence of CTL-mediated apoptosis (Hu and Kipps, 1999; Goldberg et al., 1999). These observations indicate that a CTL normally may secrete sufficient PFN for endocytic uptake but not membrane rupture. We suggest that a target cell interacts with a trimolecular complex consisting of SG, PFN, and granzymes (likely both GrA and GrB). Following binding and internalization, granzyme-SG is released to the cytosol by the endosomolytic action of PFN. Equally plausible, after exocytosis into the cleft between target and effector, PFN dissociates from the complex and acts separately to deliver the GrB-SG complex in a process that does not appear to require pore formation (Figure 7). Regardless of the mechanism, PFN assuredly does not deliver the macromolecular granzyme through transmembrane pores but rather from an intracellular compartment. Granule-mediated apoptosis, therefore, should be viewed as a modular protein delivery system whereby SG acts as a scaffold module, PFN as the delivery module, and the granzymes as the targeting/informational module.

Experimental Procedures

Reagents

L-glutamine, PMSF, leupeptin, aprotinin, EDTA, glycerol, paraformaldehyde (PFA), 3-aminopropyltrietoxy-silane (APS), and propidium iodide were purchased from Sigma (St. Louis, MO), while RPMI 1640 was from GIBCO. GELCODE blue stain reagent and Hoechst 33342 were, respectively, from Pierce (Rockford, IL) and Molecular Probes (Eugene, OR). Dynabead Protein-A and Apo-Direct kit were, respectively, from Dynal (Lake Success, NY) and Pharmingen (San Diego, CA). The anti-δG9 PFN mAb against native PFN and anti-PB2 PFN mAb against central 199 AA span of PFN have been described (Hameed et al., 1992; Geisberg et al., 1990). Horseradish peroxidase (HRP)-rat anti-mouse κ mAb was from Accurate Chemical (Westbury, NY), and ready gels, silver stain kit, Immunblot, and Sequiblot-PVDF membranes were purchased from Bio-Rad (Hercules, CA), while ECL Western blot system and Hyperfilm ECL were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). $[^{35}S]Na_2SO_4$ (specific activity 531.71 mC/mm) was from NEN Life Sciences (Boston, MA).

Isolation of GrB, PFN, and SG

Human GrB and PFN were isolated as described (Hanna et al., 1993; Froelich et al., 1996c). Human serglycin was isolated from supernatants of THP-1 and quantitated by Safranin O staining (Oynebråten et al., 2000).

Solubilization of Enriched Cytotoxic Granules and IP

Cells (YT, 2×10^6/ml) were incubated with $[^{35}S]Na_2SO_4$ (25 uCi/ml) for 20–24 hr, and cytotoxic granules isolated as described (Hanna et al., 1993). The granules were solubilized with 0.5% Zwittergent 3–12 in PBS overnight followed by preclearance and IP using either anti-GrB antibody (GB10) coupled to Sepharose (GB10-Sepharose; Spaeny-Dekking et al., 1998), the anti-δG9 PFN antibody (IgG2b, 20 μg/ml), or, as a control, polyclonal anti-BSA antibody (30 μg/ml). The latter two were incubated with Protein A-Dynal beads (4°C overnight), and beads were washed and run on 4%–15% gradient gels under reducing conditions, followed by phosphoimaging.

IP and Western Blotting of Complexes from Cytotoxic Granules

Experiments that involved IP of GrB and PFN from in vivo complexes in solubilized granules were executed as described above. To demonstrate protein interactions, anti-GrB immunoprecipitated material was probed with anti-PB2 PFN (1:1000) and vice versa (clone 2C5, 1:10000) and developed with an anti-kappa HRP antibody (1:1000, Accurate Chemicals) and the ECL.

LAK Cell Generation and Granule Exocytosis

Lymphokine activated killer (LAK) cells were generated and induced to undergo exocytosis as described (Galvin et al., 1999). Supernatants were collected, and degranulate was subjected to 100 kDa filtration (1000 g, 10 min). GrB ELISA was performed on the total degranulate fraction, the 100 kDa retentate, and filtrate fractions.

Enzymatic Digestions of Proteoglycans

Cytotoxic granules from radiolabeled YT were solubilized with Zwittergent 3–12 in PBS and immunoprecipitated with GB10-Sepharose. Degranulates from radiolabeled LAK were fractionated on 100 kDa membrane, and retentate was subjected to GB10-Sepharose IP. The immunoprecipitated proteoglycans were then exposed to chondroitinase ABC (0.5 U/ml) (C ABC, Boehringer Mannheim, Indianapolis, IN) overnight (37°C) or with heparinase or heparitinase (0.02 U/ml) (Seikagaku Corp, Tokyo, Japan) overnight (43°C). The beads were then run on 4%–15% gradient gels (reduced) and phosphoimaged.

Generation and Analysis of GrB-SG and PFN-SG Complexes

GrB-SG complexes were prepared in GrB excess (3:1 w/w) while PFN-SG complexes were in SG excess (1:3 w/w) (150 mM NaCl and 20 mM HEPES [pH 7.4]). Mixtures were chilled (20 min) and filtered through Microcon YM-100 (100 kDa exclusion) (Millipore, Bedford, MA). GrB or PFN alone served as controls to assess duration of filtration (GrB filtrate, PFN filtrate) whereby mixtures were spun until controls lacked visible retentate. The concentrations of GrB and

PFN in retentates and filtrates were assessed by ELISA (GrB) and silver staining/densitometry (PFN), respectively.

To confirm the stable interaction of GrB or PFN with SG, we performed agarose gel electrophoresis and immunoblotting of select bands. GrB-SG or PFN-SG were run on 1% agarose gels with appropriate controls in Tris-Borate-EDTA (TBE) buffer. SG and proteins were visualized by serial toluidine blue and gel code blue staining. A set of proteoglycan standards (aggrecan, $>2.5 \times 10^3$ kDa; biglycan, 200–350 kDa; CS, 30 kDa) served as MW markers. To confirm the presence of complexes, slices were removed, and proteins were extracted (Ugozzoli and Chiu, 1992) and subjected to immunoblotting for designated protein.

Interaction of PFN with Chondroitin Sulfate-Sepharose Beads

Chondroitin sulfate A (Sigma, St. Louis, MO) was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) whereby efficiency (Blyscan assay) was 50%. PFN (500 ng) was mixed with 5000 ng CS-Sepharose (150 μl–150 mM NaCl and 20 mM HEPES) at pH 7.4 or 1.0 pH decrements (7.0–4.0) for 30 min. Beads were washed at room temperature with appropriate buffer, and supernatant (20%) was subjected to 10% SDS-PAGE, silver stain, and densitometry. To determine if PFN was dissociated by Ca, PFN was mixed with CS-Sepharose (isotonic buffer [pH-7.4]), washed, and resuspended in buffer containing increasing concentrations of $CaCl_2$. Beads were then incubated at room temperature (10 min) and centrifuged, and supernatant and bead fractions were submitted to SDS-PAGE, silver stain, and densitometry.

Processing of Caspases

Experiments were performed as described (Galvin et al., 1999)

BAADT and IETD Assay for GrB Esterolytic Activity

GrB enzymatic activity was measured using Boc-Ala-Asp-thiobenzyl ester (BAADT) (Hanna et al., 1993) or Ac-Ile-Glu-Thr-Asp-pNA (IETD-paranitroanilide, Calbiochem). Cleavage was monitored at appropriate nm.

Blyscan Assay

A quantitative dye binding assay (Accurate Chemicals and Scientific, Westbury, NY) was used that detects glycosaminoglycans and proteoglycans (Wagner et al., 1998).

GrB ELISA

GrB ELISA was performed as described (Spaeny-Dekking et al., 1998).

Delivery Proteins for Induction of Cell Death

PFN (ng/ml) and PFN-SG (ng/ml) were used at concentrations causing <20% permeabilization (150 mM NaCl, 20 mM HEPES [pH 7.4], 0.5% BSA, and 1.5 mM $CaCl_2$) and were pretested before each experiment by PI or trypan blue stain. AD was employed at 50 pfu/cell. GrB and GrB-SG were used at 1–2 μg/ml. Jurkat cells (1×10^6) were treated for 4 hr unless indicated and fixed, and apoptosis was determined by Hoechst and/or TUNEL.

Apoptosis Assays

Hoechst Assay

Following treatment, cells were fixed in 4% PFA and dried on microscope slides. For analysis, cells were hydrated, stained with Hoechst 33342 (10 μg/ml), and mounted with a drop of 50% glycerol in PBS. For each sample, 200 cells were counted to determine the percentage of cells with nuclear fragmentation.

FITC-TUNEL

Target cells (10^6/ml) were treated with the appropriate reagents in microfuge tubes for the required time, and death was measured by terminal deoxyribonucleotidyl transferase catalysed labeling of DNA strand breaks with FITC-dUTP, followed by flow cytometry (TUNEL).

Yopro-1/PI Reactivity

Assay was performed according to the manufacturer's instructions (Vybrant Apoptosis Assay kit IV, Molecular Probes, Eugene, OR).

Imaging, Computers, and Software

Images of gels and immunoblots were captured either with a Kodak digital camera or Saphir Ultra 2 flatbed scanner and exported to Adobe Photoshop 6.0, after which the EPS images were placed for final presentation in Adobe Illustrator 9.0 using a Macintosh Power G4 computer.

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