A Novel Mechanism for Protein Delivery

GRANZYME B UNDERGOES ELECTROSTATIC EXCHANGE FROM SERGLYCIN TO TARGET CELLS*

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The molecular interaction of secreted granzyme B-serglycin complexes with target cells remains undefined. Targets exposed to double-labeled granzyme B-serglycin complexes show solely the uptake of granzyme B. An in vitro model demonstrates the exchange of the granzyme from serglycin to immobilized, sulfated glycosaminoglycans. Using a combination of cell binding and internalization assays, granzyme B was found to exchange to sulfated glycosaminoglycans and, depending on the cell type, to higher affinity sites. Apoptosis induced by purified granzyme B and cytotoxic T-cells was diminished in targets with reduced cell surface glycosaminoglycan content. A mechanism of delivery is proposed entailing electrostatic transfer of granzyme B from serglycin to cell surface proteins.

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells eliminate virus-infected and tumor cells. These effector cells store granzymes and perforin (PFN) complexed with the proteoglycan serglycin (SG) within secretory granules. Granule-mediated apoptosis involves, in part, the release of these proteins into the immunological synapse that is formed after specific recognition of the target cell (1, 2). The target cell is postulated to bind and internalize the granzymes to endosomes, whereas PFN is thought to disrupt the vesicles to deliver the granzymes to the cytosol (3–7). GrA and GrB are considered the predominant initiators of apoptosis. GrA is believed to induce caspase-independent cell death via cleavage of the several proteins within the SET complex (a 270–420 kDa cytoplasmic complex) leading eventually to single-stranded DNA nicks (8–10). GrB, on the other hand, appears to function as an apical caspase activating procaspases-3, which mediates, via mitochondria, downstream events that dismantle the cell (11–14). Nevertheless, depending on the amount of granzyme delivered and the levels of counter-regulatory anti-apoptotic factors, the granzyme may also initiate cell death through cleavage of the proapoptotic protein BID (15).

Whether the granzymes undergo target cell binding and internalization for the induction of cellular cytotoxicity has been a matter of intense debate. The original model proposed that granzymes would diffuse to the cytosol through perforin-induced plasma membrane pores. We have, however, recently demonstrated that GrB is secreted as a multi-molecular complex with the chondroitin sulfate (CS) proteoglycan SG (4, 16). Furthermore, in the presence of non-permeabilizing concentrations of PFN, preformed complexes readily induce apoptosis in target cells. Following the original description of specific binding sites for GrB on target cells (3), the granzyme was reported to use the mannose-6-phosphate receptor/insulin-like growth factor-2 receptor (Mpr300) for target cell entry (17). Subsequent work suggested that the uptake occurs both via the dynamin-dependent as well as independent pathways (7, 18). Finally, using cell lines derived from Mpr300 and Mpr46 knock-out mice, both receptors were found to be entirely dispensable for CTL-induced apoptosis (19) and allorejection (20). Similarly, the internalization of GrB and its capacity to induce apoptosis do not require Mpr300 and Mpr46 (19).

Taken together, the mechanism(s) of granzyme delivery into target cells remains unsolved. Studies that have described the uptake of GrB have relied on the isolated cationic granzyme and not the physiologically relevant GrB-SG complex secreted by the cytotoxic cell. The highly cationic monomer would bind with varying affinity to a number of anionic sites on the cell surface, obscuring the interaction with “binding sites” designed to efficiently deliver the granzyme intracellularly. Although GrB remains complexed to SG after degranulation, the molecular fate of the complex after contact with the target cell is unclear. Because of the tight interaction of GrB with the CS glycosaminoglycans (GAGs), the entire GrB-SG complex was hypothesized to undergo en masse uptake by target cells. However, as described here, the granzyme appears to dissociate from SG binding to cell surface molecules. Sulfated GAGs displayed by PGs are shown to be crucial participants in this
process, contributing to exchange and internalization of GrB and optimizing both GrB-mediated and effector cell-induced target cell death. Nevertheless, disruption of PG synthesis in a number of cell lines reveals the presence of higher affinity binding sites for the granzyme, which may also participate in the exchange phenomenon.

**MATERIALS AND METHODS**

**Cell Lines**—Jurkat, Raji, K562, and HL-60 cells were maintained in complete RPMI medium, MCF-7, 293, and 39(+/-) MEF cells were maintained in complete Iscove’s modified Dulbecco’s medium, and RAW cells were kept in complete DMEM. Dr. Howard Lipton (Evanton Northwestern Healthcare Research Inst., Evanston, IL) provided CHO-K1, pgpA-745 deficient in xylosyl transferase and thus lacking all GAGs (21, 22), pgpB-677 deficient in HS-polymerase and thus lacking HS-GAGs (23), and Lec2 cells deficient in sialic acid (24). All CHO cell lines were maintained in complete Ham’s F-12 K medium. RMA cells and peptide-specific CTLs from T cell receptor-transgenic OT-1 mice were employed as described recently (19).

**Reagents**—All reagents and tissue culture supplies were purchased from either Sigma or In VitroGen. Bovine kidney heparan sulfate and porcine intestinal mucosa heparin were from Sigma. Chondroitin sulfate, heparan sulfate, dermatan sulfate, and heparitin sulfate were from Seikagaku Kogyo Co. (Tokyo, Japan). A mouse anti-N-sulfated-glucosamine antibody (namely the anti-HS antibody) was from EMD Biosciences (San Diego, CA). Fluorescein isothiocyanate-labeled secondary anti-mouse Ig antibody was from BD Biosciences. Human GrB and SG were isolated as described (16, 25). Alexa Fluor 488 and Alexa Fluor 633 carboxylic acid succinimidyl esters and fluorescein isothiocyanate-dextran were from Molecular Probes Inc. (Eugene, OR). GrB was labeled per the manufacturer’s instructions. The stoichiometry of labeling was 1.2–2 moles of Alexa Fluor 488 per mole of GrB. SG was labeled with Alexa Fluor 633 hydrazide to modify the uronic acid residues on CS-GAG chains (26). Assuming a molecular mass of 250 kDa for SG, the stoichiometry of labeling for SG633 was 3.2. Alternatively, streptavidin-Alexa 488 or streptavidin-Alexa 546 was coupled to SG633. HS$_{5}$S$_{1}$6G and CS$_{5}$S$_{1}$6G were prepared by coupling biotinylated hydrazide (16, 27). The stoichiometry of labeling for SG633 was 1.53. The modified GrB and SG retained the ability to form complexes as judged by mobility shift assays (16). HS-Sepharose, heparin-Sepharose and CS-Sepharose were prepared with cyanogen bromide-activated Sepharose (Amersham Biosciences).

**Colocalization Studies of GrB$_{488}$ with SG$_{633}$**—For colocalization studies, adherent cells were plated in a chamber slide (Lab-Tek; Nalgene Nunc, Naperville, IL) overnight at 37 °C in 100% humidified, 5% CO$_{2}$, 95% air with half calf serum containing Iscove’s modified Dulbecco’s medium. The cells were then washed thrice with RPMI with 1% BSA and incubated with 1 μg/ml either GrB Alexa 488 (GrB$_{488}$) alone, SG Alexa 633 (SG$_{633}$) alone, or the doubly labeled GrB$_{488}$-SG$_{633}$ complexes in RPMI with 1% BSA at 37 °C for 120 min. The cells were then washed thrice with plain RPMI and paraformaldehyde (1% final paraformaldehyde), nonpermeabilized samples. The cells were left untreated or treated with a combination of 0.1units/ml heparinase I and heparinase III (heparitase II) (Sigma, St. Louis, MO) for 10 min at room temperature and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for imaging. For suspension lines, cells were first washed thrice with RPMI and 1% BSA and incubated with either 1 μg/ml GrB$_{488}$ alone, SG$_{633}$ alone, or the doubly labeled GrB$_{488}$-SG$_{633}$ complexes at 37 °C for 30 min. The cells were washed thrice with plain RPMI, fixed, and attached to poly-L-lysine-coated coverglass. The coverglass was then assembled into a Focht live cell chamber (FCS2; Biopets Inc., Butler, PA) for imaging.

**Confocal Laser-scanning Microscope (CLSM) Imaging**—The treated cells were imaged on a Leica CLSM System with a DMI202 inverted microscope (Leica Microsystems, Exton PA). Twelve-bit fluorescence images of the fluorophores were acquired with 63× or 100× oil-immersion objectives (numerical apertures 1.25 and 1.4, respectively) using the Leica Confocal software. Where z-series was acquired, the section step size ranged between 0.4 and 1 μm. Sequential scans were employed with all double-labeled specimens to eliminate cross-talk between channels. Differential interference contrast images were acquired simultaneously with one of the fluorescence channels. Images or image stacks (both fluorescence and digital interference contrast) were imported into Image J version 1.31 (National Institutes of Health) and processed as follows. Each stack was run through a Kalman stack filter to remove noise. Single fluorescence images were produced by performing a z-projection for maximum intensity. Each maximum projection was corrected for brightness and contrast using standard parameters. Digital interference contrast images were processed in a similar fashion except that instead of using a maximum projection, the single most in-focus image in each stack was selected. All images were overlaid using Velocity version 2.5 (Improvision, Inc., Lexington, MA). Image stacks for each fluorophore were loaded into Image J and then thresholded to segment areas of the image corresponding to the label. The “colocalize sessions” function of Velocity was used to produce spread-sheets containing percentages of pixels that were directly overlaid. Three to five different fields, each containing 5–15 cells, were analyzed for colocalization. The results were expressed as the mean of the percentage of colocalization ± S.D. Numerical calculation of colocalization indices was independently verified by the laboratory of Prof. Frederick Maxfield (Cornell University, New York, NY), using published procedures (27).

**Internalization of GrB by Flow Cytometry**—Internalization experiments were done in RPMI plus 1% BSA at 37 °C. For uptake experiments, cells, either in suspension or confluent monolayer, were incubated with 1 μg/ml GrB$_{488}$ or GrB$_{488}$-SG or a double-labeled GrB$_{488}$-SG$_{633}$ complex for 120 min. Alexa 488-labeled avidin (avidin$_{488}$) was doubly conjugated overall GAG content (28). Cells were incubated with avidin$_{488}$ at 66 μg/ml (1 μg) under conditions similar to those for the granzyme. Cells were washed three times with buffer and subjected to flow cytometry. Adherent cells were detached with trypsin. Flow cytometry was performed on a FACSCalibur with CellQuest software (BD Biosciences). The relative mean fluorescence intensity (MFI) was reported as the ratio of geometric mean of the sample to the geometric mean of the negative control.

**Binding of Gr-B-SG to Target Cells by Flow Cytometry**—For binding studies, GrB$_{488}$ or GrB$_{488}$-SG, or the complex was added to suspended cells in RPMI/BSA plus sodium azide (0.02%) for 30 min at 4 °C. Under these conditions the signal for bound GrB was negligible; therefore, amplification was achieved by staining with rabbit polyclonal anti-Alexa Fluor 488 antibody (anti-488) followed by Alexa 488-conjugated goat anti-rabbit IgG (anti-488) followed by fluorescein isothiocyanate-labeled secondary anti-mouse Ig. This process was repeated once more with Alexa 488-conjugated goat anti-rabbit IgG and then binding was reported as the MFI of bound GrB. The matched control for nonspecific antibody binding was defined as cells incubated with GrB$_{488}$-SG (1000 ng/ml) followed by polyclonal rabbit IgG and then the secondary labeled antibody. Nonspecific binding of the granzyme was assessed by detecting the interaction of the labeled GrB in the presence and absence of 100-fold excess of unlabeled protease. Under these conditions, the majority of the fluorescence associated with the bound granzyme was removed.

**Detection of Cell Surface HS**—Cells in suspension were stained with anti-HS monoclonal antibody for 60 min on ice followed by detection with fluorescein isothiocyanate-labeled secondary anti-mouse Ig for 40 min. Adherent cells were trypsinized, allowed to recover in complete medium for 2 h, and then stained with anti-HS monoclonal antibody.

**Heparinase Digestion of Cell Surface HS**—To remove bound serum proteoglycans, cells were pre-incubated with RPMI and 1% BSA for 60 min at 37 °C followed by two washes in the same buffer. The washed cells were then left untreated or treated with a combination of 0.1units/ml heparinase I and heparinase III (heparitase II) in 100 units/ml sodium acetate, pH 7.0, containing 10 mM Ca$^{2+}$ for 30 min at 37 °C. RPMI with 1% BSA was added to the cells, and incubation was continued for another 30 min. Cells were then washed thrice with RPMI and 1% BSA.

**Xyloside-Mediated Inhibition of GAG Attachment**—Cells were grown with or without 2.5 μg/ml xylosidase for 3 days before use. GAG-Sepharose Binding—GrB$_{488}$ or GrB-SG (100 μg/ml) was incubated with either control or GAG-Sepharose beads for 30 min at room temperature. The beads were then centrifuged, and the supernatants were analyzed for GrB activity.

**GrB-induced Apoptosis**—Granzyme-induced apoptosis was determined by loss of mitochondrial membrane potential as described (29). The LAK Cell-induced Cytotoxicity—LAK cells were derived from wild type 129/SVJ mice (courtesy of Timothy Ley, Washington University) and cultured for 10 days in RPMI medium with 10% fetal bovine serum and 100 units/ml interleukin 2.

**CTL-induced Apoptosis**—CTL-induced DNA fragmentation, assessed as the percentage of thymidine release, was performed as described (19).

**CTL and Target Cell Conjugate Formation**—V3yran cell-labeling solutions (Molecular Probes Inc.) were used to stain OT-1-derived CTL (Di d) and RMA target cells (DiO d) according to the manufacturer’s directions. After coculture in the presence or absence of the relevant peptide (SIINFEKL), the cells were analyzed on a FACSScan flow cytometer for conjugate formation.

**Imaging, Computers, and Software**—Images were captured either with a Kodak digital camera or Saphir Ultra 2 flatbed scanner and exported to Adobe Photoshop 6.0, after which TIFF images were placed for final presentation in Adobe Illustrator 10.0 using a Macintosh Power G4 computer.
Granzyme B-Serglycin Complex Interacts with Cell Surface PGs

**RESULTS**

**GrB Is Internalized into Target Cells without SG**—GrB is secreted as a multi-molecular complex bound to SG (4). To learn whether the intact complex was internalized, the uptake values of monomeric GrB488 (GrB labeled with Alexa 488), SG633 (SG labeled with Alexa 633), and reconstituted double-labeled GrB488-SG633 complexes were compared by flow cytometry. Typically, GrB and SG are mixed at 1:1 (w/w), producing 6–8 GrB molecules per SG molecule (16). The results (Fig. 1a) indicated that although internalization of the GrB was readily apparent in >90% of the cells, uptake of SG was minimal. These observations were consistently made for a number of cell lines including 39(+/+) MEFs and CHO cells (data not shown). The wild type 39(+/+) MEF line (19) was the single exception, showing ~15% double positive cells and higher uptake of SG633 alone (Fig. 1b). The results suggested that target cells incubated with GrB-SG complexes might preferentially internalize the granzyme but exclude the carrier PG.

Because flow cytometry studies failed to demonstrate substantial uptake of SG, the interaction of double-labeled GrB-SG was examined by CLSM. Imaging was performed with 293, RAW and 39(+/+) MEF cells. Data were collected in real time over 120 min (data not shown) or at end point in fixed cells. In comparison with GrB, a very weak signal was consistently observed for SG (Fig. 1c) and, when present, the SG633 signal resided in regions of the cytoplasm separate from GrB. Quantitation of colocalized signals by two separate algorithms (see “Materials and Methods”) failed to exceed 15.5 ± 8.3%. Similar data were obtained for GrB488-SG633-treated RAW cells (data not shown). Importantly, SG was also labeled with Alexa 546, and cells treated with these double-labeled complexes produced similar results (data not shown). The 39(+/+) MEFs contained significant levels of SG when incubated with either labeled free SG or treated with double-labeled GrB-SG (Fig. 1c). This exception served as a crucial positive control, ensuring that labeled SG could indeed be visualized intracellularly by our CLSM system. Based on image analyses, the granzyme seemed, in the majority of cell lines examined, to dissociate from SG and to undergo internalization without the proteoglycan.

**GrB Undergoes Ion Exchange from SG to Immobilized GAGs or Target Cell Surface**—The observations suggested that the granzyme might dissociate from SG and interact separately at the cell surface. We have reported, in surface plasmon resonance studies, a strong association between GrB and CS GAGs of SG (16). However, it seemed plausible that the granzyme could undergo transfer to more highly acidic cell surface GAGs. Typically, the CS on SG characterized from hematopoietic cells could indeed be visualized intracellularly by our CLSM system. Based on image analyses, the granzyme seemed, in the majority of cell lines examined, to dissociate from SG and to undergo internalization without the proteoglycan.
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compared with 947 ± 55 nM for GrB-CS. As reported previously (16), the $K_d$ for GrB-SG interaction was ~710 nM.

To establish whether GrB has the capacity to undergo ion exchange from SG to another acidic GAG molecule, an in vitro assay was designed that entailed the mixing of GrB-SG complexes with either HS or CS GAG chains that had been immobilized to Sepharose beads. GrB remaining in the fluid phase was then determined enzymatically after removal of the beads. In this model system, ~20% of the total GrB was detected in the fluid phase, indicating that the majority of the protease had associated with HS- or CS-Sepharose beads (Fig. 2b). The granzyme could also transfer from equimolar GrB-HS complexes to immobilized HS-Sepharose (data not shown). The observation that GrB could transfer to immobilized GAGs suggested that the exchange process was not merely due to differences in the $K_d$ for these sulfated carbohydrates. The Sepharose-coupled GAGs appeared to provide a topological distribution of negative charge exceeding the level displayed by the soluble GAGs favoring exchange.

The transfer phenomenon was then established for target cells by showing that addition of GrB-SG complexes to Jurkat cells resulted in detectable binding of the protease but not the proteoglycan. Using an antibody amplification technique (see “Materials and Methods”), GrB$_{asg}$ was detected on the target cell surface of Jurkat cells after incubation with GrB$_{asg}$-SG. However, when GrB-SG$_{asg}$ complexes were added to the target cells, bound SG was not observed (Fig. 2c). The results therefore suggest that GrB is transferred to the target cell, whereas SG is concomitantly excluded.

Level of Cell Surface PGs Correlates with the Internalization of Exchanged GrB—Because PGs contain the bulk of sulfated GAGs, it was then asked whether these proteins modulated GrB uptake. The studies were performed with wild type CHO-K1 and two mutant cell lines, namely pgsA-745 cells that have markedly reduced levels of all GAGs due to a deficiency in xylosyl transferase and pgsD-677 cells that have reduced levels of HS-GAGs due to the lack of HS-polymerase (23). The reduction in cell surface HS-GAGs in the mutant lines was documented with an anti-N-sulfated glucosamine antibody (31, 32) (Fig. 3a). Both the pgsA-745 and pgsD-677 cells were consistently found to internalize less granzyme (monomer or complexed) than the wild type CHO-K1 cells (Fig. 3b). In comparison with the CHO-K1 cells, GrB uptake was reduced to 46.7 ± 5.5% ($n = 9$) and 67.6 ± 6.9% ($n = 9$), respectively, for pgsA-745 and pgsD-677 cells (Fig. 3b).

Because GrB uptake in pgsD-677 cells exceeded the levels observed for pgsA-745 cells, the result suggested involvement of other GAGs in the internalization process apart from HS. However, uptake was substantial in the GAG-deficient lines, indicating that other negatively charged molecules on the cell surface may contribute to exchange and internalization of the granzyme. Because sialic acid also contributes to the net negative surface charge, its role in GrB uptake also was also examined. A comparison of Lec2, a sialic acid-deficient CHO line (24), and CHO-K1 revealed no difference in granzyme uptake (data not shown). Furthermore, neuraminidase treatment of Jurkat, CHO-K1, or pgsA-745 cells did not alter GrB uptake (data not shown). Overall, the observation that uptake of the granzyme was reduced in pgsA-745 and pgsD-677 but unaffected in sialic acid-deficient cells indicated that internalization was influenced predominantly by the levels of cell surface-sulfated GAGs and not by negative charge per se. Finally, because pgsA-745 cells exposed to double-labeled GrB-SG complexes did not internalize SG (data not shown), uptake in a GAG-deficient cell was not due to fluid phase pinocytosis. Thus, exchange and uptake of the granzyme appeared to depend on...
cell surface GAG content as well as on electrostatic exchange with undefined anionic sites.

The participation of cell surface GAGs in the exchange was then examined by learning whether free GAGs blocked the process. Exchange was compared in both Jurkat and CHO cells using a 500-fold molar excess of commercial HS, heparin, and chondroitin sulfate-E (CS-E). More heavily sulfated GAGs (heparin and CS-E) could partially block uptake of the granzyme (Fig. 3d), whereas the less sulfated HS was much less effective. These results are consistent with the level of sulfation displayed by these GAGs. For CHO-K1 cells, cell surface sulfation is \( \sim 33\% \) (33). For bovine kidney HS (average \( M_r \sim 10000 \)) and porcine intestinal heparin (average \( M_r < 20,000 \)), the estimated sulfation is \( \sim 44 \) (34) and \( \sim 85\% \), respectively (35). The results confirm that the exchange is driven by electrostatic interactions. The role of cell surface sulfation was also verified using chlorate treatment in CHO-K1, pgsA-745, and Jurkat cells. Suppression of sulfation led to a 2.6-fold reduction in the uptake of free or complexed GrB in CHO-K1 and Jurkat cells (data not shown). The contribution of cell surface HS in the uptake of the granzyme was also verified using HL-60 cells that had undergone heparinase/heparitinase digestion (Fig. 3e).

The levels of GrB uptake were then correlated with the cell surface GAG content for a panel of adherent and non-adherent cells.

**Fig. 3. Cell surface GAGs contribute to GrB uptake.** 

a, analysis of anti-HS reactivity of wild type and mutant cell lines. A representative flow cytometry profile (\( n = 3 \)) of cell surface HS expression in CHO-K1 and the PG-deficient cells, pgsA-745 and pgsD-677, determined with an anti-N-sulfated-glucosamine antibody, is shown. FITC, fluorescein isothiocyanate. b, uptake of GrB in cells displaying disrupted PG synthesis. Adherent cells were treated with GrB-SG for 120 min and processed as described under “Materials and Methods.” The uptake represents relative MFI \( \pm \) S.E. (\( n = 9 \)). c and d, ability of soluble GAGs to inhibit uptake of GrB to Jurkat and CHO is sulfation-dependent. GrB-SG, in presence or absence of a >500-fold molar excess of soluble HS, heparin (Hep), or CS-E, were incubated with cells for either 30 min (for Jurkat) or 120 min (for CHO cells) at 37 °C. Samples were processed as described under “Materials and Methods.” Results shown are mean \( \pm \) S.E. of three experiments. e, effect of heparinase digestion on GrB uptake. HL-60 cells were subjected to heparinase digestion as described under “Materials and Methods.” The effect of HS-GAG removal on the uptake of GrB-SG at the varying concentrations is shown here. Results are mean \( \pm \) S.E. of two experiments run in duplicate. f, correlation of GrB uptake with the level of GAG expression. The internalization of GrB, either monomeric or complexed, HS expression defined by anti-N-sulfated-glucosamine antibody, and avidin uptake by various cell types are shown. Experimental conditions are as described under “Materials and Methods.” The uptake is shown as relative MFI. Mean of three experiments \( \pm \) S.E. is shown.
using anti-N-sulfated glucosamine reactivity and avidin uptake. Although an anti-N-sulfated glucosamine antibody has been used to detect cell surface HS, there was a concern that this approach may not accurately reflect the variation in HS-GAG sulfation displayed by the various cell lines. Therefore, the immunological approach was complemented by the measurement of labeled avidin (pI of 10) uptake. Avidin binds to GAGs through an ionic interaction and, thereby, should reflect the content of sulfated GAGs (28).

Utilizing these probes, granzyme internalization appeared to correlate more with avidin 488 uptake than with anti-N-sulfated glucosamine reactivity (Fig. 3f). Although avidin and GrB have similar pI values, uptake of the former required 66 µg/ml (1 µM) of GrB, a concentration substantially exceeding the concentration of monomeric or complexed GrB (1 µg/ml; 31.25 nM) used in the majority of the experiments. This observation suggested that whereas electrostatic forces drive the interaction of both GrB and avidin with cell surface GAGs, specific GAG binding sequences on the GrB molecule and the fine specificity of GAG chains may contribute to the tighter interaction of granzyme with cell surface GAGs.

GrB, Depending on Cell Type, Exchanges with Varying Affinity—Developing a semi-quantitative binding assay (see "Materials and Methods"), the exchange of GrB was compared for CHO-K1, pgsA-745, and pgsD-677 cells as well as for Jurkat, Raji, and K562 cells. Using a concentration range for the granzyme that is sufficient to induce apoptosis in a variety of cells, Jurkat cells were found to bind GrB with substantially higher affinity than the CHO cell lines. Although binding of granzyme to the CHO-K1 cells was apparent at 1 µg/ml, exchange to GAG-deficient pgsA-745 cells and the HS-GAG-deficient pgsD-677 cells was minimal (Fig. 4a). In comparison with the CHO cells, Raji and K562 cells also bound the granzyme with higher affinity, consistent with previous data reported for Jurkat cells by the radioligand binding method (3). The findings therefore suggested that CHO cells lacked higher affinity binding sites for granzyme.

To learn whether the sites on Jurkat cells were GAG sequences, we studied the binding and uptake of GrB-SG in Jurkat cells treated with MX. The xyloside acts as a false substrate for galactosyl transferase-I, competitively inhibiting GAG polymerization onto endogenous core proteins. Efficacy was validated by showing that the inhibitor reduced internalization of labeled avidin by 50–70% (28) (data not shown). The
inhibitor reduced the binding (Fig. 4c) and internalization (Fig. 4d) of GrB, but the higher affinity interaction persisted. These observations suggested that, in addition to sulfated GAGs, a specific receptor might contribute to the exchange of the granzyme to Jurkat cells.

**Multiple Pathways Exist for Uptake of GrB—Cellular uptake consists of numerous biological processes, including classical receptor-mediated endocytosis and more poorly understood pathways (e.g. caveolin-dependent and fluid phase pinocytosis). Previous reports have indicated that granzyme uptake is dynamically dependent and -independent (7, 18). Internalization pathways may be distinguished by comparing the rate and concentration dependence of uptake. The kinetics (see Fig. 5a) and concentration dependence (Fig. 5b) of GrB uptake in Jurkat cells was compared with CHO-K1 cells. Although internalization of the GrB was rapid (t1/2 < 2 min) and saturable (30 min) in Jurkat cells, CHO-K1 cells displayed a linear curve without reaching saturation at 120 min. These studies indicated that kinetically distinct pathways might exist for the uptake of the bound granzyme.

**Influence of Target Cell PG Expression on GrB-, LAK-, and CTL-mediated Apoptosis—**Although cell surface PGs seemed to participate in the binding and internalization of the granzyme, it was crucial to learn whether abrogation of PG expression attenuated granule-mediated apoptosis. To assess this possibility, we first compared the susceptibility of CHO-K1 and pgsA-745 cells to GrB-induced apoptosis. The CHO cells readily succumbed to staurosporine-induced cell death but resisted the effects of the granzyme, an outcome attributed to the failure to activate caspase-3 by undefined anti-apoptotic factors (data not shown). Therefore, the susceptibility of MX-treated Jurkat cells was evaluated. As predicted by uptake studies (Fig. 4d), Jurkat cells that had been exposed to MX became partially resistant to GrB-mediated apoptosis (Fig. 6a). Next, the influence of PGs on the susceptibility of targets to killing by cytotoxic cells was examined. In the first approach, LAK cells were generated from murine splenocytes and tested against MX-treated Jurkat cells. As shown in Fig. 6b, Jurkat cells with reduced cell surface GAG content were more resistant to LAK. Finally, the role of PGs in target cell susceptibility to peptide-specific CTLs was examined. Target cells included untreated RMA cells, cells pretreated with MX for 72 h, or cells exposed to MX during the cytotoxicity assay. The various targets were incubated with CTL in the presence and absence of a cognate peptide. Untreated RMA cells and cells exposed to MX during the cytotoxicity assay expressed similar levels of sulfated GAGs, and re-expression was not substantial during the assay (data not shown). Remarkably, the xyloside also diminished antigen-specific CTL-mediated DNA fragmentation at the majority of specific binding sites that are saturable and demonstrate rapid kinetics of uptake. Because the reduction of avidin uptake did not exceed 70%, the possibility cannot be excluded that the residual binding has an electrostatic basis occurring through undefined high affinity sulfated GAGs or other anionic sites. In cells lacking the higher affinity sites (e.g. CHO cells), the granzyme clearly interacts with sulfated GAGs but with substantially lower affinity. In this instance, uptake is neither saturable nor rapid. A similar phenomenon has been reported for a protein transduction domain like HIV-TAT proteins (37, 38) or latex beads coated with HSPG-ligating antibodies (39). Importantly, the model emphasizes the generalization of the electrostatic exchange phenomenon to multiple uptake pathways, ensuring that the granzyme enters the widest variety of cell types (see Fig. 7).

GrB uptake correlated with target cell-associated GAG content when surface expression was defined by an electrostatic indicator (avidin uptake) but not by an immunological approach (anti-N-sulfated glucosamine reactivity). HS is a highly heterogeneous molecule, and accurate quantitation with an
A reduction in cell surface GAG content is associated with diminished GrB- and CTL-induced apoptosis. 

**a**, reduction in GrB-induced apoptosis in targets exposed to the PG synthesis inhibitor MX. Jurkat cells were treated with MX (2.5 mM) for 72 h and then treated with GrB-SG/AD followed by assessment of mitochondrial depolarization as described. Values for GrB-SG on vertical axis (µg/ml) represent concentrations of GrB added to targets. AD was titrated against targets to a plaque-forming unit that provides 50% β-galactosidase-positive cells. Results are the mean ± S.E. for five independent experiments. A paired t test was significant for all concentrations of GrB-SG/AD at p < 0.05. 

**b**, role of target cell-associated PGs in nonspecific LAK cell-induced cytotoxicity. Jurkat cells were incubated with MX as described and labeled with CFSE (125 nM) for 15 min. The labeled Jurkat cells were mixed with LAK cells (effector-to-target ratio, 20:1) and incubated in LAK medium (with or without MX) for 4 h. The suspensions were collected and stained with 7-amino actinomycin D for fluorescence-activated cell sorter analysis. The results represent the mean ± S.D. of two experiments. Paired t test was significant for wild type LAK with or without MX at p < 0.05. 

**c**, role of cell surface PGs on antigen specific CTL-mediated apoptosis. RMA cells were cultured in the presence or absence of 2.5 mM MX for 72 h and then used as targets for the peptide-specific T cell receptor-transgenic OT-I CTL in the presence or absence of additional MX. The presence of MX during pretreatment and its presence during assay exposure are marked as the values before and after the slash sign (e.g. 2.5/0). Specificity was demonstrated by pulsing with relevant (SIINFEKL) or irrelevant peptide (P) (data not shown). Specific [3H]thymidine release was determined (n = 3) after a 4 h assay at the defined effector-to-target ratios. The percentage of CTL-induced apoptosis of control RMA cells (0/0) at the highest effector target ratio (10:1) in each test was adjusted to 100%, and the effect of the various MX treatments at different effector-to-target ratios was calculated. Results are the mean ± S.E. for five experiments. A paired t test was used to compare 0/0 with 2.5/0 and 0/2.5 with 2.5/2.5. An asterisk marks pairs where the differences were significant with p < 0.05, and ns describes non-significant differences.
Granzyme delivery (7, 41). Nevertheless, others have shown recently that apoptosis induced by GrB-SG complexes as well as CTLs is entirely dynamin-dependent (18). Therefore, in a physiological setting the relevance of pinocytosis, either constitutive fluid phase pinocytosis or simulated macropinocytosis, as a mechanism for the internalization of GrB remains unclear.

The information described here revises the mechanism underlying PFN-mediated delivery of granule proteins. We had proposed that PFN acts primarily as an endosomolytic agent releasing an internalized granzyme. At the cytotoxic synapse, PFN might produce sufficient damage to allow the direct entry of macro-complexes to the cytosol of the target cell. However, based on the evidence supporting the exchange model, the capacity of the protease to enter the target by diffusion through PFN-generated pores becomes less plausible. The permeabilizing activity of PFN also appears to be modulated by the level of cell surface HS GAGs (42). Thus, electrostatic exchange may play a critical role within the cytotoxic synapse, focusing the mediators responsible for granule-mediated apoptosis toward the plasma membrane of the target cell. Identification of the binding system(s) for the granzyme and PFN that facilitate either endocytic or pinocytic uptake will allow formal testing of the hypothesis that the granzyme undergoes PFN-dependent endosomolytic release.

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