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Granzyme B translocates across the lipid membrane only in the presence of lytic agents

Mojca Podlesnik Beseničar [a], Sunil Metkar [b], Baikun Wang [b], Christopher J. Froelich [b], Gregor Anderluh [a,*]

[a] Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia
[b] Department of Medicine, Evanston Northwestern Healthcare Research Institute, Feinberg School of Medicine, Evanston, IL, USA

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ABSTRACT

Granzyme B (GrB), a component of the cytotoxic cell granule secretion pathway, is designed to kill infected and transformed cells after intracellular delivery by the pore forming protein, perforin. The mechanism of the delivery remains speculative. In this study we tested the hypothesis that GrB possesses capacity to bind and disrupt lipid membranes. Here in comparison to previous studies that show GrB interacts with carbohydrate moieties, the protease does not bind membrane phospholipids nor has intrinsic membranolytic properties. To study the transmembrane movement of GrB, we developed a model membrane system consisting of a high-molecular weight GrB substrate encapsulated in unilamellar vesicles. Intra-vesicle proteolysis clearly requires concentrations of lytic agents (streptolysin O, perforin or Triton X-100) that disrupt unilamellar membranes.

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Granzymes and perforin (PFN) are key components of cytotoxic T lymphocytes and natural killer cells that mediate caspase-dependent and -independent apoptosis of target cells. Despite substantial progress in our understanding of GrB-mediated killing, the mechanism underlying intracellular delivery of the protease by PFN remains enigmatic [1,2]. The original model proposed that secreted PFN binds to the target cell membrane polymerizing into transmembrane pores in a Ca^{2+}-dependent manner [3,4]. These pores then would act as the gateway to allow the soluble protease to enter the cytosol [4,5]. However, GrBs are highly cationic proteins that are predicted to avidly bind negatively charged cell surface structures that would potentially impair transmembrane movement. Support for this possibility is based on the observations that GrBs bind and enter cells through a number of clearance mechanisms [6–11]. The existence of a plasma membrane interaction step in the delivery process is also reinforced by the observation that GrB is secreted bound to the proteoglycan, serglycin, but is then able to exchange to the electrostatically more favorable plasma membrane [12].

The second model for PFN-mediated protein delivery proposes endosomolytic release of internalized GrB by PFN. Proof of principle has been demonstrated by work showing that plasma membrane bound GrB is internalized and released with adenoviral particles [5,13]. Data supporting this model are based primarily on studies, which show that GrB is able to induce cell death in the presence of concentrations of PFN that did not appear to overtly damage the plasma membrane [12,14,15]. The possibility remains that a low density of pores may be generated to allow entry of a sufficient number of GrB molecules to induce apoptosis but not for detection of membrane disruption. Taken together, the molecular mechanism for the cytoplasmic delivery of GrB is still largely unknown.

In this study, we have used large unilamellar vesicles (LUV) to study the transmembrane movement of GrB without binding to the membranes. This was monitored by cleavage of a model high-molecular weight substrate encapsulated in liposomes. Results show that GrB does not bind well to pure lipids and that a lytic agent is clearly needed for an access to the encapsulated substrate.

Materials and methods

Materials. GrB was isolated as described earlier [16]. PFN was isolated as described in [17]. All lipids were from Avanti Polar Lipids (USA). All other chemicals were from Sigma (USA).

Preparation of high-molecular weight GrB substrate. Peptide CGIETDSGAK (P), fluorescently labeled on Lys with 5,6-carboxyfluorescein was synthesized by JPT Peptide Technologies (Germany). It was covalently bound via thiol group in its N-terminal Cys to maleimido group of 20,000 Da polyethylene glycol maleimide (PEG) (Shearwater Inc., USA). P and PEG (P:PEG 5:1, mol:mol) were incubated in 100 mM phosphate buffer, pH 7.2 for 4 h in the dark at 25 °C and gentle stirring. The excess of P was removed from labeled PEG (PEG-P) with filtration through centriprep YM-10 (Amicon). The filtration was performed until

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; GrB, granzyme B; LUV, large unilamellar vesicles; P, fluorescently labeled peptide CGIETDSGAK; PEG, polyethylene glycol; PEG-P, P covalently bound to PEG; PFN, perforin; SLO, streptolysin O; TX-100, Triton X-100.

* Corresponding author. Fax: +386 1 257 33 90.
E-mail address: gregor.anderluh@bf.uni-lj.si (G. Anderluh).

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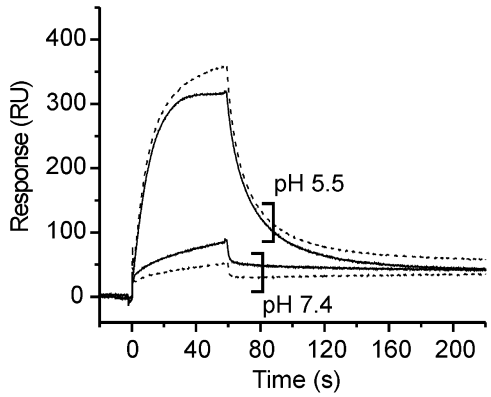

Fig. 1. Binding of GrB to model lipid membranes. GrB (250 nM) in PFN buffer with 1 mM Ca^{2+} was injected over the DOPC (solid line) or DOPC:DOPG (dotted line) LUV, immobilized on Biacore L1 sensor chip at pH 5.5 and 7.4, respectively.

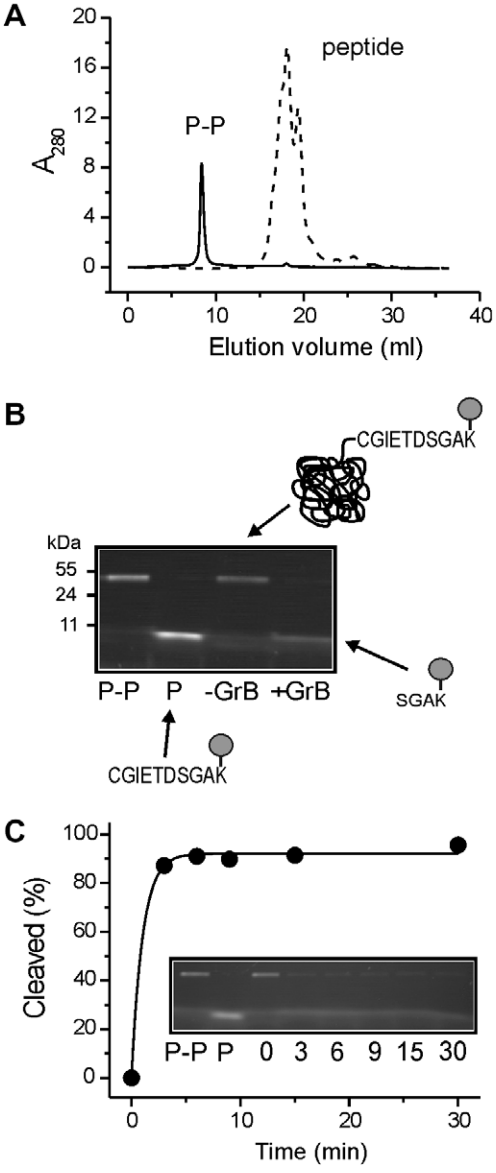

Fig. 2. The preparation of a high-molecular weight GrB substrate. (A) The quality of the synthesized substrate (P-P) was verified by size-exclusion chromatography on Superdex 75-HR column at a flow rate of 0.4 ml/min in 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.4. (B) Separation of PEG-P and P on 12% SDS gels and demonstration of GrB cleavage. Around the gel are schematic representations of the individual molecules. (C) The time-course cleavage of the PEG-P in PFN buffer, with 1 mM Ca^{2+}, by 0.25 μg GrB.

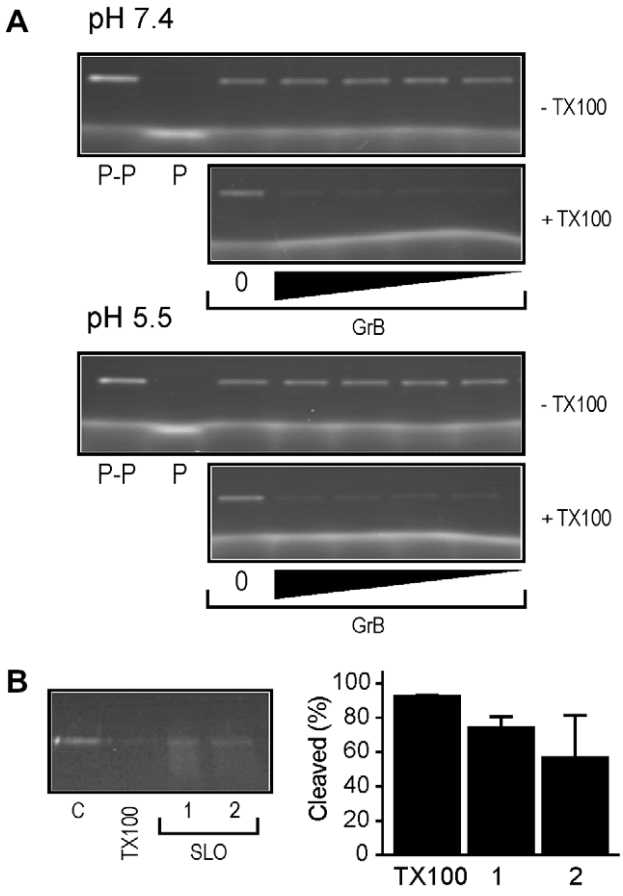

Fig. 3. The ability of GrB to cross the lipid bilayer. (A) Experiments were performed with 2-fold dilutions of GrB starting with 0.25 μg at pH 7.4 and 5.5. GrB was incubated with the DOPC LUV containing PEG-P (P-P) in the presence (+TX100) or absence (−TX100) of detergent TX-100. (B) Control experiments with two different SLO concentrations and 0.5 μg GrB in PFN buffer, with 1 mM EDTA; C, LUV with 6 mM DTT and GrB alone (negative control); TX100, LUV in the presence of 2 mM TX-100 and GrB (positive control); 1, 1.5 μM SLO; 2, 0.5 μM SLO. For these experiments membranes contain 40% (molar) of cholesterol. Next to the gel the estimation of the percentage of the cleaved substrate is shown. Data from two independent experiments are presented. Average ± SD.

no fluorescence was visible in the filtrate. The quality of PEG-P preparations was verified by size-exclusion chromatography on a Superdex 75-HR column (Akta System, Amersham Pharmacia) and SDS–PAGE. The concentration of PEG-P was measured spectrophotometrically at 490 nm by using $\varepsilon^{M}_{490} = 9 \times 10^4\ M^{-1}\ cm^{-1}$ [18].

Preparation of LUV. The lipids were dissolved in chloroform:methanol 2:1 (vol:vol) and dried under vacuum to form a lipid film. The film was resuspended either in 150 mM NaCl, 20 mM HEPES (PFN buffer), pH = 7.4 or in 110 μM PEG-P in PFN buffer by vortexing in the presence of glass beads. So formed multilamellar vesicles were freeze-thawed six times and extruded through polycarbonate membranes with 100 nm pores to form LUV. The solution of vesicles was passed across Sephacryl 400-HR column (0.7 × 50 cm) at the flow rate of 160 μl/min to remove the untrapped fluorescent probes. Lipid concentration was measured using Phospholipids B kit (Wako chemicals, Germany).

Permeabilization experiments. LUV (150 μM) were incubated in 12.5 μl of PFN buffer with 1 mM Ca^{2+} at different LUV/PFN molar ratios for 45 min at 25 °C. The vesicles were incubated without PFN or with 2 mM Triton X-100 (TX-100) to obtain negative and positive controls, respectively. After the incubation, PFN buffer was added to a final volume of 220 μl and the sample loaded onto Microcon YM-100 (Amicon). The Microcons were centrifuged at 1000*g*, for 15 min at 4 °C and the fluorescence of 200 μl of the filtrate was measured by a microplate reader (Anthos, Austria). Fluorescence values were corrected for the contribution of the buffer and TX-100 alone. The percentage of permeabilization (P) was calculated as follows:

$$P(\%) = (F - F_0)/(F_T - F_0) \times 100,$$

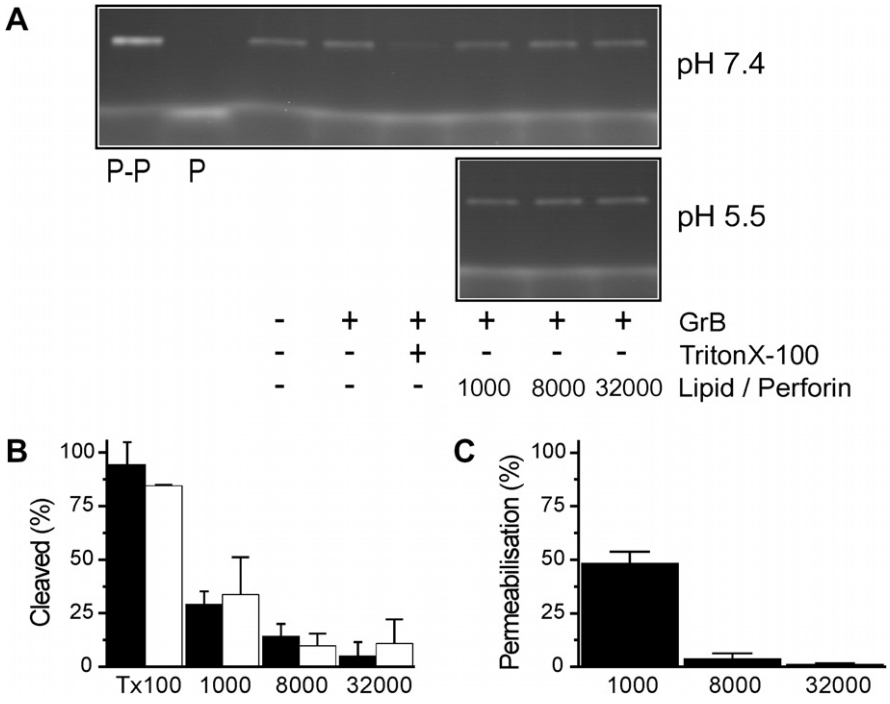

Fig. 4. Cleavage of the LUV-encapsulated PEG-P in the presence of PFN. (A) The cleavage was monitored in PFN buffer with 1 mM Ca^{2+} at pH 7.4 or 5.5. P-P, PEG-P standard; P, peptide standard. (B) The estimation of the percentage of the cleaved substrate. Black bars, pH 7.4; white bars, pH 5.5. Data from three independent experiments are presented. (C) Leakage of the PEG-P from the LUV at the same LUV/PFN molar ratios at pH 7.4. $n = 2$, average ± SD.

where F, F_0, and F_T stand for fluorescence of the filtrate, fluorescence of the vesicles without PFN, and fluorescence of the vesicles in the presence of TX-100, respectively.

Surface plasmon resonance (SPR) experiments. Biacore X system (Biacore, Uppsala, Sweden) and L1 sensor chip were used for binding experiments. LUV were immobilized on L1 chip surface as described [19]. The running buffer was PFN buffer pH 7.4 or 5.5 with 1 mM Ca^{2+}. GrB was injected for 1 min at a flow rate of 20 μl/min and the dissociation was monitored for 3 min.

Cleavage of the PEG-P substrate by GrB. The time-course cleavage of the PEG-P by GrB in solution was monitored by incubating approximately 0.2 pmol of PEG-P with 0.25 μg of GrB for different time intervals at 25 °C. The incubation was performed in the dark in PFN buffer with 1 mM Ca^{2+}. The samples were mixed with SDS–loading buffer (0.362 M Tris, 30% glycerol (vol:vol), 12% SDS (mass:vol)) and loaded onto 12% SDS–PAGE gel immediately after the end of incubation. The gel was then visualized under UV light. The intensities of the bands were estimated using GelPro.

The effect of PFN on the GrB cleavage of PEG-P encapsulated in LUV composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was checked as follows: LUV at a 200 μM final concentration were incubated with 1 μg of GrB for 45 min at 25 °C and different LUV/PFN molar ratios. TX-100 at a 2 mM final concentration was used as a positive control. For the acidic pH, the LUV were first incubated at 25 °C with PFN for 15 min in 150 mM NaCl, 1 mM HEPES, pH 7.4, and then GrB in 150 mM NaCl, 40 mM HEPES, pH 5.0 was added to a final volume of 8 μl and incubated for additional 30 min at 25 °C. The final pH was 5.5. At the end of the incubation the samples were treated as described above.

To monitor the effect of streptolysin O (SLO) on GrB cleavage of encapsulated PEG-P, SLO was first activated for 30 min on ice in the presence of 10 mM DTT. LUV (400 μM) were then incubated with 0.5 μg of GrB and SLO in PFN buffer with 1 mM EDTA for 60 min in the dark at 25 °C. The samples were then treated as described above.

Results and discussion

We first determined the binding characteristics of GrB for lipid membranes, especially the requirement for negative charge. The binding of GrB to DOPC LUV, immobilized on L1 Biacore sensor chip, is concentration dependent (not shown) becoming more extensive, but reversible, at acidic pH (Fig. 1). Since GrB is highly cationic, the protease is predicted to bind negatively charged membranes more avidly. However, no difference in the binding was observed for negatively charged lipids, i.e. 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (30 molar %) (Fig. 1). Similar behavior was observed for DOPC:cholesterol 60:40 (mol:mol) and composition mimicking early endosomal membrane (cholesterol:DOPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine:1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine:sphingomyelin:phosphoinositol 43:30:12.5:2:12:0.6 (molar ratios) [20]), i.e. GrB shows more extensive, though reversible, binding at acidic pH (not shown). Since highly cationic proteins and peptides may act as membranolytic agents, we also examined whether GrB can induce leakage of calcein encapsulated in LUV. Lipid/GrB molar ratios from 20 to 10,240 were evaluated, but no calcein leakage was observed throughout this concentration range.

A major priority was to construct a high-molecular weight GrB substrate that could serve as a marker of proteolysis mediated by GrB. We linked high-molecular weight PEG with a fluorescently labeled peptide (P) containing the GrB cleavage sequence, Ile-Glu-Thr-Asp [21]. The prepared substrate (PEG-P) was free of residual P as judged by size-exclusion chromatography (Fig. 2A). P and PEG-P are resolved on 12% SDS–PAGE gels with apparent masses of approximately 8 and 43 kDa, respectively (Fig. 2B). The PEG-P band disappears in the presence of GrB, indicating cleavage, while the P band becomes prominent (Fig. 2B). Since the P band travels with the slightly fluorescent buffer front, we relied on the disappearance of the PEG-P band to monitor GrB proteolytic activity.

Virtually all soluble PEG-P is cleaved in 3 min at the concentrations of granzyme tested, suggesting PEG-P is a sensitive indicator of GrB activity (Fig. 2C). The granzyme could not access PEG-P when encapsulated in DOPC LUV. As shown in Fig. 3A, the substrate was not cleaved at either pH tested, i.e. 7.4 or 5.5 (Fig. 3A, upper panels). However, in the presence of the detergent TX-100, the cleavage of LUV-encapsulated PEG-P at both pH values was as efficient as for soluble substrate (Figs. 2C and 3A, lower panels). The PEG-P cleavage proceeds also in

the presence of a bacterial cytolysin SLO, which forms pores of diameter (30 nm) sufficient for entry of a variety of macromolecules (Fig. 3B) [22].

We next monitored cleavage of the LUV-encapsulated PEG-P in the presence of PFN at 1 mM Ca^{2+}. Since PFN generated pores are commonly viewed to be around 5 nm, movement of GrB across the lipid bilayer through these pores would be rate limiting. Consequently, these studies were performed with higher concentration of GrB (1 μg/reaction). In the presence of PFN, modest cleavage of the encapsulated PEG-P was observed at LUV/PFN molar ratio of 1000 at acidic and neutral pH (Fig. 4A). We measured, as a marker of membrane permeabilization, the amount of PEG-P released from LUV by PFN. At LUV/PFN molar ratio of 1000, PFN released approximately 50% of the encapsulated PEG-P, whereas leakage of the substrate was not observed at higher LUV/PFN ratios (Fig. 4C). Cleavage of the substrate in the presence of PFN, therefore, correlates with PFN pore forming ability.

In summary, GrB does not possess a phospholipid membrane binding capacity and neither calcein nor PEG-P were released from LUV in its presence. Furthermore, GrB readily accesses PEG-P only when a membrane disrupting agent, e.g. TX-100, SLO or PFN, is present. This was true also for more acidic conditions that are present in endosomes. Our results further indicate that in an *in vitro* system, extremely low sublytic concentrations of PFN do not aid in membrane translocation of GrB. The presented results can, therefore, safely rule out any significant role of phospholipids in the translocation process of GrB and strongly support the prevalent view that GrB requires a translocation agent to enter the target cell.

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