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An atomic-force basis for the bacteriolytic effects of granulysin

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Abstract

While granulysin has been suggested to play an important role in adaptive immune responses against bacterial infections by killing pathogens, and molecular force for protein–protein interaction or protein–bacteria interaction may designate the specific functions of a protein, the molecular-force basis underlying the bacteriolytic effects of granulysin at single-molecule level remains unknown. Here, we produced and purified bactericidal domain of macaque granulysin (GNL). Our bacterial lysis assays suggested that GNL could efficiently kill bacteria such as *Listeria monocytogenes*. Furthermore, we found that the interaction force between GNL and *L. monocytogenes* measured by an atomic force microscopy (AFM) was about 22.5 pN. Importantly, our AFM-based single molecular analysis suggested that granulysin might lyse the bacteria not only through electrostatic interactions but also by hydrogen bonding and van der Waals interaction. Thus, this work provides a previous unknown mechanism for bacteriolytic effects of granulysin.

Keywords

AFM; Granulysin; Perforin; Bacteria; *Listeria monocytogenes*

1. Introduction

Human CD4⁺ T cells, cytotoxic T lymphocytes (CTLs) [1,2], $\gamma\delta$ T cells [3], and natural killer (NK) cells [4] play important roles in host defense against intracellular pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*. We and others have recently shown that the bacteriolytic activities of human or macaque CD4⁺ T cells, CTL, $\gamma\delta$ T cells, or NK cells are mediated by an important antimicrobial protein, granulysin [1,3,5–16] that

are stored in cytolytic granules together with perforin and granzyme B. Granulysin and perforin therefore show great potentials as antimicrobial reagents. To develop granulysin for an antimicrobial reagent, it is necessary to fully understand the bacteriolytic mechanisms of granulysin. It has been reported that the positive charges at neutral pH are crucial for lytic activity of human granulysin against bacteria and negatively charged liposomes [11,15,17–19]. However, little is known regarding the molecular-force aspects for bacteriolytic effects of granulysin.

Accumulating evidence has suggested that molecular force for protein–protein or protein–bacteria interactions may designate the specific functions of a protein [20–22]. For example, the interaction force between granulysin and bacteria at single-molecular level may determine the specificity and efficacy of bacteriolytic effects for granulysin. Elucidation of the molecular-force bases at single-molecular level underlying the bacteriolytic effects of granulysin may not only facilitate the understanding of bacteriolytic mechanisms of granulysin but also help develop an antimicrobial agent with high specificity and efficacy. Currently, there is no report regarding molecular force analyses of granulysin–bacteria interface at the single-molecular level. It is therefore important to explore the molecular basis at the single-molecular level of bacteriolytic actions of granulysin.

Thanks to its extremely high spatial and force resolution, recent progress of atomic force microscopy (AFM)-based nanotechnology provides a powerful tool for protein identification [23] and studying molecular interaction [22], molecular [24] and cellular nanostructures [25,26]. Thus AFM-based nanotechnology should provide a powerful tool to explore the molecular-force basis underlying the bacteriolytic effects of granulysin. In this study, we made use of our expertise of AFM-based nanotechnology [21,22] to elucidate the molecular-force basis of recombinant macaque granulysin at the single-molecular level. We produced and purified macaque granulysin bactericidal domain (termed as GNL) and functional domain of perforin (C terminal of perforin, termed PC). The antimicrobial effects of granulysin and perforin were determined by bacterial lysis assays. Most importantly, the AFM-based single-molecular analysis allowed us to determine the molecular-force basis underlying the bacteriolytic effects of recombinant macaque granulysin. We found that granulysin might lyse the bacteria not only through electrostatic interactions but also by hydrogen bonding and van der Waals interaction.

2. Materials and methods

2.1. Recombinant bactericidal domain of macaque granulysin

Recombinant macaque granulysin bactericidal domain (termed as GNL) was produced and purified using recombinant protein production and purification systems that have been recently described by us [27]. Briefly, macaque GNL were obtained by PCR amplification based on previously reported functional domains of the human granulysin and perforin [8,28]. Each DNA construct was transformed into *Escherichia coli* to induce expression of foreign protein with isopropyl β -D-1-thiogalactopyranoside (IPTG). The cell pellets were harvested from the different induced conditions. After lysis, the collected cytoplasm and lysates of inclusion body were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using specific antibody against granulysin.

2.2. Bacteriolytic effects of GNL determined by bacterial lysis assays

The engineered proteins were incubated with 1×10^6 CFU/well of *L. monocytogenes* (10403S strain) suspension for 2 h at 37 °C, then the 10-fold serial dilutions of each reaction were spread triplicate on LB agar plates. The bacterial colonies of each plate were enumerated in the following day to collect the lytic data.

2.3. Immobilization of *L. monocytogenes* on mica

To immobilize *L. monocytogenes* on micas for AFM measurements, the micas were first thoroughly cleaned. The micas were sonicated sufficiently in chloroform to remove the grease, and micas were then etched in HF acid for a while at room temperature to remove natural oxide layer; After that, the micas were immersed in alkaline solution ($\text{NH}_4\text{OH}:\text{H}_2\text{O}_2:\text{H}_2\text{O} = 1:1:5$, v/v) for 30 min, followed by cleaning and oxidizing by heating to 90 °C in piranha solution (98% $\text{H}_2\text{SO}_4:\text{H}_2\text{O} = 7:3$, v/v) for 30 min. The micas were thoroughly rinsed with distilled water between each step mentioned above. The cleaned micas were immediately incubated in a solution of 1.0% (v/v) APTES in toluene solution for 2 h. The unbound silane was washed away carefully with the solvent. The silanized micas were activated by immersing them in a 10% glutaraldehyde solution in PBS buffer (20 mM NaH_2PO_4 , 150 mM NaCl, pH 7.0) at room temperature for 1 h and then rinsed with buffer. The activated micas were dipped into a solution of *L. monocytogenes* at room temperature for 2 h. The unbound *L. monocytogenes* were removed by extensive PBS wash.

2.4. Functionalization of AFM tips

AFM silicon nitride tips (Veeco, CA) were functionalized following the protocol as described in our previous works [21,22]. Briefly, the Si_3N_4 AFM cantilevers (Veeco, CA) were incubated with 1% (v/v) 3-aminopropyltriethoxysilane (APTES; Sigma) in toluene for 2 h, and rinsed in toluene for 5 min. Subsequently, they were incubated with 0.2% (v/v) glutaraldehyde in distilled water for 30 min, and then rinsed with distilled water for 5 min. The activated tips were immediately transferred to a protein solution and incubated at room temperature for 30 min, followed by extensive washing to remove any unbound proteins.

2.5. AFM analysis

Before performing each force–distance (F–D) experiment, AFM cantilever spring constant was calibrated following instructions of Veeco, as we previously described [21]. Images were performed using AFM tips functionalized with proteins in PBS in contact mode on an Explorer AFM (Veeco). After localization of individual bacterial cells by imaging, AFM F–D curves were obtained on bacterial surface. All AFM F–D curves were obtained in the same loading rate (0.8×10^5 pN/s) and contact time (0.25 s).

2.6. Statistical analysis

GraphPad Prism (GraphPad Software, Inc.) was used to create graphs, and the data of each protein are presented as the mean $\pm$ SEM in this study. Student's *t*-test, as described previously [29], was used to evaluate statistical significance between groups or conditions, and differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Granulysin but not perforin has bacteriolytic effects against *L. monocytogenes*

As an initial step to characterize the single-molecular force basis for the bacteriolytic effects of granulysin, we first produced bactericidal domain of macaque granulysin (Termed GNL). Because perforin might also be very important to facilitate granulysin to lyse intracellular bacteria [1], we produced functional domain of macaque perforin (C terminal of perforin, termed PC) as well. Our SDS-PAGE analysis suggested that purified GNL and PC have the molecular weight of 12 and 17 kDa, respectively (Fig. 1a), which were consistent with the predicted molecular weight of these proteins. Furthermore, Western blot analysis also confirmed that the purified recombinant proteins preserve functional domains of granulysin and perforin (data not shown). Thus, biochemical characterization suggested that macaque

GNL and PC proteins were successfully produced, and were ready for functional characterizations and molecular force determination.

Because human granulysin has been shown to have antibacterial effects, we first asked whether our recombinant monkey GNL had the similar antibacterial effects in vitro. To address this, *L. monocytogenes* (1.0×10^6 CFU/well) were selected to incubate with recombinant protein to determine the bacteriolytic effects of recombinant GNL and perforin. Bacterial colonies were then enumerated to determine the bacteriolytic effects of GNL and PC. Interestingly, as shown in Fig. 1b, the GNL, but not perforin, displayed remarkable antimicrobial activity by significantly decreasing bacterial colonies of *L. monocytogenes*. As a control, no significant bacteriolytic effect against *L. monocytogenes* was observed when the heat shock protein 70 (HSP70) was used to incubate with *L. monocytogenes* (Fig. 1b). Collectively, these pieces of data suggested that the GNL but not PC could specifically kill *L. monocytogenes*.

3.2. Granulysin but not perforin could specifically interact with *L. monocytogenes*

Given the fact that our GNL could specifically kill bacteria, we then asked the mechanisms underlying the bacteriolytic actions of granulysin. While the previous studies have suggested that electrostatic interaction [19] might play an important role for bacteriolytic ability of granulysin, the mechanisms associated with the electrostatic interaction between granulysin and bacteria surface alone cannot provide full explanation for the bacteriolytic ability of granulysin since a lot of proteins bearing positive net charges cannot efficiently lyse bacteria. Thus, there are might be other interaction forces between granulysin and bacterial cell surface governing the interactions between granulysin and bacterial cell surface for the bacteriolytic actions of granulysin. However, the possibility to determine molecular-force bases for the antimicrobial effects of recombinant granulysin cannot be tested by traditional biochemical and immunological approaches. To address this issue, we made use of our expertise of AFM-based single-molecule force measurement [21,22], and choose *L. monocytogenes* as a model prototype to determine the single-molecular force bases underlying the bacteriolytic actions of recombinant granulysin.

Because the rod-shape of bacteria such as *L. monocytogenes* are extremely difficult to be immobilized on the substrate in buffer for AFM images and AFM-based F–D measurements, micas were functionalized [22] so that *L. monocytogenes* could be immobilized on the substrate in buffer with minimum denaturation of the bacterial cells (Fig. 2a). After initial AFM imaging of the surface, individual bacterial cell was selected for AFM-based force–distance measurement to determine the binding force between granulysin and *L. monocytogenes*. As shown in Fig. 2b, two single *L. monocytogenes* could be clearly observed on the mica surface.

To assess the specificity of single-molecule force bases underlying the bacteriolytic effects of granulysin, recombinant PC protein was first used to probe the cell surfaces of *L. monocytogenes* (Fig. 2c). As shown in Fig. 2c, no interaction between PC and cell surface of *L. monocytogenes* was detected, indicating that PC itself interact very weakly with cell surface of *L. monocytogenes*. Consistently, bacteriolytic assay also indicated that PC protein alone has no ability to kill *L. monocytogenes*, as shown in Fig. 1b. In contrast, strong binding affinity between GNL and *L. monocytogenes* were demonstrated when GNL-functionalized AFM tips were used to probe the *L. monocytogenes* cell surface (Fig. 2d), indicating that GNL could specifically bind to cell surface of *L. monocytogenes*. Importantly, this piece of data suggested that GNL could specifically bind to *L. monocytogenes* cell surface to perform bacteriolytic actions rather than through non-specific accumulation or absorption on the *L. monocytogenes* cell surface.

To further determine the specificity for the interaction between granulysin and *L. monocytogenes*, we also probed the interaction between GNL and yeast cells. The round-shape yeast cells were trapped on polycarbonate filter membrane with a pore diameter ($\approx 8 \mu\text{m}$) very close to the diameter of yeast cells with minimum denaturation of yeast cells, as we and others previously described [20,21,26,30] (Fig. 2e). However, no specific interactions between GNL and yeast cells were observed when we used AFM tips functionalized with GNL with the same concentration used for probing *L. monocytogenes* cell surface (Fig. 2f), suggesting that GNL at this concentration has either no specific or very weak interaction force with yeast cells. Consistently, GNL at this concentration has no significant lytic ability against yeast cells (data not shown). These data therefore suggested the specificity and reliability of the system that we set up for the measurement of interaction force between granulysin and *L. monocytogenes*.

3.3. A single-molecular force basis for antimicrobial effects of recombinant GNL

Given the specificity and reliability of our system for probing the interaction between granulysin and *L. monocytogenes*, we then sought to determine the single-molecular force basis underlying the bacteriolytic actions of granulysin through using a combined method of AFM-based force–distance (F–D) measurement and Poisson statistics. The combined method of AFM-based F–D measurement and Poisson statistics has the advantage of discriminating the chemically specific and non-specific interaction forces and providing accurate individual molecule bond rupture forces with less noise [31–34]. The Poisson statistics using to decipher specific molecular force was developed based on the assumptions that the measured total adhesive force under AFM-based F–D measurement is composed of a finite number of discrete interacting molecular pairs [31–34]. Therefore, the distribution of the numbers of the interacting pairs should follow the Poisson distribution, and an equation can be derived as follows:

$$\sigma_m^2 = \mu_m F_i - F_i F_0 \quad (1)$$

where F_i is the individual molecule rupture force for the chemically specific interactions between granulysin and *L. monocytogenes*, and F_0 is the possible chemically non-specific and long-range interactions which can be derived from the slope and intercept of the linear regression curve of the variance (σ_m^2) versus the mean (μ_m) of the pull-off force from the measurements.

We underwent four different sets of AFM force measurements (each set composed of 46–66 AFM force measurements) for GNL-*L. monocytogenes* interactions at different locations on the surface of *L. monocytogenes*, as shown in Fig. 3a. The mean force and variance were then calculated based on Eq. (1). The results are summarized in Table 1. A histogram of the number of bonds from one set of the measurements (Set A in Table 1) and the theoretical curve of the Poisson distribution with the same mean are shown together in Fig. 3b. As shown in Fig. 3b, we found that the experimental AFM force data fit the theoretical Poisson distribution curve well, providing evidence demonstrating that the Poisson statistical method is suitable to determine the molecular-force basis for the interactions between GNL and *L. monocytogenes*. The force variance versus the mean force plotted from the data of AFM force measurement suggested that they had a good linear relationship (Fig. 3c). Notably, the individual rupture force (i.e. F_i) for the interaction between GNL and *L. monocytogenes* was calculated to be $\approx 22.5 \text{ pN}$. It is also worth to note that such interaction force (i.e. 22.5 pN) between GNL and *L. monocytogenes* is comparable to other interactions in biological systems which has very strong binding ability such as CD4 antigen and anti-CD4 antibody interaction that is about 25.45 pN [35]. While granulysin is regarded as an effective antimicrobial molecules secreted by some active immune cells such as CD8^+ and CD4^+ T

cells [14,36,37], such strong interaction force between granulysin and bacteria implicates a previously unknown biomechanical mechanism underlying the bacteriolytic effects of granulysin.

Most importantly, through the application of a combined method of AFM-based F–D measurement and Poisson statistics, the individual components of the overall interaction between GNL and *L. monocytogenes* could be broken into two fundamental parts: (i) chemically specific interactions and (ii) chemically non-specific interactions. Because there are no chemical reactions to be expected between GNL and cell surface of *L. monocytogenes*, the part for the chemically specific interaction force between GNL and *L. monocytogenes* should be attributed to hydrogen bonding [38]. Another part of interaction components between GNL and *L. monocytogenes* should therefore be attributed to van der Waals and electrostatic interactions. Thus, these results suggested that granulysin interacted with *L. monocytogenes* not only through electrostatic interaction but also through van der Waals and hydrogen bonding. It is worth to point out that van der Waals and hydrogen bonding between granulysin and bacteria were not expected by previous studies based on traditional biochemical or immunological approaches [11,19]. Thus, based on these results, we proposed a new model for the bacteriolytic effects of granulysin, as shown in Fig. 4. In this model (Fig. 4), we could see that granulysin might lyse the bacterial cells through hydrogen bonding, electrostatic and van der Waals interactions.

4. Conclusion

This work suggests that recombinant GNL but not PC could specifically lyse *L. monocytogenes*. Importantly, our AFM-based force analysis suggests that granulysin could specifically interact with bacteria not only using electrostatic interactions but also by van der Waals interactions and hydrogen bonding. Thus, this study uncovers a previous unknown mechanism for bacteriolytic effects of granulysin.

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Fig. 1.

Granulysin but not perforin specifically lysed *L. monocytogenes*. (a) SDS-PAGE analysis of bactericidal domain of macaque granulysin (GNL) and C terminal of perforin (PC). The recombinant proteins were purified by passing through a Ni-NTA agarose and S-protein agarose column. SDS-PAGE results showed that the each purified recombinant protein has the molecular mass consistent with its anticipated molecule. Lane 1: GNL (12 kDa); lane 2: PC (17 kDa); Marker: Pageruler Prestained Protein Ladder (Fermentas). (b) Detection of the bactericidal abilities of GNL against *L. monocytogenes*. Recombinant GNL (50 μ M), PC (50 μ M) or HSP70 (50 μ M) protein was incubated with 1×10^6 CFU of *L. monocytogenes*. Bactericidal effects of each protein were measured by the CFU assays. Data showed representative of at least three independent experiments. Error bars represent SEM. *** $p < 0.001$; NS, no statistical significance.

Fig. 2.

Granulysin but not perforin could specifically bind to *L. monocytogenes*, and granulysin could specifically bind to *L. monocytogenes* but not yeast cells. (a) Experimental design to measure the interaction forces between protein and bacteria surface. (b) A representative AFM topographic image showing two *L. monocytogenes* immobilized on mica surface in PBS buffer. (c) A representative force-distance (F-D) curve showing no interaction between PC and *L. monocytogenes*. (d) A representative F-D curve showing interaction between GNL and *L. monocytogenes*. (e) A representative AFM topographic image showing single yeast cell trapped in filter membrane. (f) A representative F-D curve showing no interaction between GNL and yeast.

Fig. 3.

A single-molecule force basis was elucidated by AFM for the bacteriolytic effects of granulysin. (a) Experimental scheme to determine the interaction force between granulysin and *L. monocytogenes* in PBS buffer at pH 7.4 (tip modified with GNL). A–D: Four different experimental sets for AFM F–D measurements. (b) Histogram of the number of bonds from one set of measurements (set A from a) between the granulysin-modified AFM tips and *L. monocytogenes* within a fixed contact area. (c) Force variance vs mean force for the granulysin-*L. monocytogenes* system acquired in PBS buffer at pH 7.4 (tip modified with GNL). Each point represents a data set taken with a different location on *L. monocytogenes*, as shown in (a).

Fig. 4.

A proposed model shows the bacteriolytic effects of granulysin. The charge cluster of the granulysin molecule in solution orients the granulysin toward the negatively charged surface of the bacterial cell (line with dashes). Granulysin may then bind to bacterial cells via electrostatic interactions, van der Waals force and hydrogen bonding. These interactions may allow granulysin to undergo conformation change or rolling to bring more charge amino acid of granulysin closer to bacterial cells. Furthermore, granulysin may then bend and tear the bacterial cell surface to lyse the bacterial cells.

Table 1

Poisson statistics-based results of binding force between granulysin and *L. monocytogenes*.

Set	Mean force μ_F (nN)	Variance σ_F^2 (nN ²)	Size of set N	Mean no. of bonds
A	0.047412698	0.000369	63	2.1
B	0.050719298	0.000609	59	2.25
C	0.041863636	0.000429	66	1.86
D	0.040934783	0.000291	46	1.82