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Inactivation of the budded virus of *Autographa californica* M nucleopolyhedrovirus by gloverin

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Abstract

Antimicrobial peptides are generated in insects exposed to pathogens for combating infection. Gloverin is a small cationic antibacterial protein whose expression is induced in the hemocytes and fat body cells of *Trichoplusia ni* larvae exposed to bacteria. The purpose of this study was to determine the role of gloverin during baculovirus infection. We found that gloverin expression is induced in *T. ni* systemically infected with the baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV). Two gloverin genes were cloned using RNA isolated from the hemocytes of *T. ni* larvae that were systemically infected AcMNPV budded virus (BV) and C-terminal 6x-His and V5 epitope tags were incorporated to facilitate gloverin isolation, detection and functional studies. The supernatants of *Sf9* cells stably transfected with the two gloverin expression plasmids and affinity purified gloverin proteins reduced the quantity of infectious AcMNPV BV as measured *in vitro* by plaque assay with untransfected *Sf9* cells. Nanomolar concentrations of affinity column purified gloverin protein caused calcein to be rapidly released from unilamellar vesicles comprised of phosphatidylglycerol, but not from vesicles made up of phosphatidylcholine, suggesting that gloverin interaction with membranes is rapid and affected by membrane charge. Both the BV inactivation and calcein release activities of gloverin increased with higher concentrations of gloverin. These results demonstrate that gloverin is an antiviral protein that interacts with vesicle membranes to cause the contents to be released.

Keywords

antiviral; gloverin; baculovirus; AcMNPV; *Autographa californica* M nucleopolyhedrovirus

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6. Conflict of Interest

The authors declare no conflict of interest.

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1. Introduction

Antimicrobial proteins are typically comprised of less than 200 amino acids and are key factors of insect innate immunity that function to combat infectious bacteria and fungi (Jiang and Vilcinskis, 2011). The majority of these small proteins are amphipathic and have a high number of positively charged amino acids that interact with the negatively charged phospholipids of microbial membranes (Peters et al. 2010). Once bound, the peptides increase membrane permeability and permit the efflux of ions to cause cell death (Ganz, 2003). Antimicrobial proteins produced by insects have been isolated from the hemolymph (blood plasma) of lepidopterans (Lavineet al. 2005) and include cecropin (Ourth et al. 1994), moricin (Hemmiet al. 2002), lebecin (Liu et al. 2000), melittin, tenecin 4 (Chae et al. 2011), attacin (Carlsson et al. 1991; Hultmark et al. 1983) and gloverin (Axen et al. 1997; Lundström et al. 2002; Mackintosh et al. 1998). Of these, cecropin and melittin exert an antiviral activity in cultured human cells infected with human immunodeficiency virus 1 (HIV-1) by suppressing viral gene expression (Wachinger et al. 1998). Gloverin expression is upregulated in *Plutellaxylostella* parasitized by an ichneumonid wasp harboring a polydnavirus (Etebari et al., 2011). However, there is little known of the roles antimicrobial proteins play during viral infection in insects.

The expression of antimicrobial peptides is upregulated in lepidopteran larvae exposed to a wide range of pathogens (Brown et al. 2009; Freitaket al. 2007; Huang et al., 2009; Silva et al. 2010; Wang et al. 2010a). Gloverin is produced by the fat body and hemocytes of *Trichoplusia ni* larvae as a pro-protein that is proteolytically processed in the hemolymph, removing a short signal peptide from the N-terminus to form the mature protein (Lundström et al. 2002). Micromolar concentrations of the pro- and mature gloverin proteins potently inhibit growth of the bacteria *Escherichia coli* (Axen et al. 1997; Lundström et al., 2002). Of note, a gloverin-like protein in *Helicoverpa armigera* is upregulated 3 h after the larvae are systemically infected with the budded virus (BV) of the baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV) (Wang et al. 2010a). There are two *gloverin* genes reported for *T. ni* which are highly expressed in larvae exposed to bacteria and encode proteins having predicted molecular weights of 18.9 kDa (Lundström et al. 2002) and 17.5 kDa (Freitak et al. 2007). To study the role of gloverin during baculovirus infection, both *gloverin* genes were cloned from RNA isolated from the hemocytes of AcMNPV-infected *T. ni* and the proteins were expressed in cultured *Sf9* cells. The gloverin proteins permeabilized vesicles and reduced the infectivity of AcMNPV BV, suggesting that gloverin is an antiviral protein that may act by disrupting the viral envelope.

2. Materials and Methods

2.1 Overview of Study Design

The experiments described herein were designed to study the potential antiviral activities of gloverin protein. Briefly, two *gloverin* genes were cloned from cDNA generated from RNA isolated from *Trichoplusia ni* larvae systemically infected with AcMNPV. To produce and isolate gloverin protein from *Sf9* cells, the resulting gloverin genes were epitope tagged and inserted into protein expression vectors. The antiviral activity of the purified gloverin protein was assessed by exposing AcMNPV budded virus to gloverin protein and subsequently quantifying the infectivity of the virus using a plaque assay with untransfected *Sf9* cells. To determine whether gloverin interacted with membranes and affected membrane permeability, calcein-encapsulated vesicles were incubated with purified gloverin protein and the amount of fluorescent calcein released from the vesicles was quantified.

2.2 Primer Design

PCR primers were designed to amplify two *T. ni gloverin* genes (GenBank accession numbers and AF233590.1 (*TnGlv1*) and EU016389.1 (*TnGlv2*); Table 1). Because of concerns with low levels of *TnGlv1* expression in *Sf9* cells, a Kozak translation initiation sequence was produced immediately after the start codon for the forward *TnGlv1* primer to increase protein expression in insect *Sf9* cells, as described in the pIB/V5-His vector manual (Invitrogen). This resulted in a nonsynonymous amino acid substitution of glutamine for valine in the second amino acid of *TnGlv1*. Reverse PCR primers were designed to remove the stop codon while maintaining the sequence in frame for fusion of a C-terminal V5 epitope and 6x-His tag.

2.3 RNA Isolation, RT-PCR and PCR

Total RNA was isolated using Trizol (Invitrogen) from the hemocytes of *T. ni* larvae intrahemocoelically inoculated as fourth instars with *AcMNPV-hsp70lacZ* BV or media vehicle (complete TNM-FH cell culture medium containing 10 % FBS) and subsequently treated with DNase (Sigma Aldrich), according the manufacturer product inserts. To produce cDNA from the isolated RNA, a reverse transcription polymerase chain reaction (RT-PCR) was performed using the Titan One-tube kit (Roche) and *GAPDH*- or *TnGlv2*-specific primers (30 cycles; primer sequences in Table 1), as described in the product insert. To determine whether viral or cellular DNA was present in the RNA isolated from *T. ni* larvae, RT-PCR was performed as described above using GoTaq Flexi DNA polymerase (Promega) or Titan One-tube kit enzyme that was heat inactivated (94 °C for 2 min). PCR products were analyzed using standard gel electrophoresis methods, visualized with a GelDoc XR gel documentation system (Bio-Rad) and analyzed using Quantity One software (Bio-Rad). The predicted molecular weight of the PCR products was 345 bp for *GAPDH* and 665 bp for *TnGlv2*. The band intensities for the *GAPDH* and *TnGlv2* RT-PCR products were quantified using Quantity One software (Bio-Rad) and fold difference in *TnGlv2* expression for vehicle and BV-injected samples calculated relative to the *GAPDH* expression in each sample. High Fidelity Platinum Taq polymerase (Invitrogen) was used to PCR amplify reverse transcribed cDNA for cloning *TnGlv1* and *TnGlv2*. Prior to cloning, PCR Clean-Up spin columns (Promega) were used to remove primers and unincorporated nucleotides.

2.4 Cloning *TnGlv1* and *TnGlv2*

TnGlv1 and *TnGlv2* cDNA was ligated into the pENTR3D-TOPO TA vector as described by the manufacturer (Invitrogen). One Shot TOP10 chemically competent *E. coli* (Invitrogen) were transformed with the resulting plasmids to generate the entry clones pENTR-*TnGlv1* and pENTR-*TnGlv2*. Chemically transformed cells were plated onto solid Luria broth (LB) media containing kanamycin (50 µg/ml), incubated overnight at 37 °C, colonies selected and cultured overnight in liquid LB containing kanamycin (50 µg/ml). Plasmid DNA was isolated from liquid cultures using QIAprep Spin Mini Prep Kit (Qiagen) and screened for the *TnGlv1* or *TnGlv2* inserts using PCR with the M13 forward primer and *TnGlv1* or *TnGlv2* specific reverse primers. Positive clones with inserts of the correct size for *TnGlv1* or *TnGlv2* were sequenced. Entry clones with the highest similarity (> 97 %) to the published GenBank sequences were selected for recombination with pIB/V5-His-DEST vector (Invitrogen). One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) cells were transformed as described by the manufacturer to produce the expression vectors pIB/V5-His-DEST-*TnGlv1* and pIB/V5-His-DEST-*TnGlv2*. Colonies were selected using LB media containing ampicillin (100 µg/ml), screened using PCR with *OpIE2* forward primers and reverse primers specific for *TnGlv1* or *TnGlv2*. The resulting positive clones, pIB/V5-His-DEST-*TnGlv1* c112.c and pIB/V5-His-DEST-*TnGlv2* c110.a, were sequenced to ensure the

gloverin expression plasmids were correctly constructed and did not contain any unintended mutations.

2.5 Transfection and gloverin expression

Sf9 cells cultured in complete TNM-FH media containing 10 % FBS (Lonza) were transfected with pIB/V5-His-DEST-*TnGlv1* c112.c or pIB/V5-His-DEST-*TnGlv2* c110.a using Cellfectin II Reagent (Invitrogen) and stably transfected cells selected and maintained using Blasticidin S (InvivoGen), as described by the manufacturer. *TnGlv1* and *TnGlv2* protein expression was analyzed using standard SDS-PAGE and western blot methods. Molecular weight markers were ProSieveQuadColor Protein Marker (Lonza) and/or Precision Plus Protein WesternC Standards (Bio-Rad). Mini Protean TGX precast 4–20 % gels (Bio-Rad) were run at 180 V for 30 min, the proteins transferred to Immobilon Transfer Membranes (Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) run at 20 V for 35 min. The resulting blots were stained with Ponceau S to visualize the quantity of protein transferred. The membranes were subsequently destained and probed using the Anti-V5 Epitope Tag Mouse Monoclonal Antibody (Pierce) and Goat Anti-Mouse IgG (H+L) HRP Human Adsorbed Antibody (Southern Biotech). HRP-reactive bands were developed using the chemiluminescence substrate SuperSignal West Pico (Pierce) and the blot imaged using a Nikon D700 digital single-lens reflex camera, as previously described (Khoury et al. 2010). The colors of the resulting images were inverted to generate black-colored bands with a white background, straightened and cropped using Adobe Lightroom 3 software. No other modifications were made to the blot images. The relative quantity of His-tagged protein present in blots was assessed by measuring the intensity and area of chemiluminescent bands using Quantity One software (Bio-Rad), as described by the manufacturer.

2.6 Gloverin protein purification

Sf9 cells stably transfected with pIB/V5-His-DEST-*TnGlv1* c112.c and pIB/V5-His-DEST-*TnGlv2* c110.a were cultured for 96 h in a shaking incubator (125 rpm, 28 °C, 300 ml, $1-2 \times 10^6$ cells/ml) and subsequently frozen at -80 °C. Immediately after thawing, the serine protease inhibitor PMSF was added (1 mM), the culture media centrifuged ($3000 \times g$, 30 min), the supernatant (SN) decanted, the cell pellet suspended in 5 ml of PBS and solubilized for 10 min with cell lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10 % glycerol, 1 % NP-40), the lysed cells centrifuged ($10,000 \times g$, 15 min) and the resulting SN combined with the culture media SN. The combined SN was concentrated 20-fold at 4 °C using a Minimate TFF Omega 3 kDa MWCO Membrane Filter (Pall Corporation) and Varistaltic Pump Plus (Vera). His-tagged gloverin protein was isolated from the concentrated SN (cSN) using His-Pure Ni-NTA 3mL Spin Columns (Thermo Scientific). Proteins not tightly bound to the Ni-NTA resin were removed with three sequential washes of two resin-bed volumes of 25 mM imidazole. His-tagged proteins that remained bound to the column were eluted twice with one resin-bed volume of 250 mM imidazole and a third time with 300 mM imidazole. The resulting elutions were combined, dialyzed overnight at 4 °C in PBS using 2 kDa MWCO Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific), concentrated 10-fold further using 3 kDa MWCO Ambicon centrifugal filter devices (Millipore) and filtered using a 0.45 µm syringe filter. Protein concentration was quantified using the BCA assay (Thermo Scientific), as described by the manufacturer. Western blot was used as described above to assess the quantity of His-tagged protein present in the material that flowed through the Ni-NTA columns after applying the concentrated supernatant, washes and elutions.

2.7 Virus inactivation assay

The antiviral activity of *TnGlv1* and *TnGlv2* protein was quantified using standard plaque assay with untransfected *Sf9* cells. *AcMNPV-hsp70/lacZ* BV was serially diluted in TNM-FH media containing 10 % FBS (vehicle), the cell supernatants of *Sf9* cells transfected with pIB/V5-His-DEST-*TnGlv1* c112.c or pIB/V5-His-DEST-*TnGlv2* c110.a, or affinity column purified *TnGlv1* or *TnGlv2* protein. The diluted BV was incubated for 3 h at 28 °C and plaque assayed using untransfected *Sf9* cells. Briefly, 10 µl of the incubated BV was added to the wells of a 96-well plate containing a confluent layer of *Sf9* cells and 40 µl of TNM-FH media (4–6 wells per BV dilution). After incubation for 1 h at 28 °C, the inocula was removed, methyl cellulose (1.5 g/ml) dissolved in TNM-FH media added to each well and the plate incubated in a humidified chamber at 28 °C. After 3 days, the cells were fixed with formaldehyde, rinsed three times with PBS, processed using X-gal to develop the blue lacZ-positive plaques, as previously described (Chikhalya et al., 2009) and the plaques counted using a Leica MZ12.5 stereomicroscope. This experiment was repeated at least three times on separate days and the resulting data combined to calculate the mean and SEM for each treatment. For one replicate experiment, the wells of the X-gal developed plate were imaged using a MicroPublisher 3.3 cooled CCD digital color camera (QImaging) mounted to a Leica MZ12.5 stereomicroscope and QCapture Suite Imaging software (QImaging). To evaluate the concentration dependent activity of gloverin protein, decreasing concentrations of Ni-NTA purified *TnGlv1* or *TnGlv2* protein or vehicle (complete TNM-FH media) was incubated for 3 h in the presence of *AcMNPV-hsp70/lacZ* BV (10^5 PFU) and the resulting PFU quantified using plaque assay as described above. Statistical analysis was conducted using Prism 5.0d (GraphPad).

2.8 Calcein release from vesicle assay

The calcein release from vesicle assay employs a self-quenching dye (*i.e.* calcein) to quantify vesicle leakage. It is a long-standing method for measuring pore-formation and the permeabilizing activity of antimicrobial peptides (Matsuzaki et al., 1995; Prenner et al., 2001; Patel et al., 2009). Five mg of egg phosphatidylglycerol (PG) or egg phosphatidylcholine (PC) (Avanti Polar Lipids Inc.) was dissolved in 0.5 mL 3:1 chloroform/methanol (v/v) in phosphate-free glass tubes washed with Contrad 70 (Decon Labs) and dried with a stream of N₂ gas. The lipid film was suspended in Buffer A (20 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, pH 7.4) containing 100 mM calcein (Sigma-Aldrich). After rigorous vortexing for 1 min, the lipid suspension was extruded with a mini extruder (Avanti Polar Lipids Inc) at 45 °C through a membrane with a pore size of 100 nm to form unilamellar vesicles with encapsulated calcein. The vesicles containing trapped calcein were then separated from free calcein using a PD-10 desalting column (GE Healthcare). Phospholipid concentrations were determined using the Ames phosphate assay (Ames, 1966). Changes in the fluorescence emission intensity, resulting from membrane leakage of entrapped calcein, was measured at 0.01 min intervals using a LS 55 Fluorescence Spectrometer (PerkinElmer). The excitation and emission wavelength were set at 490 and 520 nm, respectively (slit width 5 nm). The fluorescence emission intensity was continuously monitored for 12 min. Vesicles (10 µM) were equilibrated for 2 min in a 1 mL reaction cuvet containing Buffer A at 37 °C, after which the protein was added at varying lipid-protein molar ratios (100:1 to 10,000:1), which corresponded to 1 – 100 nM of protein. The percentage of protein-mediated calcein release from vesicles was quantified by measuring the fluorescence intensity change after gloverin addition, and correction by subtracting the baseline fluorescence intensity at 2 min. Maximum calcein release was determined by the fluorescence intensity change after addition of detergent (Triton-X-100, 0.1 % final concentration). This resulted in the complete solubilisation of the phospholipid vesicles and the fluorescence intensity is then associated with fully dequenched calcein. The following formula was used to calculate the calcein released from the vesicles as a

percentage of the maximum: $R = [(I_p - I_2)/(I_{max} - I_2) * 100]$. R is calcein released from the vesicles as a percentage of the maximum; I_p is the fluorescent intensity of protein-mediated calcein release, I_2 is the calcein fluorescence intensity at 2 min, before protein was added; I_{max} is the calcein fluorescence intensity at 10 min, after the detergent was added. Each experiment was conducted at least three times to generate the mean value \pm standard deviation.

2.9 Illustration Software

Illustrator and Photoshop CS5 software (Adobe) was used to construct the illustration of the hypothesized mechanisms for the antiviral activity of gloverin.

3. Results

3.1 Cloning *TnGlv1* and *TnGlv2* for protein expression

To study the role of gloverin during baculovirus infection, two *gloverin* genes were cloned in top ENTR3D-TOPO TA vectors using cDNA generated in a RT-PCR reaction from RNA isolated from the hemocytes of *T. ni* larvae infected with *AcMNPV-hsp70/lacZ* BV. These two gloverin genes are distinct (Figure 1A), however the gene names submitted to GenBank do not easily distinguish between the two. Therefore, we refer to GenBank accession number AF233590.1 as *TnGlv1* and EU016389.1 as *TnGlv2*. PCR analysis of the resulting vectors (pENTR-*TnGlv1* c112 and pENTR-*TnGlv2* c110) using M13 forward and reverse primers showed single bands near the predicted sizes of 702 bp and 665 bp, respectively (Figure 1B), indicating that *TnGlv1* and *TnGlv2* were correctly inserted into the pENTR3D-TOPO TA vectors. To generate gloverin-containing vectors for protein expression in insect cells and to fuse V5 and 6x-His tags to the cloned *TnGlv1* and *TnGlv2*, pENTR-*TnGlv1* c112 and pENTR-*TnGlv2* c110 were recombined with pIB/V5-His-DEST vectors to generate pIB/V5-His-DEST-*TnGlv1* c112.c and pIB/V5-His-DEST-*TnGlv2* c110.a.

Computational analysis of the predicted amino acid sequences of *TnGlv1* and *TnGlv2* using the Membrane Protein Explorer (MPEx) algorithm (<http://blanco.biomol.uci.edu/mpex/>) (Snider et al. 2009) indicate that both proteins may have a transmembrane domain contained within the putative signal peptide near the N-terminus (Figure 1A). The predicted molecular weights of the *TnGlv1* and *TnGlv2* protein fused to the V5/6x-His tags are 25.2 kDa and 23.5 kDa, respectively.

PCR analysis and sequencing of pIB/V5-His-DEST-*TnGlv1* c112.c and pIB/V5-His-DEST-*TnGlv2* c110.a demonstrated that: *TnGlv1* and *TnGlv2* were inserted in the correct orientation in top IB/V5-His-DEST, the 6x-His and V5 tags were correctly fused, the stop codons prior to the 6x-His and V5 tags were removed, greater than 97 % of the nucleotides of *TnGlv1* and *TnGlv2* were unchanged from the sequences reported in NCBI. Additionally, sequence analysis demonstrated that there were no unintended nonsynonymous base substitutions that changed the predicted amino acid sequence (not shown).

We attempted to express *TnGlv1* and *TnGlv2* protein in bacteria by recombining pENTR-*TnGlv1* c112 and pENTR-*TnGlv2* c110 with the arabinose-inducible pBAD-DEST49 bacteria expression vector. While bacteria were stably transformed with the resulting gloverin expression vectors and could be cultured in the absence of arabinose, the transformed bacteria did not grow robustly under standard inducing conditions, suggesting that *TnGlv1* and *TnGlv2* may be toxic to bacteria (not shown).

3.2 Isolating TnGlv1 and TnGlv2 protein

TnGlv1 and *TnGlv2* protein was isolated from the supernatant and solubilized cell pellet of *Sf9* cells stably transfected with pIB/V5-His-DEST-*TnGlv1* c112.c or pIB/V5-His-DEST-*TnGlv2* c110.a. The highest quantity of gloverin was present in the cell culture supernatant (not shown), suggesting that the V5-His-tagged gloverin proteins were secreted from transfected *Sf9* cells. While gloverin-expressing *Sf9* cells cultured in serum-free SFX medium grew robustly to high densities, high quantities of gloverin were not detected in the cell pellets or supernatants (not shown). This suggests that complete TNM-FH culture medium containing 10 % FBS was required for high expression and/or stability of *TnGlv1* and *TnGlv2* in transfected *Sf9* cells.

Because the *Sf9* cells efficiently produced stable *TnGlv1* and *TnGlv2* protein only when cultured in complete media, the concentrated supernatant (cSN) contained high quantities of serum proteins, as indicated by the dark red-stained spots on the Ponceau S-stained blot (Supplementary Figure 1A). As a result, we could not detect the V5-tagged *TnGlv2* on the blot with the anti-V5 antibody in the cSN (Supplementary Figure 1A 1B). Similar results were observed for *TnGlv1* (not shown). When Ni-NTA columns were used to isolate the 6X-His tagged gloverin proteins, the serum proteins were removed by the third wash of the column (W3) as indicated by the lack of dark-red spots in the W3 lane of the Ponceau S-stained blot (Supplementary Figure 1A). A high quantity of *TnGlv2* was observed in the first two elutions of the column (E1 and E2; Supplementary Figure 1B). Multiple distinct low molecular weight bands that were reactive with the mouse anti-V5 antibody were detected in the eluted *TnGlv2* (Supplementary Figure 1B) and *TnGlv1* (not shown), suggesting that some protein was degraded. To estimate the relative quantity of degradation in the eluted protein, densitometry was used to quantify the chemiluminescence and area of each band on the blot containing the eluted gloverin protein (Supplementary Figure 1B). We found that the large band of the molecular weight expected for V5/His-tagged *TnGlv2* (23.5 kDa) comprised 32 % of the epitope-tagged protein for elution 1 (E1) and 44 % for elution 2 (E2). Very little epitope-tagged protein was detected in elution 3 (E3). Because a serine protease inhibitor (PMSF) was added at each step of protein isolation, these low molecular weight bands may represent gloverin that was cleaved by proteases while the transfected cells were cultured or during protein isolation by non-serine proteases that were not inhibited by PMSF.

A western blot with known quantities of Ni-NTA affinity column purified gloverin was used to estimate the concentrations of *TnGlv1* and *TnGlv2* in the serum-containing supernatants (Figure 1C). Comparing the chemiluminescence intensity and area of the gloverin bands for each sample (Figure 1C) with the known loaded quantity of Ni-NTA column purified gloverin (85 µg/ml of *TnGlv1* and 525 µg/ml of *TnGlv2*), there was approximately 22 µg/ml of *TnGlv1* and 40 µg/ml of *TnGlv2* in the serum-containing supernatants. While gloverin protein isolated with the Ni-NTA column may have been degraded as it was produced by *Sf9* cells or during protein isolation with affinity chromatography (Supplementary Figure 1B), band densitometry demonstrated that the final dialysis step used to purify gloverin removed most of the smaller molecular weight epitope-tagged protein (Figure 1C; 86 % and 77 % of *TnGlv1* and *TnGlv2*, respectively, was of the predicted molecular weight for gloverin that was not degraded). Both *TnGlv1* and *TnGlv2* protein was present on the western blot at molecular weights that were approximately 2 kDa less than what was predicted from the amino acid sequences (Figure 1C).

3.3 Inactivation of BV by TnGlv1 and TnGlv2

To determine if *TnGlv1* or *TnGlv2* could inactivate baculovirus, *AcMNPV-hsp70/lacZ* BV was incubated for 3 h in the presence of supernatants containing unpurified *TnGlv1* (1 µM)

or *TnGlv2* (2 μM) or Ni-NTA affinity column purified *TnGlv1* (4 μM) or *TnGlv2* (4 μM). Approximately 0.03 mg/ml of gloverin (2.17 μM) was isolated from *H. gloveri* pupae injected with gram-negative bacteria (Axen et al., 1997), which is similar to or greater than the gloverin concentrations used for these studies. To aggregate results from multiple experiments ($N \geq 3$), the observed BV plaque forming units (PFU) for each treatment was normalized to the vehicle treatment for each experiment, which was set to 100% infectious BV (Figure 2A). When BV was incubated with gloverin-containing supernatants, the quantity of infectious BV, as measured by PFU, was reduced to $26.6 \pm 3.4\%$ and $8.3 \pm 1.6\%$ for *TnGlv1* and *TnGlv2*, relative to that of BV incubated in the presence of vehicle (Figure DAB). A higher quantity of infectious virus was detected for BV incubated in the presence of *TnGlv1* and *TnGlv2* purified using Ni-NTA affinity columns ($60.8 \pm 4.5\%$ and $36.3 \pm 5.8\%$ of vehicle, respectively; Figure 2A). These results suggest that *TnGlv1* and *TnGlv2* inactivate baculovirus BV *in vitro* and that *TnGlv2* has greater antiviral activity than *TnGlv1*. The observation that purified gloverin protein had lower activity relative to unpurified gloverin present in cell culture supernatants suggests that the protein purification process may have affected gloverin activity or that additional elements in the supernatants may exert antiviral activity.

To determine whether the activity of gloverin was concentration dependent, increasing concentrations of affinity column purified *TnGlv1* or *TnGlv2* were incubated for 3 h with *AcMNPV-hsp70/lacZ* BV (10^5 PFU) and the quantity of infectious BV quantified using plaque assay. The results were normalized to the quantity of infectious BV present when *AcMNPV-hsp70/lacZ* BV (10^5 PFU) was incubated for 3 h in the presence of vehicle (TNM-FH media). While there was no observable difference between vehicle and low concentrations of *TnGlv1* (0.008 μM and 0.4 μM ; Figure 2C), low concentrations of *TnGlv2* moderately reduced the quantity of infectious BV ($82.0 \pm 12.3\%$ for 0.008 μM and $85.0 \pm 11.8\%$ for 0.04 μM ; Figure 2C). In contrast, higher concentrations of *TnGlv1* and *TnGlv2* produced substantial reductions (*TnGlv1*: $46.1 \pm 14.3\%$ for 4 μM and $87.5 \pm 8.8\%$ for 0.8 μM ; *TnGlv2*: $49.2 \pm 5.5\%$ for 4 μM and $76.5 \pm 19.1\%$ for 0.8 μM ; Figure 2C). As the concentration of *TnGlv1* or *TnGlv2* was increased, the quantity of infectious BV decreased (Figure 2C), suggesting that the antiviral activity of gloverin was concentration dependent.

3.4 Vesicle lysis by gloverin

To determine whether gloverin could interact with membranes and induce changes in membrane permeability, unilamellar vesicles encapsulated with the fluorescent probe calcein were incubated with affinity column purified *TnGlv1* or *TnGlv2* and the amount of calcein released quantified. The maximum release of calcein from the vesicles was determined by solubilizing the vesicles with detergent 10 min after gloverin addition. This is a long-standing method to assess the pore-forming activity of antimicrobial peptides (Matsuzaki et al., 1995; Prenner et al., 2001; Patel et al., 2009). Phosphatidylglycerol (PG) vesicles were used to evaluate the interaction of gloverin with negatively charged membranes. Nanomolar concentrations of *TnGlv1* and *TnGlv2* caused greater than 85% of the encapsulated calcein to be released from PG vesicles within two minutes of being added to the reaction cuvette (*TnGlv1*: $87.3 \pm 2.1\%$ for 100nM; *TnGlv2*: $95.6 \pm 3.7\%$ for 10nM; Figure 3). Decreasing the concentrations of gloverin resulted in proportional decreases in calcein released from the PG vesicles (*TnGlv1*: $69.5 \pm 6.1\%$ for 33 nM and $21.4 \pm 1.5\%$ for 3.3 nM; *TnGlv2*: $42.4 \pm 4.5\%$ for 3.3 nM and $20.1 \pm 4.5\%$ for 1.0 nM; Figure 3A), suggesting that the vesicle perforation activity of gloverin was concentration dependent. When the quantity of calcein released from PG vesicles was compared for similar concentrations of *TnGlv1* and *TnGlv2* (ex. 3.3 nM), two to three times more calcein was released from vesicles incubated with *TnGlv2* (Figure 3A). The release of calcein from PG vesicles was almost completed within 30 s after addition of *TnGlv1* or *TnGlv2* and was close

to maximum within 2 min after addition of protein (Figure 3B 3C), suggesting that the interaction between gloverin and PG vesicles occurred rapidly and with high efficiency. The higher pore-forming activity of *TnGlv2* relative to *TnGlv1* (Figure 3A) correlated with the relatively higher antiviral activity of *TnGlv2* (Figure 2A).

Vesicles comprised of phosphatidylcholine (PC) and encapsulated with calcein were used to determine if the phospholipid charge affected the interaction between gloverin and the membrane. In contrast to PG, the PC impart a neutral charge to the vesicles. Three separate trials using high concentrations of *TnGlv1* and *TnGlv2* (100nM) incubated with PC vesicles showed no or less than 2 % of the calcein released during the 8 min incubation period (Figure 3D 3E).

3.5 *TnGlv2* expression in *T. nilarvae*

Expression of *TnGlv2* in hemocytes isolated from *T. ni* 12 h after inoculation with 500 PFU of *AcMNPV-hsp70/lacZ* BV or vehicle (TNM-FH media) was analyzed using semi-quantitative RT-PCR. When normalized to *GAPDH* expression, *TnGlv2* expression was increased 5.7-fold in hemocytes isolated from larvae inoculated with BV relative to vehicle-inoculated larvae (Figure 4). Control amplifications using only Taq DNA polymerase or heat-inactivated reverse transcriptase and *GAPDH* or *TnGlv2* primers did not amplify the template (Supplementary Figure 2) demonstrating the absence of contaminating DNA in the RNA isolated from *T. ni* hemocytes.

4. Discussion

Although the antibacterial activity of gloverin is well appreciated, understanding its impact on viruses is of interest because gloverin expression is upregulated in lepidopteran larvae systemically infected with baculoviruses (Wang et al., 2010a). In the present study, we demonstrate that gloverin reduced the infectivity of baculovirus BV *in vitro* and that gloverin perforated vesicles. Together, these results suggest that gloverin inactivates BV by disrupting the viral membrane surrounding the nucleocapsid (Figure 5).

Gloverin proteins are small, less than 200 amino acids in size, and their expression is potently stimulated in several species of lepidopteran larvae and pupae infected with bacteria (Axen et al., 1997; Brown et al., 2009; Kawaoka et al., 2008; Lundström et al., 2002; Mackintosh et al., 1998; Silva et al., 2010; Zhou et al., 2008). The induction of gloverin expression is higher in larvae inoculated intrahemocoelically with the Gram-negative bacteria *E. coli*, compared to challenge with the fungus *Candida albicans* (Kawaoka et al., 2008). Gloverin and gloverin-like proteins inhibit growth of *E. coli* (Lundström et al., 2002) but have little effect on Gram-positive bacteria or fungi (Mackintosh et al., 1998). Fat body cells and hemocytes mediate essential immune system functions and both express gloverin in response to infection by bacteria (Lundström et al., 2002; Tamezguerra et al., 2008). Although a high quantity of gloverin is present in the hemolymph of insects (Axen et al., 1997) and in the medium of *Sf9* cells transfected with gloverin-expressing plasmids (Figure 1C), this does not appear to negatively affect cell growth.

As with many antimicrobial proteins, gloverin is produced as a pro-protein that contains a N-terminal signal peptide that is proteolytically removed to produce the mature form (Axen et al., 1997). Computational analysis of *TnGlv1* and *TnGlv2* suggest that the signal peptide of these proteins contain a transmembrane domain (Figure 1A). When expressed in *Sf9* cells, the N-terminal signal peptide of gloverin is not removed (Lundström et al., 2002). However, gloverin cloned from *T. ni* and expressed in *Sf9* cells displays a high antibacterial activity that is similar to the mature form isolated from *Hyalophora gloveri* larvae (Lundström et al.,

2002), suggesting that removing the signal peptide is not essential for the antibacterial activity. When the total peptide content of cell-free hemolymph isolated from *Galleria mellonella* was analyzed using liquid-chromatography mass spectrometry, the region containing the N-terminal signal peptide was detected (Brown et al., 2009), suggesting that the pro-protein is secreted into the hemolymph and may be active during the immune response against bacteria.

The 6x-His and V5 epitope tags that were engineered into the C-termini of *TnGlv1* and *TnGlv2* are widely employed individually or in combination to isolate proteins for functional studies. The 6x-His tag is comprised of 6 histidine amino acids. When incorporated into the C- or N-termini of proteins, the His tag is reported to not significantly affect protein structure (Carson et al., 2007). The V5 epitope sequence is formed from 14 amino acids of the RNA polymerase subunit from simian virus 5 (Southern et al. 1991). Although inserting the V5 epitope tag into the middle of the coding region of polymerases can reduce the polymerase activity (Shi and Elliott, 2009), the V5 epitope has been successfully engineered into the C- and N-termini of proteins without affecting protein function (Alarcon-Chaidez et al. 2003; Morais and Costa, 2003; Oezbey et al. 2007). However, because there is no means for removing the V5/6x-His epitope tag from the gloverin proteins that were expressed in *Sf9* cells, it remains possible that the biological activity of the proteins was affected.

The predicted amino acid sequences of *TnGlv1* and *TnGlv2* show that the proteins have 27 and 29 positively charged arginine or lysine amino acids, respectively, which impart an overall net positive charge to the proteins. The outer membranes of Gram-negative bacteria, including *E. coli*, contain lipopolysaccharide (LPS) that increases the negative charge of the outer membrane. The specificity of gloverin to Gram-negative bacteria is hypothesized to result from electrostatic interactions between the positively charged gloverin and negatively charged LPS (Axen et al., 1997). Once attached to the outer membrane of *E. coli*, gloverin is reported to stimulate a reduction in the biosynthesis of the outer membrane proteins OmpA and OmpF without affecting the production of other proteins (Axen et al., 1997). OmpA is a multifunctional protein that aids in maintaining membrane structure and normal cell shape (Sonntag et al., 1978) while also contributing to the initial steps of bacterial adhesion and invasion into tissues (Prasadarao et al., 1996). OmpF is a porin that forms a large beta-barrel to allow passive diffusion of molecules across the outer membrane (Nikaido, 2003). However, the down regulation of OmpF and OmpA observed for *E. coli* exposed to gloverin (Lundström et al., 2002) may instead be a response initiated by the microbe to limit the efflux of intracellular contents and prevent cell lysis.

The work presented in Axen *et al* (1997) is consistent with hypothesis that gloverin binds to LPS and inhibits outer membrane protein synthesis to increase membrane permeability. However, our results demonstrate that gloverin reduces the infectivity of baculovirus BV *in vitro* and acts alone to permeabilize membranes. The BV membrane is generated from the plasma membrane of an infected cell as the virus buds and is released. Because BV is metabolically inactive and eukaryotic cells lack known homologs of OmpA and OmpF, it is unlikely that antiviral activity of gloverin only results from an inhibition of protein synthesis. Many antibacterial proteins, including defensins produced by vertebrates (Wimley et al. 1994) and cecropins produced by insects (Christensen et al. 1988), act against microbes by disrupting membranes and forming pores (Peters et al. 2010). Although gloverin contains a transmembrane domain, it is removed if the signal peptide is cleaved from gloverin. However, the transmembrane domain of gloverin may not be essential to form pores because defensin binds to the outer membrane of microbes to cause membrane strain and pores to form that permit molecules to leak across the membrane (Ganz, 2003). Additionally, the human alpha defensin HNP-1 lacks a transmembrane domain, yet it

inactivates several enveloped viruses (Daher et al. 1986). Whether or not gloverin retains the transmembrane signal peptide, the overall positive charge of gloverin may allow it to accumulate on the surface of microbes or enveloped viruses to cause membrane strain and pores to form. Pores in the envelope of baculovirus BV may allow small proteins or molecules to leak across the viral envelope or disrupt the assemblage of envelope-associated proteins (Figure 5).

The interaction of positively charged gloverin with AcMNPV BV may be driven by charge-based interactions between gloverin and the BV envelope protein GP64 (Figure 5). GP64 is one of the three most abundant proteins that are present in AcMNPV BV (Wang et al., 2010b). GP64 is responsible for attaching BV to the cell surface and mediating fusion of the BV envelope with the endosome membrane to cause entry of the virus into the cell (Blissard and Wenz, 1992). The region of GP64 displayed on the surface of BV (amino acids 21–483) has a predicted isoelectric point (pI) of 5.59, indicating that this region has an overall negative charge at a physiological pH and may interact with the positively charged gloverin. The measured pI of gloverin isolated from *H. gloveri* pupae is 8.5 (Axen et al., 1997) while the pH of hemolymph in lepidopteran larvae was measured at 6.45 and 6.8 (Wang and Haunerland, 1993; Wyatt, et al., 1956). The crystal structure of post-fusion GP64 places two clusters of negatively charged amino acids (aspartic acid and glutamic acid) on the solvent-exposed surface of GP64 and near the transmembrane domain (residues 114–133 and 410–418) (Kadlec et al. 2008). The negatively charged amino acids of residues 114–133 are contained within the fusion domain of GP64 (residues 67–166); the fusion domain plays a crucial role in triggering fusion between viral and cellular membranes (Kadlec et al., 2008). Gloverin binding to the fusion domain of GP64 may restrict the protein movements required for membrane fusion, thereby reducing BV infectivity. Alternatively, this charge-based interaction may direct gloverin to the surface of BV to cause membrane strain or pores to form that disrupt the BV envelope and reduce infectivity of the virus (Figure 5). Future studies of truncated gloverin and GP64 will provide detailed analysis of the mechanisms that mediate the reduced infectivity of BV exposed to gloverin.

Gloverin expression is induced in response to infection and reduces the growth of Gram-negative bacteria and the infectivity of baculovirus BV. The current models proposes that gloverin inhibits the synthesis of specific proteins localized to the outer membrane of bacteria (Lundström et al., 2002). Because gloverin also displays antiviral activity and perforates vesicles, we suggest an alternative mode of action in which gloverin strains the BV envelope to form pores (Figure 5), and in this regard, may be mechanistically similar to defensin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Gloverin expression is upregulated in *Trichoplusia ni* infected with the baculovirus AcMNPV.

Gloverin protein reduced the infectivity of AcMNPV budded virus.

Vesicles comprised of phosphatidylglycerol were perforated by gloverin.

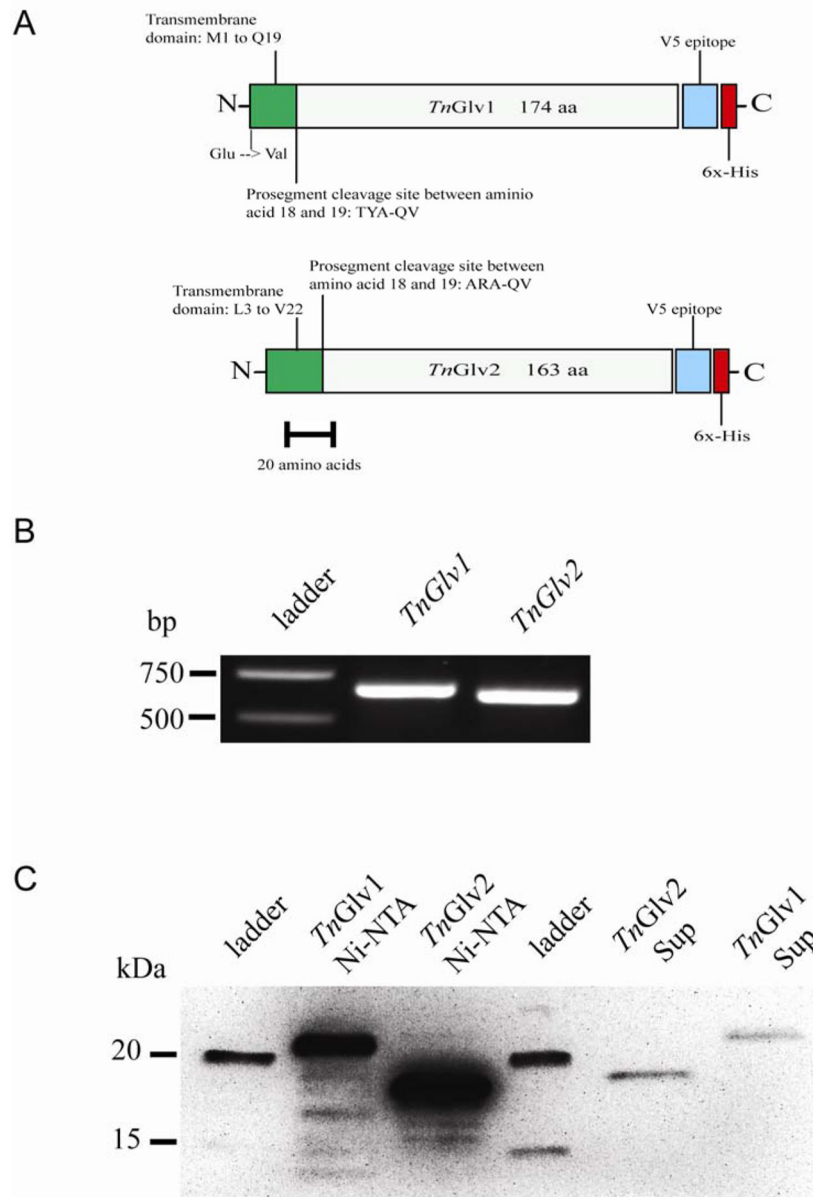


Figure 1. Cloning and expression of *TnGlv1* and *TnGlv2*

(A) Diagram of *TnGlv1* (top) and *TnGlv2* (bottom) with the N-terminal signal peptide, putative transmembrane domains noted, and locations of C-terminal 6x-His and V5 epitope tags. (B) PCR analysis of the cloned *TnGlv1* and *TnGlv2* inserted into expression vectors. Bands of the predicted molecular weights were observed for *TnGlv1* and *TnGlv2* inserted in top ENTR-*TnGlv1* c112 and pENTR-*TnGlv2* c110 TA vectors (702 bp and 665 bp, respectively) using PCR with M13 forward and reverse primers. (C) Western blot of *TnGlv1* and *TnGlv2* protein present in the supernatants of *Sf9* cells stably transfected with gloverin expression vectors (*TnGlv1* Sup, *TnGlv2* Sup) and of Ni-NTA affinity column purified gloverin protein (*TnGlv1* Ni-NTA and *TnGlv2* Ni-NTA; 60 s exposure).

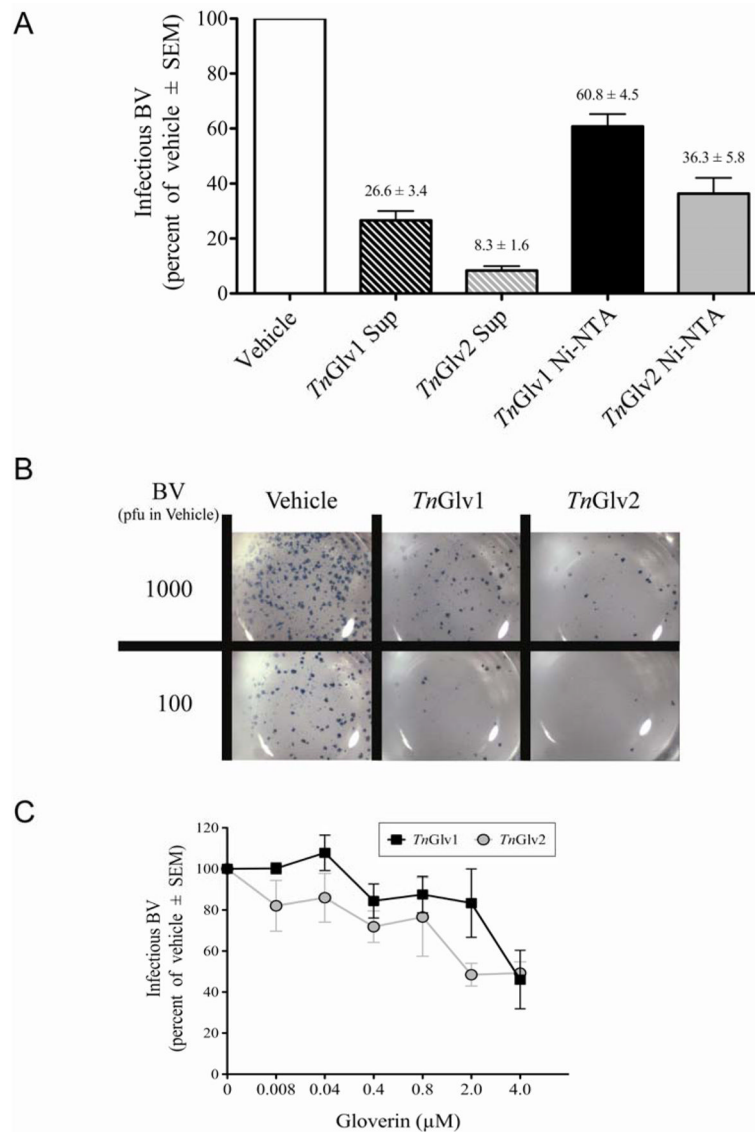
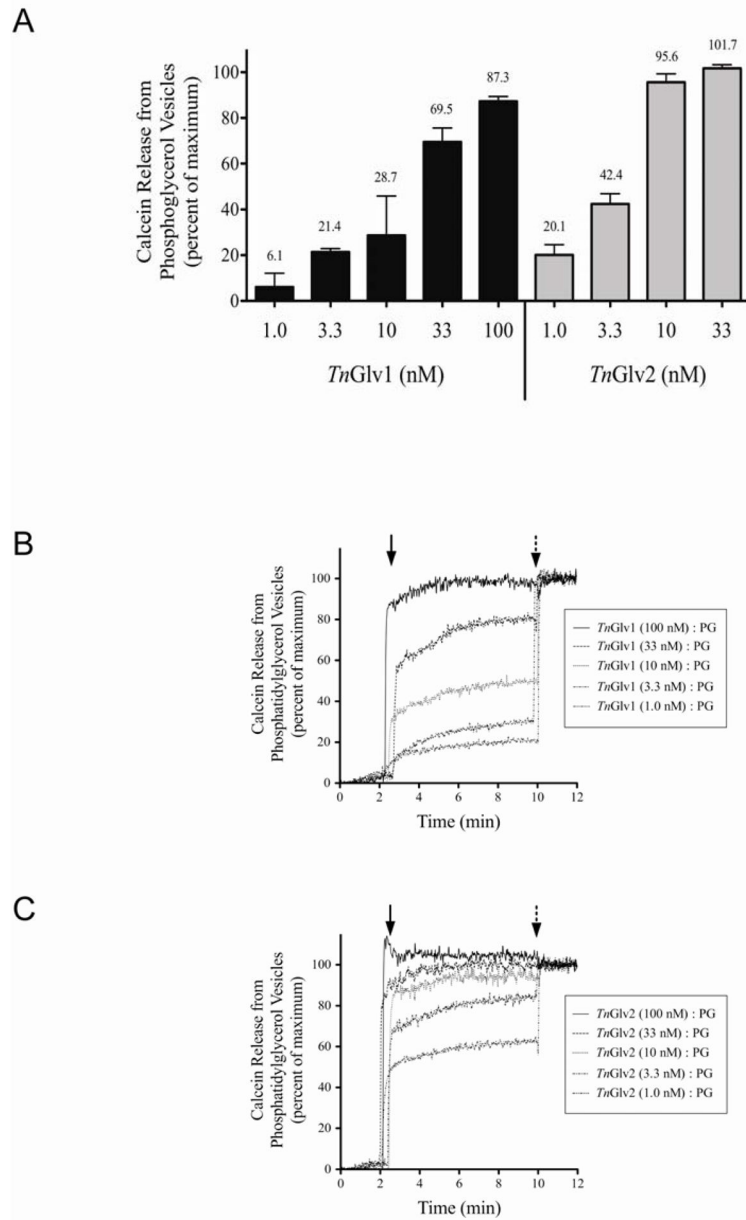


Figure 2. *TnGlv1* and *TnGlv2* reduced the quantity of infectious *AcMNPV-hsp70/lacZ* BV (A) Infectious BV was reduced when incubated in the presence of *Sf9* cell culture supernatants containing *TnGlv1* (*TnGlv1* Sup), *TnGlv2* (*TnGlv2* Sup) or Ni-NTA affinity column purified *TnGlv1* (*TnGlv1* Ni-NTA) or *TnGlv2* (*TnGlv2* Ni-NTA). The results from replicated plaque assay experiments were normalized to the vehicle treatment (TNM-FH media) for each experiment, which was set to 100 % infectious BV, and the change in the quantity of infectious BV reported as the percent of the vehicle treatment. Means and SEM were calculated from $N \geq 3$ replicates per treatment. (B) Plaque assay of *Sf9* cells showing blue LACZ-positive plaques of *AcMNPV-hsp70/lacZ* BV incubated with cell culture supernatants containing *TnGlv1*, *TnGlv2*, or vehicle. (C) Concentration dependent antiviral activity of *TnGlv1* and *TnGlv2* on *AcMNPV-hsp70/lacZ* BV. Plaque assays of *AcMNPV-hsp70/lacZ* BV incubated with increasing concentrations of affinity column purified *TnGlv1* and *TnGlv2* protein was correlated with decreasing quantities of infectious BV relative to vehicle treatments (BV incubated with TNM-FH media; $N = 4$ replicates per treatment).



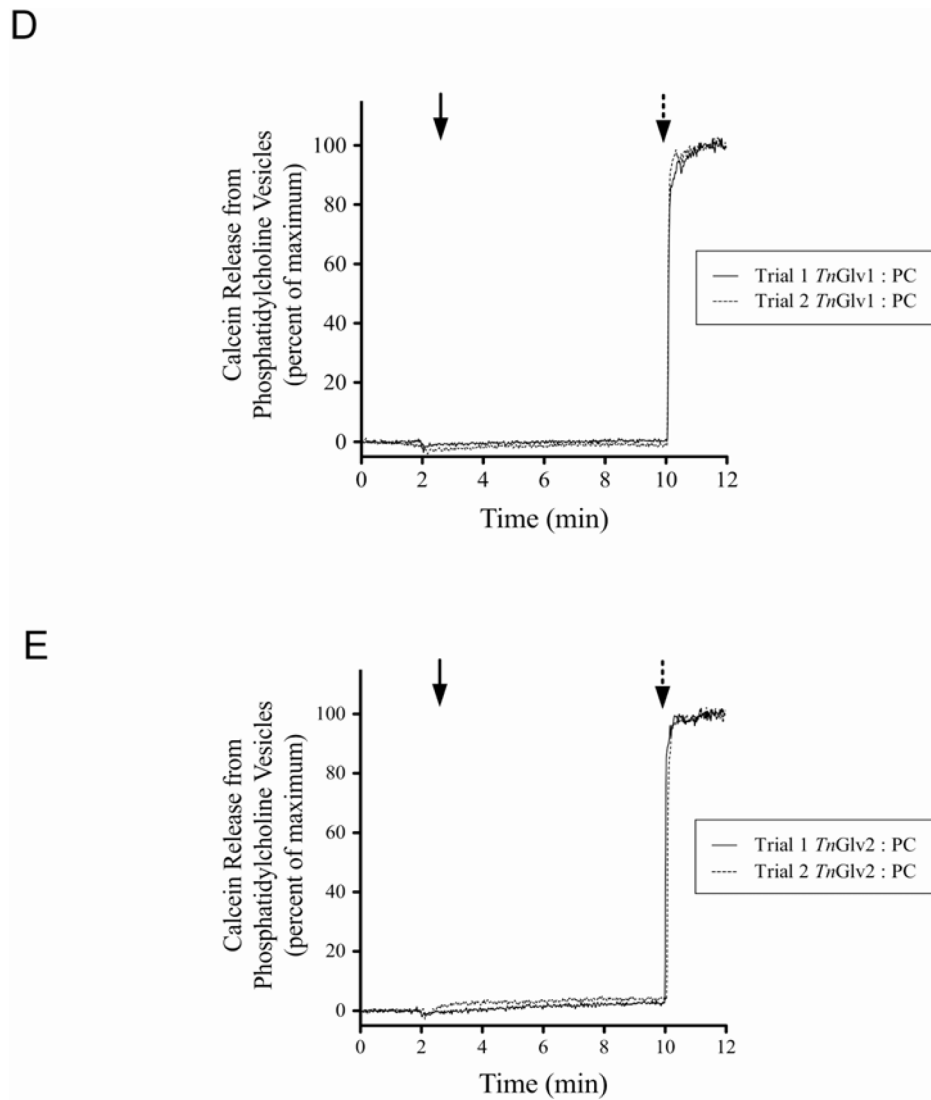


Figure 3. Gloverin-mediated release of calcein from phosphatidylglycerol (PG) or phosphatidylcholine (PC) vesicles

(A) Unilamellar vesicles comprised of PG and encapsulated with calcein were incubated with nanomolar concentrations of affinity purified *TnGlv1* or *TnGlv2* and the quantity of calcein released quantified using a fluorescence spectrophotometer (N = 3 replicates per concentration and treatment). Increasing concentrations of *TnGlv1* or *TnGlv2* protein resulted in concomitant increases in calcein released from the PG vesicles. (B and C) Individual trials showing the release of calcein over time from PG vesicles incubated with multiple concentrations of *TnGlv1* (B) or *TnGlv2* (C). Maximum gloverin-mediated calcein release from PG vesicles occurred within 2 min of the gloverin addition to the reaction cuvette. (D and E) *TnGlv1* and *TnGlv2* (100 nM) did not cause calcein release from PC vesicles after an 8 min incubation period. Solid arrows indicate the time that test proteins were added to the reaction cuvettes and broken arrows indicate the time that detergent was added to each reaction cuvette to cause full release of the encapsulated calcein from the vesicles.

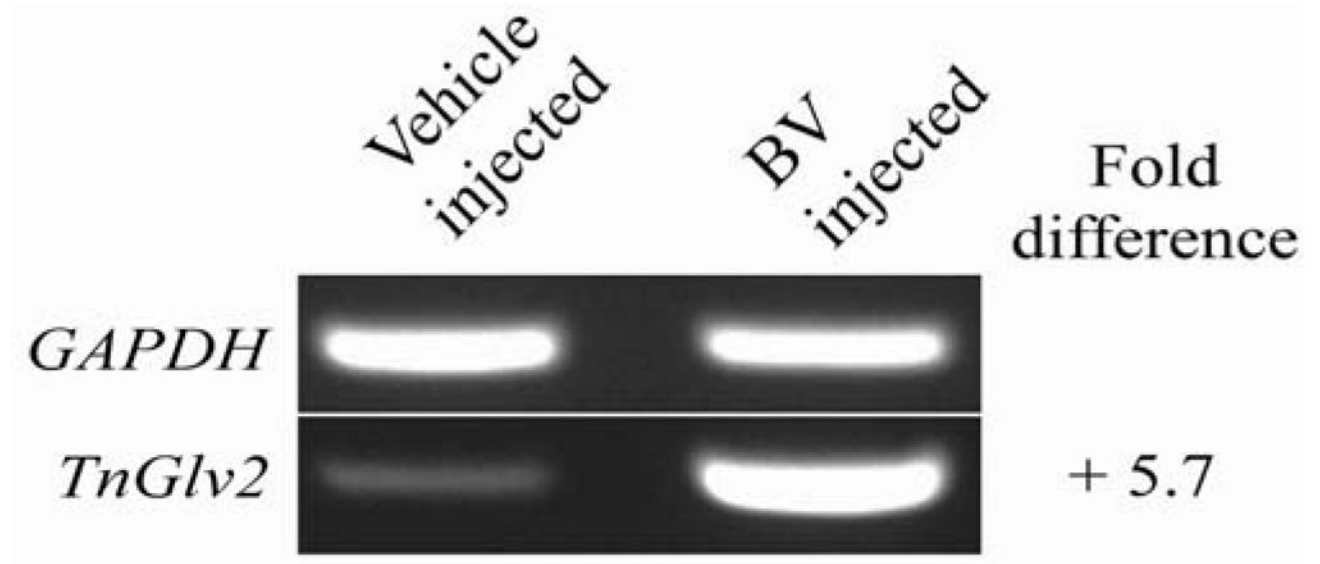


Figure 4. Transcriptional analysis of *gloverin* in hemocytes isolated from *AcMNPV*-infected *T. ni* larva

Gene expression was analyzed using semi-quantitative RT-PCR for hemocytes isolated from *T. ni* 12 h after inoculation with *AcMNPV-hsp70/lacZ* BV or vehicle (TNM-FH). Relative to *GAPDH* expression, *TnGlv2* was upregulated 5.7-fold.

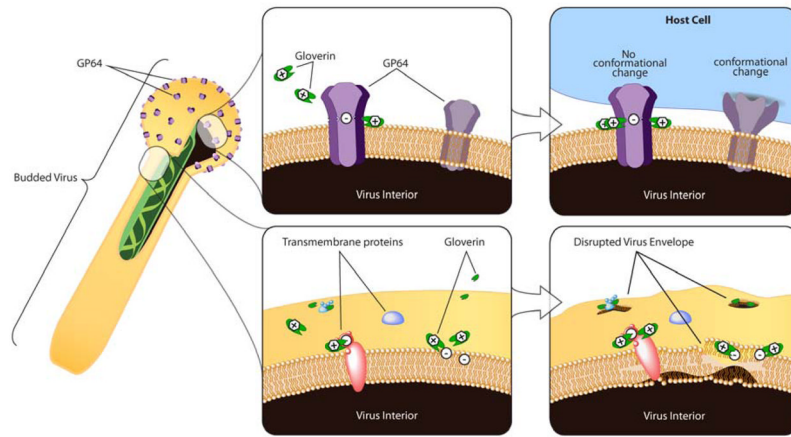


Figure 5. Hypothetical mechanisms of action for the observed antiviral activities of gloverin
Top panels. GP64 is the attachment and fusion protein of AcMNPV BV. The overall negative charge of GP64 or clusters of negatively charged amino acids localized near the transmembrane domain of GP64 may attract the positively charged amino acids of gloverin to the surface of BV. Gloverin may subsequently hinder the conformational changes of GP64 needed to initiate an infection. **Bottom panels.** Gloverin may interact with negatively charged amino acids of other proteins localized to the envelope or with negatively charged phospholipids of the envelope membrane. Accumulated gloverin on the surface of BV may cause membrane strain or pores to form that disrupt the BV envelope. Illustration credit: Emily L. Ling.

Table 1

Primers used for cloning.

Gene	Primer name	Bases	Sequence
<i>TnGlv1</i>	Glv1Q2V-L	25	CACCATGGTGTGTCGTCTATTTATTA
<i>TnGlv1</i>	Glv1-R	24	AAAATCATGTTCAATTTCCCTTG
<i>TnGlv1</i>	Glv1-L	24	CACCATGCAGTCGTCTATTTATT
<i>TnGlv2</i>	Glv2-L	22	CACCATGCAGTTATCAACCATC
<i>TnGlv2</i>	Glv2-R	24	TTTAAAATCATGTTCAATTTTCGC
<i>GAPDH</i>	GAPDH-L	22	GACGGACCCTCTGGAAAACCTG
<i>GAPDH</i>	GAPDH-R	23	ACCAGCTGATGAGCTTGACGAA