Expression of the Cydia pomonella granulovirus matrix metalloprotease enhances Autographa californica multiple nucleopolyhedrovirus virulence and can partially substitute for viral cathepsin

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The Cydia pomonella granulovirus open reading frame 46 (CpGV-ORF46) contains predicted domains found in matrix metalloproteases (MMPs), a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins. We showed that CpGV-MMP was active in vitro. Autographa californica multiple nucleopolyhedrovirus (AcMNPV) expressing CpGV-ORF46 replicated similarly to a control virus lacking CpGV-ORF46 in cultured cells. The effects of AcMNPV expressing CpGV-MMP on virus infection in cultured cells and Trichoplusia ni larvae in the presence or absence of other viral degradative enzymes, cathepsin and chitinase, were evaluated. In the absence of cathepsin and chitinase alone, larval time of death was significantly delayed. This delay was compensated by the expression of CpGV-MMP. CpGV-MMP was also able to promote larvae melanization in the absence of cathepsin and chitinase. In addition, CpGV-MMP partially substituted for cathepsin in larvae liquefaction when chitinase, which is usually retained in the endoplasmic reticulum, was engineered to be secreted.

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Introduction

Matrix metalloproteases (MMPs) are a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins. They are characterized by the presence of a conserved zinc-binding motif (HEXGHXXGXXHS/T) within the catalytic domain. In addition, most MMPs have an N-terminal propeptide domain with a cysteine residue that maintains MMPs latency as it interacts with the zinc ion in the catalytic domain. Finally, a C-terminal hemopexin-like domain contributes to substrate binding, substrate recognition and binding to tissue inhibitors of MMPs (TIMPs) (Murphy et al., 1992; Van Wart and Birkedal-Hansen, 1990). MMPs are found in a wide range of organisms from invertebrates to vertebrates and plants, and they play a crucial role in extracellular matrix remodeling during processes such as embryonic development, angiogenesis, wound healing and metamorphosis (Nagase and Woessner, 1999; Page-McCaw et al., 2007). However, MMPs have also been implicated in a number of pathological processes, including cancer, neurological diseases, arthritis, bacterial and viral infections, and tumor invasion (Elkington et al., 2005; Frantz et al., 2010).

Baculoviruses are arthropod-specific viruses which primarily infect lepidopteran larvae. They contain relatively large circular double-stranded DNA genomes (80–180 kbp) which are packaged into rod-shaped capsids and replicate in the nucleus of cells (Rohrmann, 2013). Baculoviruses have been classified into four genera based on phylogeny: Alphabaculovirus, Betabaculovirus, Gammabaculovirus and Deltabaculovirus (Jehle et al., 2006). Baculoviruses produce two different types of virions during their replication cycle, budded virions (BV) and occlusion-derived virions (ODV). BV allow cell-to-cell virus transmission; they are exported into the cytoplasm and bud from the cell plasma membrane from which they acquire their envelope. ODV are responsible for host-to-host virus transmission. They are produced in the final phases of virus replication, where nucleocapsids accumulate in the nucleus and acquire envelopes from the host cell nuclear membrane. ODV are then packaged in a protein matrix to form occlusion bodies. Occlusion bodies are released by lysis of infected cells and spread into the environment after disintegration and liquefaction of the host (O’Reilly et al., 1994). Larval disintegration and liquefaction require viral encoded enzymes.

Pathogenesis from members within Alphabaculovirus and Betabaculovirus may differ, although fewer studies have described betabaculovirus pathogenesis. Briefly, alphabaculoviruses and betabaculoviruses infect the midgut epithelium of insect hosts. In alphabaculoviruses, virions reach tissues in the insect hemocoel producing more virions. The product of the very late gene P10 is responsible for nuclear lysis (van Oers et al., 1993), releasing ODV in the environment after the insect cadaver liquefies. In betabaculoviruses, the nucleus of infected
cells enlarges, followed by nuclear membrane breakage. The genes and mechanisms affecting this early nuclear membrane rupture are not known. Nucleocapsids are then enveloped and occluded in this nucleocyttoplasmic compartment. Betabaculovirus pathogenesis differs in tissue tropism, from viruses being midgut restricted, to infecting midgut epithelium and fat body tissue, to infecting several tissues of the host. In addition, dispersal of ODV may differ from dispersal in diarrheal secretions to dispersal following complete insect liquefaction (reviewed in Federici, 1997).

Degradative enzymes have been reported in baculoviruses, including viral-chitinase (v-chitinase) which digests chitin, the main component of the insect exoskeleton; and viral-cathepsin (v-cathepsin), which is involved in the degradation of internal larval tissues (Ohkawa et al., 1994; Slack et al., 1995). The concerted activity of these two enzymes enables host liquefaction which allows virus release from the infected cadaver and dissemination to other hosts (Hawtin et al., 1997; Kang et al., 1998a). The betabaculovirus Cydia pomonella granulovirus (CpGV) v-cathepsin was shown to be a functional protease and necessary for larval melanization and liquefaction or softening of larval cadavers, depending on the virus background in which it was tested (Hilton et al., 2008; Kang et al., 1998b). Similarly, the CpGV v-chitinase expressed from a Bombyx mori NPV (BmNPV) recombinant virus allowed larval liquefaction and interacted with BmNPV v-cathepsin (Daimon et al., 2007).

The role of another degradative enzyme, the viral MMP, found in betabaculoviruses, has not been studied extensively. To date, there is only one functional study on baculovirus MMPs, characterizing the Xestia c-nigrum granulovirus (XcGV) MMP (XcGV-MMP). XcGV-MMP is a functional MMP involved in larval melanization and thought to have a role in degradation of host basement membranes during the late stages of infection (Ko et al., 2000). Open reading frame ORF46 encoded in the CpGV genome predicts a protein, CpGV-MMP, which shows significant similarity to MMPs (Luque et al., 2001). However, its role in viral pathogenesis has not been described.

In this study, we characterized the CpGV-MMP in silico, in vitro, and in vivo to determine its function independently and in conjunction with the viral degradative enzymes, v-cathepsin and v-chitinase. To this end, we analyzed the phylogenetic relationships between CpGV-MMP and other baculovirus MMP homologs and tested CpGV-MMP enzymatic activity in vitro. We also determined the effects of Autographa californica M nucleopolyhedrovirus (AcMNPV) expressing CpGV-MMP on cultured cells and insects in the presence or absence of v-chitinase and v-cathepsin and their interactive roles in insect mortality, liquefaction, and melanization.

Results

MMPs in betabaculoviruses

We screened for putative viral MMPs in the GenBank® protein database. Results revealed the presence of putative mmps in the virus families Baculoviridae, Ascosviridae, Iridoviridae, Hytrosaviridae, Poxviridae (only in the subfamily Entomopoxvirinae) and Nudiviridae. Viruses within these (sub)families contain diverse large double-stranded DNA viruses that infect invertebrates. In baculoviruses, MMP homologs are present in all species from the genus Betabaculovirus (granuloviruses) sequenced to date but absent in species from Alphabaculovirus, Gamma baculovirus and Deltabaculovirus. Baculovirus MMPs cluster more closely with insect MMP-3 than with insect MMP-1 or MMP-2 (Fig. 1A). Insect MMPs are classified into these three major groups (MMP-1, MMP-2 and MMP-3), based on phylogenetic analyses.

All previously identified cellular MMPs, except the human MMP-23 which contains a membrane anchor sequence and is membrane-associated (Ohnishi et al., 2001), contain an N-terminal signal sequence and are secreted. Signal peptide prediction programs uncovered a putative signal peptide in all baculovirus MMPs except in XcGV, Pieris rapae GV and Epinotia aporema GV, which do not contain signal sequences or N-terminal anchor sequences. Thus, the secretion patterns of viral MMPs may differ and may be correlated to their function.

Similar to cellular MMPs characterized to date, baculovirus MMPs have a conserved zinc-binding motif (HEGXHXXGXXH/S/T) within the predicted catalytic domain (Fig. 1B). The three underlined histidine residues act as ligands of a zinc ion (Zn2+) cofactor necessary for catalysis. The baculovirus MMP catalytic domain also predicts a conserved methionine-turn (Met-turn) region, where a methionine residue is located seven residues C-terminal to the zinc-binding motif. The Met-turn is thought to enable correct folding of the MMP catalytic domain (Bode et al., 1993).

Although baculovirus MMPs have the conserved catalytic domain, comparison of the predicted baculovirus and cellular MMP sequences revealed that baculovirus MMPs lack other hallmark MMP structural features. First, baculovirus MMPs do not have a cysteine sequence (PRCGV/NPD) necessary for the cysteine switch mechanism. This sequence is conserved within the N-terminal propeptide domain of most cellular MMPs and is involved in maintaining MMP latency. Interaction between the cysteine residue in the conserved cysteine switch motif and the Zn2+ in the catalytic domain renders the active-site cleft unavailable for substrate binding; thus, keeping the enzyme inactive. Disruption of the Cys–Zn2+ complex (e.g., proteolytic cleavage of prodomain, conformational perturbation, etc.) allows activation of latent MMPs (Vann Wart and Birkedal-Hansen, 1990). In addition, baculovirus MMPs lack a hemopexin-like domain at their C terminus. This domain which mediates substrate recognition, specificity, and binding to TIMPs (Murphy et al., 1992) is present at the C-terminal region of most cellular MMPs except human MMP-23, –7 and –26 and some plant MMPs (e.g., Arabidopsis thaliana; Maidment et al., 1999). The region downstream of the catalytic domain is short or almost non-existent in some cellular MMPs (e.g., MMP-23, –7, and –26) (Velasco et al., 1999; de Cognac et al., 2000). Baculovirus MMPs, as most cellular MMPs, have longer C-terminal sequences downstream of their catalytic domain; however, no functional domain could be identified in that region using the Simple Modular Architecture Research Tool (SMART) online program (Letunic et al., 2012). Overall, these analyses indicate that even though baculovirus-encoded MMPs lack some conserved cellular MMP domains, they still preserve homology to the MMP family of proteins to be classified as MMPs.

Enzymatic activity of CpGV-MMP

CpGV-MMP is 545 amino acids long with a predicted molecular mass of 65 kDa. The predicted sequence contains an 18-amino acid long signal peptide at its N terminus and a 178-amino acid long (amino acids 125–303) MMP conserved catalytic domain.

To determine if CpGV-MMP is a functional MMP, CpGV-ORF46 was cloned into the pET-32a bacterial expression vector and fused in frame to a polyhistidine tag at the C terminus. A CpGV-MMP mutant, CpGV-MMP-Mut, in which the conserved glutamic acid in the zinc binding motif was changed to glutamine was also constructed as a control. The glutamic acid residue is thought to be necessary for catalytic activity by enabling nucleophilic attack in a zinc-bound water molecule that cleaves peptide bonds (Dhanaraj et al., 1996). The recombinant proteins were expressed in Escherichia coli, purified using metal-affinity columns, and detected following immunoblotting using an anti-His antibody (Fig. 2A and data not shown). Imidazole elution (50 mM) fractions were used for MMP activity assays using a synthetic quenched fluorescently-labeled generic MMP substrate (5-FAM/ QXL520). Upon cleavage, the substrate emits fluorescence that...
can be detected using a fluorescence plate reader set at excitation and emission wavelengths of 485 nm and 535 nm, respectively. A gradual and linear increase in relative fluorescence units was measured over time in samples containing CpGV-MMP (Fig. 2B). CpGV-MMP-Mut did not show an increase in fluorescence (Fig. 2B). Proteins purified from pET32a vector-transformed bacteria were used as a negative control to confirm that substrate cleavage was due to CpGV-MMP activity and not background bacterial proteases. Activity of CpGV-MMP-containing lysates and that of a positive control enzyme, human MMP-9, was abolished...
by the broad-spectrum MMP inhibitor GM6001 (Galardy et al., 1994) and the metal chelator EDTA. These results provide evidence that CpGV-MMP is an active MMP enzyme in vitro.

**Synthesis of CpGV-MMP during virus infection**

To investigate the effects of CpGV-MMP during virus replication in cell culture and insect hosts, a recombinant AcMNPV containing HA-tagged CpGV-ORF46 under the Drosophila hsp70 gene promoter, Ac-CpGV-ORF46-PG, was generated (Fig. 3A). In addition, the corresponding control AcMNPV bacmid, AcWT-PG, was constructed (Fig. 3B). AcMNPV, like other alphabaculoviruses, does not encode an mmp, so any changes between Ac-CpGV-ORF46-PG and AcWT-PG can be attributed to the presence of mmp.

Sf9 cells were infected with Ac-CpGV-ORF46-PG and production of CpGV-MMP over the course of infection was monitored by immunoblotting using an anti-HA antibody. An HA-immunoreactive band was observed in immunoblots from pellet and extracellular media lysates derived from Ac-CpGV-ORF46-PG-infected cells (Fig. 4A). Detection of CpGV-MMP in the extracellular media confirmed secretion of CpGV-MMP. An immunoreactive protein was not detected in mock- or AcWT-PG-infected Sf9 cells collected at 12 h postinfection (p.i.) (Fig. 4A).

CpGV-MMP migrated as a protein with molecular mass of approximately 72 kDa, higher than the predicted mass of 65 kDa. In silico CpGV-MMP analyses, using NetNGlyc 1.0 (Chuang et al., 1994) and the metal chelator EDTA. These results provide evidence that CpGV-MMP is an active MMP enzyme in vitro.

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CpGV-MMP migrated as a protein with molecular mass of approximately 72 kDa, higher than the predicted mass of 65 kDa. In silico CpGV-MMP analyses, using NetNGlyc 1.0 (Chuang et al., 2012), indicated eight potential N-glycosylation sites. The increased molecular mass observed in immunoblots may be explained by protein N-glycosylation. In order to confirm N-glycosylation of CpGV-MMP, we treated cell pellets and extracellular media collected from Ac-CpGV-ORF46-PG-infected cells with PNGase F, a glycosidase that cleaves N-linked oligosaccharides from glycoproteins, and analyzed the results by immunoblotting. We observed a significant reduction in molecular mass when both cellular and extracellular CpGV-MMP-containing lysates were treated with PNGase F (Fig. 4B). The molecular mass of PNGase F-treated CpGV-MMP was closer to the predicted mass of 65 kDa. These findings indicate that CpGV-MMP is secreted as an N-glycosylated protein.

**Effects of CpGV-MMP on virus replication in cultured cells**

In order to determine the effects of CpGV-MMP on virus replication, we infected Sf9 cells with Ac-CpGV-ORF46-PG or AcWT-PG at a multiplicity of infection (MOI) of 5 PFU/cell. Infection was monitored by fluorescence microscopy (eGFP fluorescence) and occlusion body formation. There was no noticeable difference in the number and intensity of fluorescent cells or the timing in the appearance, number, and gross morphology of occlusion bodies between the two viruses (Fig. 5A). BV from Ac-CpGV-ORF46-PG- or AcWT-PG-infected cells (5 PFU/cell) were collected at selected time points and titers were determined by 50% tissue culture infective dose (TCID50) endpoint dilution assays. There were no significant differences in BV production kinetics between Ac-CpGV-ORF46-PG and AcWT-PG (Fig. 5B).

The two degradative enzymes v-cathepsin and v-chitinase have been previously characterized in baculoviruses. Similar to MMPs, some cathepsin proteases are known to degrade extracellular matrix components (Buck et al., 1992; Nosaka et al., 1999). In baculoviruses, v-cathepsin is involved in the degradation of larval tissues (Slack et al., 1995), v-chitinase digests chitin, a component of the insect exoskeleton. These two proteins are necessary for larval liquefaction which enables occlusion body release from the larval cadaver, allowing efficient virus transmission to other hosts (Hawtin et al., 1997; Kang et al., 1998a).

We determined the effects of CpGV-MMP on virus pathology in insects in the absence or presence of degradative viral enzymes, v-cathepsin and v-chitinase, which have potentially similar or overlapping functions to that of CpGV-MMP. To this end, we constructed...
additional recombinant viruses. First, Ac-Δcath-CpGV-ORF46-PG, a virus lacking v-cathepsin and expressing CpGV-ORF46, was constructed from Ac-CpGV-ORF46-PG by replacing v-cathepsin with the zeocin resistance gene through homologous recombination (Fig. 3C). Second, the AcMNPV v-chitinase/v-cathepsin double knockout bacmid, AcBacΔCC (Kaba et al., 2004), was used as the backbone to construct two other bacmids, Ac-Δcath-CpGV-ORF46-PG and Ac-ΔCC-PG. Ac-ΔCC-CpGV-ORF46-PG carries CpGV-ORF46 under the Drosophila hsp70 gene promoter control. Ac-Δcath-CpGV-ORF46-PG, Ac-ΔCC-CpGV-ORF46-PG, and Ac-ΔCC-PG encode polh and egfp at the polh locus.

We first analyzed and compared BV production kinetics of the recombinant viruses by infecting Sf9 cells (5 PFU/cell), collecting BV at different times p.i., and titering the collected virions. All viruses exhibited a consistent increase in BV production comparable to that of AcWT-PG over the course of the infection (Fig. 5B).
Finally, we confirmed expression of CpGV-MMP in Sf9 cells infected with the recombinant viruses following immunoblotting using anti-HA antibody (Fig. 5C). CpGV-MMP was expressed in all bacmids that carried its gene. Overall, CpGV-MMP had no obvious effects on virus replication in infected Sf9 cells, in the presence or absence of v-chitinase or v-cathepsin.

Effects of CpGV-MMP on insects

To evaluate the effects of CpGV-MMP on insect larvae, we performed a bioassay in Trichoplusia ni using recombinant viruses, AcWT-PG, Ac-CpGV-ORF46-PG, Ac-Δcath-CpGV-ORF46-PG, Ac-ΔCC-CpGV-ORF46-PG, Ac-ΔCC-PG, and Ac-p6.9-chiA. Ac-p6.9-chiA does not express v-cathepsin but expresses v-chitinase under the control of the late p6.9 promoter (Hodgson et al., 2007). Early 4th instar larvae (55 larvae per virus) were individually infected by the oral route with 500 occlusion bodies of bacmids that carried its gene. Overall, CpGV-MMP had no obvious effects on virus replication in infected Sf9 cells, in the presence or absence of v-chitinase or v-cathepsin.

### Table 1

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a SE: standard error.

(b) (a), (b) and (c) indicate groups with non-overlapping 95% fiducial limits.

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Compared to larvae infected with AcWT-PG, a significant delay in LT50 (~ 10 h) was observed with either a virus containing dual v-chitinase and v-cathepsin deletion or one with only a v-cathepsin deletion (Ac-ΔCC-PG- and Ac-p6.9-chiA, respectively; Table 1). However, the delay in time of death could be compensated by expression of CpGV-ORF46 (Ac-Δcath-CpGV-ORF46-PG and Ac-ΔCC-CpGV-ORF46-PG; Table 1). In contrast, significant acceleration in mortality was observed when CpGV-ORF46 and v-cathepsin were co-expressed in Ac-CpGV-ORF46-PG compared to AcWT-PG. In summary, the bioassay survival results suggest that lack of the AcMNPV v-chitinase can be compensated for by CpGV-ORF46 and expression of CpGV-ORF46 can enhance larval mortality in combination with v-cathepsin and v-chitinase.

During bioassays we monitored melanization and liquefaction of virus-infected larvae. Dark spots appeared on the cuticles of larvae infected with viruses carrying CpGV-ORF46 and/or v-cathepsin (AcWT-PG, Ac-CpGV-ORF46-PG, Ac-ΔCC-CpGV-ORF46-PG or Ac-Δcath-CpGV-ORF46-PG) a few hours prior to death. These dark patches are characteristic of early stages of melanization. Following the appearance of dark molting, the larval cuticles turned brownish, and a few hours after death turned black (Fig. 6). Larvae infected with viruses lacking v-cathepsin or CpGV-ORF46 (Ac-ΔCC-PG and Ac-p6.9-chiA), on the other hand, did not melanize prior to death or soon thereafter, retaining their light green color for a longer period of time (more than 24 h) after death (Fig. 6).

After death, the epidermis of AcWT-PG- and Ac-CpGV-ORF46-PG-infected larvae became very brittle. The flaccid larvae tissues then liquefied. The epidermis of all the other virus-infected larvae, Ac-ΔCC-CpGV-ORF46-PG, Ac-ΔCC-PG, Ac-Δcath-CpGV-ORF46-PG, and Ac-p6.9-chiA, on the other hand, retained a firm consistency after death and did not liquefy. As previously reported (Hawtin et al., 1997; Kang et al., 1998a), larval liquefaction occurs only in the presence of both v-cathepsin and v-chitinase (e.g., AcWT-PG). CpGV-ORF46 alone (in Ac-ΔCC-CpGV-ORF46-PG) or in the presence of v-chitinase (Ac-Δcath-CpGV-ORF46-PG) did not appear to promote larvae liquefaction. CpGV-MMP facilitated host melanization without v-cathepsin, but it could not complement the inability of larvae to liquefy when co-expressed with just v-chitinase. Viruses carrying CpGV-ORF46 instead of v-cathepsin could not fully substitute for the
v-chitinase/v-cathepsin co-dependent effects on host larval melanization, mortality, and liquefaction, indicating that these baculoviral degradative enzymes have non-overlapping roles during virus infection.

**Inefficient release of v-chitinase from infected cells correlates with the lack of larvae liquefaction**

In AcMNPV, v-chitinase is trafficked to the endoplasmic reticulum where it is retained, as it contains a C-terminal KDEL endoplasmic reticulum retention motif (Hodgson et al., 2011; Saville et al., 2002). v-chitinase is released from the endoplasmic reticulum only after lysis of infected cells, and its release is a requirement for larvae liquefaction (Thomas et al., 1998). The CpGV v-chitinase, as predicted for all other betabaculoviruses, does not have a KDEL sequence or equivalent motif and is predicted to be constitutively secreted from cells. We hypothesized that the inability of Ac-Δcath-CpGV-ORF46-PG virus-infected larvae to liquefy may be due to delayed or reduced release of v-chitinase from infected cells.

To investigate the amount of v-chitinase released from AcWT-PG-, Ac-CpGV-ORF46-PG-, Ac-ΔCC-PG-, or Ac-Δcath-CpGV-ORF46-PG-infected cells, cell pellets and extracellular media from infected cells were collected at 72 h p.i. and lysates were immunoblotted using a chitinase-specific antibody (Daimon et al., 2006). In addition, Cath (-), a virus expressing v-chitinase under its native promoter but lacking v-cathepsin, was used as an additional control. In Cath (-), v-cathepsin was disrupted by insertion of a p10/β-galactosidase cassette (Slack et al., 1995). There was less v-chitinase detected in the extracellular media of Cath (-) and Ac-Δcath-CpGV-ORF46-PG-infected cells compared to AcWT-PG- and Ac-CpGV-ORF46-PG-infected cells (Fig. 7A). Thus, it appeared that less v-chitinase was released from infected cells when v-cathepsin was deleted, and replacing v-cathepsin with CpGV-ORF46 (Ac-Δcath-CpGV-ORF46-PG) did not seem to restore v-chitinase release to levels observed with AcWT-PG- or Ac-CpGV-ORF46-PG-infected cells. Thus, the reduction of v-chitinase release into the extracellular media of Ac-Δcath-CpGV-ORF46-PG- compared to AcWT-PG-infected cells may explain why no liquefaction occurred when larvae were infected with Ac-Δcath-CpGV-ORF46-PG.

To test this hypothesis, we investigated whether constitutive secretion of v-chitinase from infected cells in the presence of CpGV-MMP and absence of v-cathepsin would promote larvae liquefaction. To this end, we generated two recombinant viruses in the AcBacΔCC (Kaba et al., 2004) genetic background that expressed either native v-chitinase (Ac-ΔCC-ChiA-PG) or v-chitinase lacking the C-terminal KDEL motif (Ac-ΔCC-ChiΔKDEL-PG) under the Drosophila hsp70 gene promoter control. v-chitinase activity was detected in cell pellet lysates from Ac-ΔCC-ChiA-PG- and Ac-ΔCC-ChiΔKDEL-PG-infected cells (results not shown), consistent with a previous report (Saville et al., 2002).

The secretion patterns of v-chitinase in Sf9 cells infected by these recombinant viruses were compared at different times p.i. In both Ac-ΔCC-ChiA-PG- and Ac-ΔCC-ChiΔKDEL-PG-infected cells, v-chitinase was comparably detected in the cell pellet starting at 24 h p.i. (Fig. 7B). In contrast, the accumulation of v-chitinase in the extracellular media of Ac-ΔCC-ChiAΔKDEL-PG-infected cells was more abundant than in that of Ac-ΔCC-ChiA-PG-infected cells at any time p.i. In addition, v-chitinase accumulation in the media was delayed in Ac-ΔCC-ChiA-PG-infected cells (Fig. 7B). Consistent with prior studies (Hodgson et al., 2011; Saville et al., 2002; Saville et al., 2004), our results stress that deleting the C-terminal KDEL motif in v-chitinase enhances v-chitinase secretion in Ac-ΔCC-ChiAΔKDEL-PG-infected cells.

Finally, we tested if v-chitinase secreted in the absence of v-cathepsin was able to liquefy T. ni larvae and if CpGV-MMP could have a role in this process. To this end, early 4th instar T. ni larvae were orally infected with 500 occlusion bodies of the designated virus and photographed at the indicated times post-mortem. Representative larvae are shown. Melanization or liquefaction of all larvae tested is indicated by a “Yes” or “No” under the indicated row.

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**Fig. 6.** Infected larvae melanization. Early 4th instar T. ni larvae were orally infected with 500 occlusion bodies of the designated virus and photographed at the indicated times post-mortem. Representative larvae are shown. Melanization or liquefaction of all larvae tested is indicated by a “Yes” or “No” under the indicated row.
v-chitinase secretion alone in Ac-ΔCC-ChiAΔKDEL-PG (i.e., in absence of v-cathepsin and heterologous mmp) was not sufficient for larval liquefaction. Liquefaction was partially restored in larvae coinfected with Ac-ΔCC-CpGV-ORF46-PG and Ac-ΔCC-ChiAΔKDEL-PG but no liquefaction was observed in larvae coinfected with Ac-ΔCC-CpGV-ORF46-PG and Ac-ΔCC-ChiA-PG (Table 2). These results indicate that both secretion of v-chitinase and production of heterologous CpGV-MMP were necessary for larval liquefaction in the absence of v-cathepsin.

**Discussion**

**Baculovirus unconventional MMP domain conservation and classification**

Sequence alignments revealed that baculovirus MMPs have a conserved zinc-binding motif and Met-turn but lack two commonly conserved cellular MMP domains (Fig. 1B and data not shown): the cysteine switch and the hemopexin-like domain. The cysteine switch is conserved in all cellular MMPs except human MMP-23 (Velasco et al., 1999). The absence of a recognizable cysteine switch which maintains an MMP in an inactive state by the interaction of the prodomain cysteine and the zinc ion at the catalytic site suggests that baculovirus MMPs have evolved to bypass a regulatory control mechanism and may enable them to be active immediately after synthesis. Despite the lack of conserved cellular MMP domains, the presence of a conserved MMP catalytic domain in baculovirus-encoded MMPs should be sufficient to classify them into the MMP family.
Based on amino acid composition and/or domain organization, MMPs have been traditionally subdivided into five major subclasses (Collier et al., 1992; de Coignac et al., 2000; Fillmore et al., 2001; Freije et al., 1994). Baculovirus MMP predicted sequences lack the key features required to be classified into the gelatinase (substrate binding fibronectin repeats), collagenase (conserved tyrosine, aspartic acid, and glycine in the catalytic domain), stromelysin (insertion of nine hydrophylic residues, XPLVPTXXXV, at the end of the catalytic domain), membrane-type MMP (transmembrane domain), and matrilysin (truncated C-terminal region) MMP subclasses. However, there are MMPs, including a number of human (MMP-12, -19, -20, -21, -23, -27, and -28) and insect (Drosophila melanogaster, Tribolium castaneum, Manduca sexta) MMPs, that do not fit into the prescribed MMP subclasses. These differing MMPs are usually referred to as “other MMPs”. We suggest that baculovirus MMPs should also be included into the “other MMPs” category.

CpGV-MMP characterization, activity, and effects on AcMNPV replication in cultured cells

Bacterially purified CpGV-MMP had MMP activity in vitro that was abolished with MMP and metal chelator inhibitors (Fig. 2B). HA-tagged CpGV-MMP expressed in a recombinant AcMNPV was detected in the extracellular media of infected Sf9 cells (Fig. 4A), confirming that CpGV-MMP is a secreted protein. MMP secretion is also predicted in most baculovirus MMPs, implying a common extracellular function. The molecular mass of CpGV-MMP was significantly reduced after treatment with PNGase F, indicating that CpGV-MMP was N-glycosylated (Fig. 4B). Whether N-glycosylation is a requirement for function was not investigated. Finally, CpGV-MMP does not appear to have any obvious effect on AcMNPV BV production or occlusion body formation in vitro (Fig. 5A and B).

Effects of CpGV-MMP on larval mortality

To determine the functional relationships, if any, between CpGV-MMP, v-chitinase, and v-cathepsin, and their potential synergistic effects on insects, we constructed recombinant viruses expressing CpGV-MMP in the absence of v-chitinase and/or v-cathepsin. Survival times from infected T. ni revealed that in the absence of v-cathepsin there was a significant delay in the time of larval death that could be compensated by CpGV-ORF46. The presence of v-cathepsin, v-chitinase and CpGV-ORF46 accelerated mortality relative to a control virus lacking CpGV-ORF46 only. These results imply that CpGV-MMP can rescue the delay in time of death observed when v-cathepsin was absent and could potentially be used in combination with v-chitinase and v-cathepsin in pest control management to enhance mortality of baculovirus-infected larvae. CpGV-MMP may be helping virus spread by degrading larval basement membranes and other matrix proteins. Basement membrane injury was suggested for the XcGV-MMP, leading to local melanization (Ko et al., 2000). In insects, basement membranes underlie epithelia and surround muscle fiber and fat body cells while collagenous fibrous tissues support internal organs (Ashhurst, 1982). Like MMPs, cathepsins are able to degrade extracellular matrix proteins. Baculovirus v-cathepsin, however, is not secreted, but accumulates in the endoplasmic reticulum of infected cells as an inactive enzyme where it is retained due to its interaction with v-chitinase (Hodgson et al., 2011, 2013). In AcMNPV, v-cathepsin is only activated and released extracellularly after lysis of infected cells (Hodgson et al., 2011; Hom et al., 2002). This indicates that v-cathepsin may not be present in the extracellular environment during the early stages of infection to degrade extracellular matrix components. CpGV-MMP, on the other hand, is secreted from infected cells (Fig. 4A) and may enhance virus spread via basement membrane remodeling or another mechanism. Alternatively, since GV chitinases do not have a KDEL sequence, it is possible that GV cathepsins and chitinases are co-secreted as it occurs with AcMNPV chitinase lacking KDEL and pro-cathepsin (Hodgson et al., 2011). The XcGV-MMP has been previously characterized (Ko et al., 2000). It is important to note that in contrast to CpGV-MMP, XcGV-MMP does not have a signal peptide and, as indicated by Ko et al., it is likely that XcGV-MMP is released after lysis of infected cells. This implies that XcGV-MMP, in contrast to CpGV-MMP, may function at later stages of infection (e.g., after lysis of infected cells) or it may be playing a role intracellularly, although intracellular regulation of an active XcGV-MMP is not well known.

Effects of CpGV-MMP on larvae melanization and liquefaction

T. ni bioassays showed that CpGV-MMP promotes larval melanization. Melanization did not occur in the absence of v-cathepsin but was restored when CpGV-MMP was expressed in v-cathepsin-negative viruses. Melanization is triggered by the proteolytic activation of prophenoloxidase (proPO) into active phenoloxidase (PO). This activation is mainly carried out by serine proteases (Cerenius and Soderhall, 2004) which are regulated by endogenous serpins. Although melanization plays an important role in innate immunity and wound healing, excessive and uncontrolled PO activity can have deadly consequences for the insect. Previous studies have shown that v-cathepsin promotes larvae melanization (Kan et al., 2008; Slack et al., 1995). It is possible that CpGV-MMP, like v-cathepsin, directly or indirectly activates the PO cascade which leads to melanization. Similar effects on melanization were observed with XcGV-MMP (Ko et al., 2000). Likewise, a metallo-protease secreted by the gram-negative insect pathogen Photorhabdus luminescens was able to induce melanization (Held et al., 2007). To date, only one baculovirus encodes a serpin gene (Rohrmann et al., 2013), and it is functional (Ardisson-Araujo et al., 2015). Thus, it is not clear if there are functional relationships between MMPs, cathepsins, and serpins (Ishimwe et al., 2015).

We observed that when v-cathepsin and v-chitinase were expressed (AcWT-PG and Ac-CpGV-ORF46-PG) infected larvae liquefied after death. It is known that both v-chitinase and v-cathepsin are required for degradation of the insect cadaver, leading to complete host liquefaction (Hawtin et al., 1997). However, v-chitinase has an endoplasmic reticulum retention motif (KDEL) and it is only released from the endoplasmic reticulum upon virus-induced lysis of infected cells (Hawtin et al., 1997). v-chitinase alone was not able to support larvae liquefaction. In our bioassays (Fig. 6), liquefaction only occurred when v-cathepsin and v-chitinase were both expressed or when v-cathepsin was deleted and v-chitinase was engineered to be secreted constitutively. Our results also showed that less v-chitinase appeared to be released into the extracellular media of infected cells when v-cathepsin was absent than from a control virus (Fig. 7A). Replacing v-cathepsin with CpGV-MMP (Ac-Δcath-CpGV-ORF46-PG) did not result in increased v-chitinase release (Fig. 7A), suggesting that v-chitinase release from infected cells may be aided by v-cathepsin. Curiously, granuloviruses encode v-chitinases without the KDEL motif (Daimon et al., 2007), but it is not clear whether this is related to their also encoding MMPs. It is possible the GV MMPs may activate their cathepsins. Since cell lysis seems to be a prerequisite for v-chitinase release (Hawtin et al., 1997; Thomas et al., 1998), our results suggest that v-cathepsin facilitates lysis of infected cells. The effects of v-cathepsin on lysis of infected cells and release of occlusion bodies are currently being investigated more directly. Taken together, CpGV-MMP only partially substitutes for v-cathepsin functions tested in our assays. Since CpGV-ORF46 was expressed from the Drosophila heat shock 70 gene promoter, it is possible that different expression levels and timing of expression affected its native activity.
We wanted to know if engineering v-chitinase to be constitutively secreted from cells could lead to liquefaction of infected larvae in the absence of v-cathepsin. Expression of v-chitinase lacking the KDEL motif results in better v-chitinase secretion than a KDEL-containing v-chitinase virus. Regardless of whether v-chitinase was produced, retained in cells by the KDEL peptide, or secreted due to KDEL deletion, absence of v-cathepsin resulted in poor melanization and liquefaction in studies described here and previously (Ohkawa et al., 1994; Slack et al., 1995). However, when larvae were coinfected with Ac-ΔCChiaΔKDEL-PG and Ac-Δcath-CpGV-ORF46-PG, liquefaction was restored although not as efficient as that of AcWt-PG-infected larvae (Table 2). Cultured cells infected with Ac-ΔCChia-PG secreted some v-chitinase very late in infection (72 h p.i.), but it appears that this was not sufficient for efficient liquefaction in the presence of CpGV-MMP. In summary, CpGV-MMP is only able to aid in larvae liquefaction when significant amounts of v-chitinase are available in the extracellular space. Perhaps this is true for other GVs that encode KDEL-deficient chitinases, cathespins and mmps. In the insect cuticle, chitin, forms long chains that are linked to sclerotized proteins (Merzendorfer and Zimoch, 2003). Hawtin et al. (1997) suggested that proteases are required to “unmask” the insect cuticle in order to allow v-chitinase to degrade chitin. Our results support that when v-chitinase was present in the extracellular milieu, a protease, either native v-cathepsin or heterologous CpGV-MMP, was required to liquefy the insect exoskeleton. The availability of a CpGV bacmid (Hilton et al., 2008) may be a better tool to characterize the effects of v-cathepsin, v-chitinase, and MMP on infection of the insect host, Cydia pomonella, from which CpGV was isolated. These results show that a number of enzymes, their interplay, concentrations, and cellular localization are all important for efficient virus dispersal. Baculoviruses encode a number of degradative enzymes that may optimize virus infection and dispersal (Ishimwe et al., 2015).

Materials and methods

Cells lines, bacmid maintenance and bacterial strain, and insects

The Spodoptera frugiperda-derived insect cell line Sf9 (clonal isolate 9 from cell line IPLB-SF21-AE) was purchased from ATCC and cultured at 27 °C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), penicillin G (60 μg/ml), streptomycin sulfate (200 μg/ml), and amphotericin B (0.5 μg/ml).

DH10B E. coli cells with helper plasmid pM0N7124, encoding a transposase, were purchased from Invitrogen. Recombinant bacmids were maintained in DH10B cells (Invitrogen).

Third instar T. ni larvae were purchased from Benzon Research (Carlisle, PA). Larvae were reared with artificial diet (Southland Products, Inc.) in a 27 °C chamber with a 12 h/12 h light/dark cycle and infected shortly after they reached 4th instar.

E. coli expression and purification of CpGV-MMP and the CpGV-MMP active site mutant protein

CpGV-ORF46 was PCR-amplified from CpGV genomic DNA using primers Ncol-ORF46 CpGV (5’-CCATGGTAATGACGGACGCTTAATTTTCTGTTGTTGCTGA-3’) and NotI-HA-ORF46 CpGV (5’-CCGCCGCCGCGCTAAATCTCGGACGTAGCTATGTCGCTCA-3’) and NotI-HA-ORF46 CpGV (5’-CCGCCGCCGCGCTAAATCTCGGACGTAGCTATGTCGCTCA-3’) and NotI-HA-ORF46 CpGV (5’-CCGCCGCCGCGCTAAATCTCGGACGTAGCTATGTCGCTCA-3’). The PCR product, CpGV-ORF46-HA, containing the coding sequence of CpGV-ORF46 from ATG to the codon immediately prior to the stop codon and followed by the sequence for an HA tag, was ligated into the pCR®II vector (Invitrogen) to generate pCRII-CpGV-46-HA. The correct insert was confirmed by nucleotide sequencing.

Plasmids that expressed CpGV-ORF46 (pET32a-CpGV-46-HA w/o sig. seq.) or CpGV-46-Mut (pET32a-CpGV-46-Mut-HA), carrying a glutamic acid to glutamine substitution in the zinc binding motif, in E. coli, were constructed. pET32a-CpGV-46-HA w/o sig. seq. was generated by using primers Ncol-CpGV46HAw/o/sig-seq-F (5’-TACGGGCGGGCTGTTGTAACATGTCGCTCA-3’) and NotI-CpGV46HAw/o/sig-seq-R (5’-ATAATATATATCTGCCGCGCCGCTAATTTGCGGCTA-3’) to generate a PCR product of CpGV-ORF46-HA without its signal sequence (54 nucleotides) using pCRII-CpGV-46-HA as a template. The PCR product was digested with Ncol and NotI, and the fragment was inserted into the pET32a vector (Novagen). pET32a-CpGV-46-Mut-HA was constructed by substituting a cysteine nucleotide at position 37692 in the CpGV genome (NC_002816.1) to guanine by site-directed mutagenesis (Chiu et al., 2004), using pET32a-CpGV-46-HA w/o sig. seq. as a template and the following four mutagenesis primers: CpGV-46-781C-R (5’-TACGGGCGGGCTGTTGTAACATGTCGCTCA-3’), CpGV-46-781C-F-S (5’-TACGGGCGGGCTGTTGTAACATGTCGCTCA-3’), CpGV-46-781C-F-S (5’-TACGGGCGGGCTGTTGTAACATGTCGCTCA-3’), and CpGV-46-781C-R-S (5’-TACGGGCGGGCTGTTGTAACATGTCGCTCA-3’). DNA sequencing was performed on the final clone to verify the incorporation of the mutation.

To produce CpGV-MMP and CpGV-MMP-Mut, BL21 (DE3)/pLysS competent cells were transformed with plasmid pET32a-CpGV-46-HA w/o sig. seq. or pET32a-CpGV-46-Mut-HA, respectively, and grown at 37 °C in Luria Bertani (LB) broth, containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol, until they reached an optical density (OD600) of 0.6. Gene expression was induced by adding isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM and incubating at 18 °C overnight. To purify proteins, BL21 (DE3)/pLysS cells (2 l) with pET32a-CpGV-46-HA w/o sig. seq. or pET32a-CpGV-46-Mut-HA were harvested by centrifugation at 5000 × g for 15 min at 4 °C. The cell pellet was resuspended in 50 ml of cold extraction buffer (500 mM NaCl, 50 mM Na2HPO4, pH 7.5), containing a protease inhibitor cocktail tablet (Roche), and cells were disrupted by 12 10-s sonication bursts at maximum intensity. The lysate was clarified by centrifugation at 15,000 × g for 30 min at 4 °C and mixed with 500 μl Talon metal affinity resin (Clontech) that had been pre-equilibrated with extraction buffer. The lysate-resin mixture was incubated for 3 h at 4 °C with gentle agitation, transferred to a column, washed three times with extraction buffer, and protein eluted sequentially with 600 μl of extraction buffer containing 50, 100, 200, or 300 mM imidazole. Concentrations of eluted proteins were determined using a BCA protein assay kit (Thermo Scientific).

MMP activity assays

MMP activity was measured using the SensoLyte 520 generic MMP assay kit (AnaSpec), according to the instructions of the manufacturer. Briefly, purified proteins (30 ng), containing His-tagged CpGV-MMP, His-tagged CpGV-MMP-Mut, induced pET32a empty vector proteins, or control human MMP-9 (30 ng; AnaSpec) were diluted in MMP assay buffer (200 mM NaCl, 5 mM CaCl2, 20 μM ZnSO4, 0.05% Brij 35, and 50 mM Tris–HCl, pH 7.5) to a final volume (50 μl). Proteins were mixed with generic fluorescent MMP substrate (50 μl; 5-FAM/QLL[Ms20]) in 96-well microtiter plates and incubated for 15 min at 37 °C. To test for MMP specific activity, GM6001 (400 μM; broad-spectrum MMP inhibitor; Millipore) and EDTA (metal chelator) were used to inhibit MMP activity. Fluorescence measurements were performed every 5 min for 2 h using a Wallac Victor3™ (PerkinElmer) plate reader at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Bacmid construction

The bacmid Ac-CpGV-ORF46-PG was constructed by inserting CpGV-ORF46, polyhedrin (polyh) and enhanced green fluorescent
protein (egfp) genes into the polh locus of the AcMNPV bacmid bMON14272 (Invitrogen) by site-specific transposition (Luckow et al., 1993). To this end, primers Bsu36I START-CpGVorf46HA F (5'-TCCGACACCTCCTGTAAGGACGGCGCTAATTTCGGTGTGATTCTTGTCG-3') and CpGVorf46HA-STOP Bsu36I R (5'-CTGAAACCTCTCCTAAGAAGCCGCGCTAATTTCGGTGTGATTCTTGTCG-3') were used to PCR-amply CpGV-ORF46-PG from pCRII-CpGV-46-HA. The PCR product was digested with Bsu36I, and the resulting fragment was inserted into pHSGFP (Crouch and Passarelli, 2002), which had been digested with the same enzyme, to generate pHS-CpGV-46-pA. pHSGFP contains egfp under the control of the Drosophila heat shock protein 70 (hs70) gene promoter and a polyadenylation signal derived from the Orgyia pseudosugata MNPV inhibitor of apoptosis gene (Oiap-pA). The egfp was replaced with CpGV-ORF46-PA to generate pHS-CpGV-46-pA. Next, the hs70 promoter-CpGV-46-PG helper plasmid region was replaced with the v-chitinase promoter and replaced with the CpGV-46-pA to generate Ac-CpGV-ORF46-PG. Transposon vectors pFB-HSP70-ChiAΔKDEL-PG and pFB-HSP70-ChiA-PG were used to produce Ac-ΔCC-ChiAΔKDEL-PG and Ac-ΔCC-ChiA-PG, respectively. In both vectors, v-chitinase was under the control of the Drosophila hs70 gene promoter. The transposition vector pFB-HSP70-ChiAΔKDEL-PG was constructed by cloning the Drosophila hs70 gene promoter with Xbal at both ends into the multiple cloning site of pFB-PG-pA (Wu and Passarelli, 2010). The CHIAΔKDEL vector (v-chitinase without the KDEL coding sequence) was amplified from the AcMNPV genome using primers ChiAfSac (5'-AAAGGCTCAATTGTTAATTTGTTAACAG-3') and ChiAKDEL rev Xbal (5'-TTTTTCTAGAATGTGTTAATTTGTTAACAG-3') and cloned into the SacI and Xbal sites at the multiple cloning sites in pFB-PG-pA. The transposition vector, pFB-HSP70-ChiA-PG, used to generate Ac-ΔCC-ChiA-PG was constructed in a similar manner; however, the entire v-chitinase sequence was amplified from AcMNPV using primers ChiAsac (5'-AAAGGCTCAATTGTTAATTTGTTAACAG-3') and ChiAWtKDEL rev Xbal (5'-TTTAATCTAGAATGTGTTAATTTGTTAACAG-3').

**Budded virus production from transfected bacmid DNA**

SF9 cells (1.0 × 10^6 cells/35-mm-diameter dish) were transfected with 1 μg of bacmid DNA using a non-commercial liposome reagent (Crouch and Passarelli, 2002). Cells were incubated at 27 °C for 5 h with the DNA/liposome mixture and then washed twice with TC-100 medium and replenished with 0.5 ml of fresh TC-100 medium. Optionally, media were added at 27 °C for 100 medium and replenished with 2 ml of fresh TC-100 medium supplemented with 10% fetal bovine serum. BV in the media was harvested after 72 h and passed twice to scale up virus before titers were determined by TCID50 endpoint dilution assays (O'Reilly et al., 1994). This stock was used in further experimentation.

**Virus growth curve analyses**

SF9 cells were infected at an MOI of 5 PFU/cell with the indicated viruses. Supernatant aliquots containing BV were collected at different times p.i. and titers were determined by TCID50 assays. Two independent virus growth curves for each virus were performed. The results were analyzed using GraphPad Prism, version 5.01 (GraphPad Software, Inc.).

**Analysis of CpGV-MMP synthesis during virus infection**

SF9 cells (1.0 × 10^6 cells/35-mm diameter dish) were infected at an MOI of 5 PFU/cell with Ac-CpGV-ORF46-PG or control AcWT-PG. At different time points, cells or extracellular media aliquots were collected. Extracellular media was centrifuged at 1000 × g for 5 min at 4 °C and the cleared extracellular media was transferred to different tubes. Cell pellets were washed twice with phosphate buffered saline (PBS; 1 mM Na2HPO4/7H2O, 10.5 mM KH2PO4, 140 mM NaCl, 40 mM KCl, pH 6.2) and resuspended in PBS. Cell pellets or media were mixed with an equal volume of 2 × Laemmli buffer (0.25 M Tris–Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue). Proteins were analyzed by SDS–10% PAGE followed by immunoblotting using anti-HA antibody.
N-glycosylation analysis of CpGV-MMP

Sf9 cells were infected at an MOI of 5 PFU/cell with Ac-CpGV-ORF46-PG. Cells and extracellular media were separately collected at 24 h p.i. and centrifuged at 1000 x g for 5 min at 4 °C. Cell pellets were washed twice with PBS, pH 6.2, and resuspended in sterile distilled water. Resuspended cells and extracellular media devoid of cellular debris were individually incubated for 10 min at 100 °C with glycoprotein denaturing buffer (New England Biolabs). After incubation, G7 buffer (50 mM NaPO4, pH 7.5; New England Biolabs), NP-40, and PNGase F (New England Biolabs) were added and the mixture incubated at 37 °C for 4 h. Proteins in the treated samples were separated by SDS-PAGE, followed by immunoblotting using anti-HA antibody.

Immunoblotting

Protein samples were mixed with equal volume of 2 x Laemmli buffer and incubated at 100 °C for 10 min. Proteins were resolved on SDS-10% PAGE and then transferred to a PVDF membrane (Millipore). Membranes were incubated with the indicated primary antibodies: mouse monoclonal anti-His (Santa Cruz Biotechnology), mouse monoclonal anti-HA, (Covance), rabbit polyclonal VP39 antisemur (Li et al., 2007) or a specific v-chitinase antibody (provided by Susumu Katsuma; Daimon et al., 2006). Membranes were then treated with horseradish peroxidase-conjugated secondary antibodies (Sigma). SuperSignal West Pico chemiluminescent substrate (Pierce) and X-ray films were used for protein immunodetection and visualization.

Imaging of cells expressing eGFP

Cells were visualized with a Nikon Eclipse TE200 microscope (40x lens). Images were obtained with a Nikon Coolpix 955 camera at maximum resolution.

Oclusion body purification and bioassays with Trichoplusia ni larvae

T. ni larvae (4th instar) were fed with artificial diet contaminated with occlusion bodies that had been purified from virus-infected Sf9 cells (O'Reilly et al., 1994). Infected larvae were used to obtain a larger stock of occlusion bodies from insect cadavers. Purified occlusion bodies were resuspended in distilled water and enumerated using a hemocytometer. For bioassays, early 4th instar T. ni larvae (55 larvae per virus) were maintained in individual 1-oz plastic cups and fed with a small cube of artificial diet containing 500 occlusion bodies of the designated virus (AcWT-PG, Ac-CpGV-ORF46-PG, Ac-ΔCC-CpGV-ORF46-PG, Ac-ΔCC-P-G, Ac-Δcat-CpGV-ORF46-PG or Ac-p6.9-chiA. Ac-p6.9-chiA expresses v-chitinase under the control of the late core protein p6.9 gene promoter and does not express v-cathepsin due to a deficiency in its native promoter; Hodgson et al., 2007). Mock-infected larvae (55) were fed uncontaminated food. Larvae that consumed all of the diet after 12 h were transferred to uncontaminated diet and kept in the incubator until death or pupation. Mortality, melanization, and liquefaction were scored at 12-h intervals. Two independent bioassays were conducted and results were analyzed using probit analysis in SAS 9.2 (SAS Institute, Cary, NC).

Phylogenetic analysis

Sequence alignments and a phylogenetic tree were derived from MEGA6 (Tamura et al., 2013). MMP accession numbers used for the analyses were as follows: Cydia pomonella granulovirus (CpGV: NP_148830.1), Cryptophlebia leucotreta granulovirus (CrlgGV: NP_891890.1), Pieris rapae granulovirus (PrGV: YP_0034293611.1), Choristoneura occidentalis granulovirus (ChocGV: YP_6544541), Chlorella anachoretica granulovirus (CancGV: YP_004376233.1), Phthorimaea operculina granulovirus (PhopGV: NP_66-32061), Helicoverpa armigera granulovirus (HearGV: YP_001649-0201), Agrotis segetum granulovirus (AgseGV: YP_006303.1), Adoxophyes orana granulovirus (AdorGV: NP_872491.1), Pseudoletia unipuncta granulovirus (PsunGV: YP_0034223771.1), estia c-nigrum granulovirus (XcGV: NP_0591881.1), Spodoptera litura granulovirus (SplGV: YP_001259688.1), Plutella xylostella granulovirus (PxGV: NP_0682541), Epiinopa aporema granulovirus (EapGV: YP_006908552.1), Clostera anastomosis (L) granulovirus (CaLGV: YP_008719974.1), D. melanogaster (Dm: MMP-1: YP_7264732.1, MMP-2: YP_9957881.1), T. castaneum (Trib: MMP-1: YP_001157646.1, MMP-2: EFA09264.1, MMP3: XP_9721461.1), Anopheles gambiae (Ag: MMP-1: XP_001688107.1, MMP-2: XP_320653.4, MMP-3: XP_55330.4, M. sexta (Mand: MMP-1: AEQ27775.1, B. mori (Bm: MMP-1: YP_001164991.1, MMP-2: YP_0049322981.1, MMP-3: XP_004920581.1), and Hydra vulgaris (Hydra: AAD58041.0).

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