FUNCTIONAL CHARACTERIZATION OF THE *CYDIA POMONELLA* GRANULOVIRUS MATRIX METALLOPROTEASE

by

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ABSTRACT

Cydia pomonella granulovirus (CpGV) is a member of the *Baculoviridae* family of viruses. The CpGV open reading frame 46 (CpGV-ORF46) predicts a 545 amino acid protein that shares homology with matrix metalloproteases (MMPs), a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins. *In silico* analyses revealed the presence of putative *mmp* genes in all species from the *Betabaculovirus* genus, while no *mmps* were identified in members of the *Alphabaculovirus*, *Gammabaculovirus* or *Deltabaculovirus* genera. Unlike most cellular MMPs, baculovirus MMPs do not have a propeptide domain, a domain involved in regulating MMP activation, or a hemopexin-like domain, which is necessary for substrate binding and specificity in many MMPs. However, *Betabaculovirus* MMPs do contain a predicted conserved zinc-binding motif (HEXGHXXGXXHS/T) within their catalytic domain. The function of CpGV-MMP and its effects on baculovirus replication in cultured cells and insect larvae were investigated. CpGV-MMP was expressed in and purified from *Escherichia coli*, and activity was measured using a generic MMP substrate *in vitro*. CpGV-MMP had *in vitro* activity and its activity was specifically inhibited by MMP inhibitors. To study the effects of CpGV-MMP on virus replication and dissemination, CpGV-MMP was expressed from *Autographa californica* nucleopolyhedrovirus (AcMNPV) under the control of a strong and constitutive promoter, the *Drosophila* heat shock 70 protein promoter. Expression of CpGV-MMP did not affect virus replication in cultured cells. The effects of expressing CpGV-MMP from AcMNPV during larval infection were evaluated in the presence or absence of the AcMNPV chitinase and cathepsin genes. Insect bioassays showed that the absence of cathepsin resulted in a

significant delay in larval time of death; however, this delay was compensated by expression of CpGV-MMP. In addition, larval time of death was accelerated when cathepsin, chitinase, and CpGV-MMP were all expressed. Finally, we determined the effects of CpGV-MMP on larvae melanization and liquefaction. CpGV-MMP was able to promote larvae melanization in the absence of cathepsin. CpGV-MMP, in the absence of cathepsin, was not able to promote larvae liquefaction. When chitinase was engineered to be secreted from cells, CpGV-MMP rescued liquefaction in the absence of cathepsin. In conclusion, CpGV-MMP is a functional MMP which can enhance larvae mortality with the presence of cathepsin. In addition, CpGV-MMP can promote larvae melanization; however, it can only promote liquefaction when chitinase is engineered to be secreted from cells.

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Chapter 1- Literature Review

1. BACULOVIRUSES

Baculoviruses are a large group of arthropod-specific viruses which primarily infect lepidopteran larvae. They contain large circular double-stranded DNA genomes with sizes ranging from 80 to over 180 kbp. Baculovirus genomes can encode up to 181 distinct open reading frames (ORFs) from either strand. Their genomes are packaged into an enveloped rod-shaped capsid, hence their name “baculovirus” (Rohrmann, 2013). Baculoviruses can produce two different types of virions: budded virions (BV) and occlusion-derived virions (ODV). BVs bud from infected cells to infect other cells; therefore, they allow the virus to spread from cell to cell. ODVs are embedded in a matrix protein to form occlusion bodies. Occlusion bodies remain inside infected cells and are only released after cell lysis. ODVs in occlusion bodies are the form of the virus found in the environment and are responsible for initiating midgut infection. Based on the morphology of their occlusion bodies, baculoviruses are divided into two major groups: nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). NPVs form large occlusion bodies (0.15 to 3 μm in size) containing one (SNPV) or multiple (MNPV) nucleocapsids per envelope. The enveloped nucleocapsids are embedded in a polyhedrin matrix. GVs, on the other hand, form smaller ovoid occlusion bodies (0.13× 0.50 μm in size); their envelope contains a single nucleocapsid embedded in a granulin matrix (Ackermann and Smirnoff, 1983; van Oers and Vlak, 2007). Polyhedrin (in NPVs) and granulin (in GVs) are viral proteins that constitute the major structural component of occlusion bodies. They form a lattice that surrounds embedded virions. The polyhedrin matrix has a crystalline appearance while the granulin matrix has a granular appearance (Funk et al., 1997)

The *Baculoviridae* family is comprised of four genera based on phylogenetic analyses, host specificity and virion morphology: *Alphabaculovirus* (lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs) (Jehle et al., 2006). Most baculoviruses characterized to date are alphabaculoviruses and betabaculoviruses. The most studied baculovirus is *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). It belongs to the *Alphabaculovirus* genus and its 130 kbp genome contains more than 150 ORFs (Ayres et al., 1994). AcMNPV is usually considered the type baculovirus species.

Contrary to many viruses that are studied because of their detrimental effects on a host, baculovirus studies are mainly motivated by their use as biopesticides and expression vectors. Baculoviruses have long been considered a safe and selective alternative to chemical pesticides. They have the advantage of infecting a select number of insect species and being harmless to vertebrates and other invertebrates. They have been used for many years to control pests in crops, forests, and pastures (Szewczyk et al., 2006). In addition, baculoviruses, especially AcMNPV, have been used as gene expression vectors to generate large quantities of recombinant proteins that can be used in biomolecular research (Smith et al., 1992).

1.1. Baculovirus infection and pathogenesis

Infection starts when larvae ingest occlusion bodies. Occlusion bodies are dissolved in the alkaline midgut, releasing ODVs. The released ODVs must then cross the peritrophic membrane that protects midgut epithelial cells. Some baculoviruses are

known to encode a protein called enhancin, a metalloprotease which concentrates in occlusion bodies and helps digest the peritrophic membrane thus facilitating infection of midgut epithelial cells (Lepore et al., 1996; Wang et al., 1994). However, not all baculoviruses encode enhancin, therefore it is not well understood how baculoviruses cross the peritrophic membrane. After crossing the peritrophic membrane, virions attach to and enter midgut epithelial cells by fusion of viral and cellular membranes. Nucleocapsids are then transported to the nucleus via actin cables where they are unpackaged and release their genome (Goley et al., 2006). In the nucleus, viral genes are expressed and viral DNA is replicated. Viral genes with early promoters are transcribed at early times post infection by the host RNA polymerase II complex. Later in the infection, transcription is carried out by the viral RNA polymerase (Fuchs et al., 1983).

Baculoviruses are characterized by a biphasic replication cycle, which results in the production of two virion phenotypes: BVs and ODVs. The first BVs are produced approximately 12 to 18 hours post infection; they are exported into the cytoplasm and bud from the cell plasma membrane from which they acquire their envelope. BVs envelopes contain a fusion protein (GP64 or F protein) which mediates the attachment and entry of BVs (Liang et al., 2005; Pearson and Rohrmann, 2002). BVs allow the virus to spread beyond the initial site of infection and infect other larvae tissues thus causing a systemic infection. Once released into the extracellular space, BVs are thought to spread to other larval tissues through the tracheal (respiratory) system (Engelhard et al., 1994). When they reach the hemocoel, BVs can easily be spread via the circulatory system. ODV production begins very late in the infection process (at approximately 24

to 72 hours post-infection). Nucleocapsids start to accumulate in the nucleus and acquire envelopes from the host cell nuclear membrane (Braunagel and Summers, 1994). By acquiring envelopes, nucleocapsids become ODVs. ODVs are then packaged in a protein matrix (polyhedrin for NPVs and granulin for GVs) to form occlusion bodies. In most GVs, occlusion bodies are produced in both the nucleus and the cytoplasm (Vialard et al., 1995). Occlusion bodies are released by lysis of infected cells and spread into the environment after disintegration and liquefaction of the host.

Larvae liquefaction is facilitated by two virus-encoded proteins: viral cathepsin (v-cathepsin) and viral chitinase (v-chitinase). v-chitinase and v-cathepsin are not essential for virus replication; however, they are required for larvae liquefaction and thus virus horizontal transmission (Hawtin et al., 1997; Ohkawa et al., 1994).

It is important to note that unlike lepidopteran baculoviruses (alphabaculoviruses and betabaculoviruses), deltabaculoviruses and gammabaculoviruses infection is restricted to midgut epithelial cells. These viruses do not mount a systemic infection; they instead cause diarrhea which helps spread occlusion bodies to new hosts (Fisher et al., 1999; Moser et al., 2001).

1.2. *Cydia pomonella* granulovirus

Cydia pomonella granulovirus (CpGV) is a member of the *Baculoviridae* family; belonging to the *Betabaculovirus* genus. CpGV is highly pathogenic to the codling moth (*Cydia pomonella*) which causes significant damage to apples, pear and walnut trees (Cross et al., 1999). The CpGV double-stranded DNA genome is 123,500 bp long and contains 143 ORFs (Luque et al., 2001).

It is important to note that most baculovirus studies have been done with NPVs because cell lines to efficiently propagate and purify GVs are lacking. Few studies of *in vitro* GV infection have been reported and in all cases the cell lines had low susceptibility and/or were unstable (Dwyer et al., 1988; Granados et al., 1986; Miltenburger et al., 1984; Naser et al., 1984). Therefore, not much is known about GV replication One major difference between NPV and GV replication is that GVs cause disruption of the nuclear membrane in the late phases of the replication process; occlusion body formation occurs in the mixed nuclear and cytoplasmic region (Winstanley and Crook, 1993).

2. MATRIX METALLOPROTEASES

Matrix metalloproteases (MMPs) are a family of zinc-dependent (hence, the prefix "metallo") proteases that are involved in the degradation and turnover of extracellular matrix proteins. MMP genes are found in a wide variety of organisms; they have been identified in vertebrates, invertebrates and plants. Most of our knowledge on MMPs has been gathered from studies in mammals; however, a number of studies have also been conducted in other organisms such as *Drosophila melanogaster* (Page-McCaw et al., 2003), *Tribolium castaneum* (Knorr et al., 2009), *Caenorhabditis elegans* (Wada et al., 1998), sea urchin (Lepage and Gache, 1990), green alga (Kinoshita et al., 1992) and soybean (Pak et al., 1997).

By remodeling the extracellular matrix, MMPs play a central role in normal physiological processes such as embryonic development, morphogenesis, tissue resorption, angiogenesis, wound healing, bone remodeling, and limb regeneration. However,

accelerated and uncontrolled MMP activity can contribute to pathologies. MMPs have been implicated in numerous pathological processes including tumor progression, fibrosis, chronic inflammation, cancer, cardiovascular and neurological diseases, bacterial and viral infections, liver fibrosis, arthritis and more (Parks and Mecham, 1998).

2.1. General MMP domain organization

MMPs are secreted or membrane-associated proteins. They therefore possess a signal peptide sequence at their N-terminal end that targets them to the secretory pathway. The majority of MMP family members contain three distinctive and well-conserved domains.

A) The propeptide domain: This domain consists of approximately 80 to 90 amino acids. It is located at the N-terminal end of the MMP polypeptide sequence downstream of the signal peptide sequence. This domain contains a conserved cysteine switch motif (PRCGV/NPD). This motif is involved in maintaining MMP latency. The cysteine residue within the conserved sequence interacts with the zinc ion (Zn^{2+}) in the catalytic site rendering the active site unavailable for substrate binding (Van Wart and Birkedal-Hansen, 1990). MMPs are therefore synthesized as zymogens and remain inactive until the cysteine-zinc interaction is removed by proteolysis or by conformational perturbation.

B) The MMP catalytic domain: The catalytic domain is about 170 amino acids long and is present immediately downstream of the propeptide domain. The catalytic domain is characterized by a conserved zinc binding motif (HEXGHXXGXXHS/T). The three histidine residues in the consensus sequence act as ligands of the zinc ion (Zn^{2+}) cofactor. The glutamate residue activates a zinc-bound H_2O molecule providing the nucleophile that cleaves peptide bonds (Dhanaraj et al., 1996). In addition, the MMP catalytic domain has a conserved methionine-containing turn (Met-turn) region downstream of the zinc binding motif. The methionine residue in the Met-turn region 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). The zinc binding domain and the Met-turn are also found in three other metalloprotease families: serralysins, astacins and ADAMs. Together with MMPs, these enzymes form the larger metzincin family. MMPs differ from the other family members by having a serine or threonine residue following the third histidine in the zinc binding domain (Hooper, 1994).

(C) The hemopexin-like domain: This domain structurally resembles the human hemopexin protein which binds heme and transports it to the liver for breakdown. The MMP hemopexin-like domain consists of four hemopexin repeats. It is approximately 210 amino acids long and located at the C-terminal end of the MMP polypeptide sequence. The hemopexin-like domain contributes to proper substrate recognition, substrate specificity and binding to tissue inhibitors of MMPs (TIMPs) (Murphy et al., 1992).

The hemopexin domain is connected to the catalytic domain by a proline rich linker or hinge region (~75 amino acids long) whose function is not known.

2.2. MMP subfamilies

MMPs have been classified into five main subfamilies based on their primary structure, cellular localization and substrate specificity. The five MMP subfamilies are: collagenases, stromelysins, gelatinases, membrane-type MMPs and matrilysins.

Collagenases are characterized by their ability to cleave interstitial collagens I, II, and III; they contain three conserved residues (Tyrosine, Aspartic acid and Glycine) within their catalytic domain that are necessary for their enzymatic activity (Freije et al., 1994).

Stromelysins can degrade a wide range of extracellular matrix proteins. They possess an insertion of nine mainly hydrophobic residues (XPLVPTXXV) in the C-terminal end of their catalytic domain (Murphy et al., 1991).

Gelatinases digest denatured collagens and gelatin. They are characterized by three repeats of a type II fibronectin domain inserted in the catalytic domain. The fibronectin domains mediate binding to gelatin, collagen, and laminin (Allan et al., 1995).

Membrane-type MMPs (MT-MMPs) are a subgroup of cell-associated MMPs, which are anchored to the cell membrane by a C-terminal transmembrane domain or by a glycosylphosphatidylinositol (GPI) anchor. Localization of MMPs at cell surfaces allows efficient pericellular proteolysis (Fillmore et al., 2001).

Matrilysins, the smallest MMPs identified to date, are characterized by the lack of a hemopexin-like domain. They have a minimal domain organization consisting of a propeptide and a catalytic domain (Marti et al., 1992).

There are a number of MMPs which cannot be classified in any of these defined MMP subclasses. These MMPs are commonly referred to as “other MMPs”. They include MMP such as human MMP12, 19, 20, 21, 23, 27, and 28, *Drosophila melanogaster* MMP1 and 2 (Page-McCaw et al., 2003), *Tribolium castaneum* MMP1, 2 and 3 (Knorr et al., 2009)*, Galleria mellonella* MMP (Altincicek and Vilcinskas, 2008) and *Manduca sexta* MMP (Vishnuvardhan et al., 2013).

2.3. Regulation of MMP Activity

The proteolytic activities of MMPs are tightly controlled during activation from their precursors and inhibition by endogenous inhibitors.

MMP activation

In latent MMPs, the Zn^{2+} at the catalytic site is bound to the three histidine residues within the conserved zinc binding motif; in addition, the zinc ion is bound to the cysteine residue in the cysteine switch motif. This conformation makes the catalytic site unavailable for substrate binding. Destabilization or removal of the propeptide-domain makes the active site available to cleave substrates (Fig. 1.1) (Springman et al., 1990). Most MMPs are secreted as inactive proenzymes and activated extracellularly. MMPs become proteolytically active upon the removal of the prodomain or part of it by active MMPs or other proteases such as serine proteases (Van Wart and Birkedal-Hansen, 1990).

Some MMPs including MT-MMPs have a furin recognition sequence (KXR/KR) at the C-terminal end of their propeptide domain. These MMPs are activated intracellularly in the

Golgi apparatus through cleavage of the furin recognition motif by furin-like proteinases (Suzuki et al., 1990).

In vitro, MMPs can be activated by chemical agents such as thiol-modifying agents (4-aminophenylmercuric acetate, $HgCl_2$, and N-ethylmaleimide), oxidized glutathione, sodium dodecyl sulfate (SDS), chaotropic agents, and reactive oxygens. These chemicals activate MMPs by causing a conformational change which disrupts the Cysteine-Zn^{2+} interaction allowing substrate binding (Nagase, 1997).

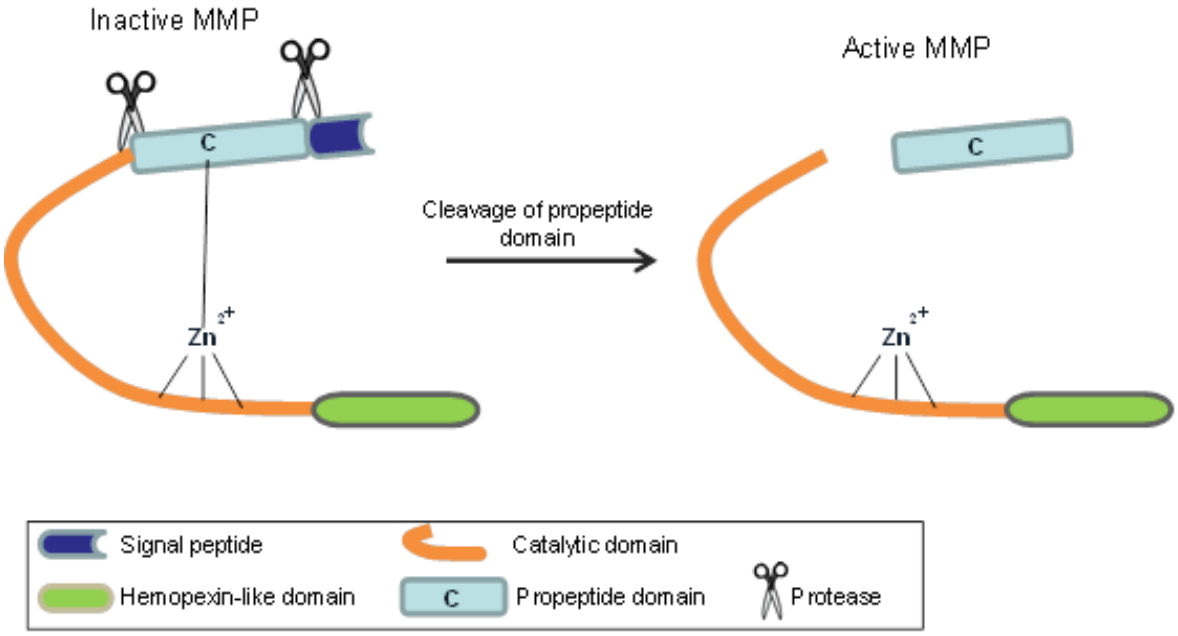

Figure 1.1- Schematic representation of MMP activation

MMPs are synthesized as inactive enzymes. In latent MMPs, the Zn^{2+} at the catalytic site is bound to the three histidine residues in the zinc binding motif and to the cysteine residue in the cysteine switch motif. This conformation renders the active site unavailable for substrate binding. MMPs become active after removal of the propeptide domain by proteases.

MMP inhibitors

Once activated, MMP proteolytic activity is regulated by tissue inhibitors of metalloproteinases (TIMPs). TIMPs are the major regulators and natural inhibitors of MMPs. They play a primary role in maintaining the balance between extracellular matrix breakdown and regeneration (Gomis-Ruth et al., 1997). TIMPs are structurally composed of two domains: an N-terminal inhibitory domain (~125 amino acids) and a C-terminal domain (~65 amino acids). TIMPs form 1:1 noncovalent, inhibitory complexes with MMPs. The TIMP inhibitory domain binds to the MMP active site; in addition, for some MMPs, the TIMP C-terminal domain binds to sites in the MMP hemopexin-like domain. The additional C-terminal interaction provides a stronger affinity between TIMPs and MMPs. (Gomez et al., 1997).

Several other proteins have been reported to inhibit MMPs. The plasma proteinase α2-macroglobulin is a general endopeptidase inhibitor that inhibits most proteinases (Herman et al., 2001). Other MMP inhibitors include the tissue factor pathway inhibitor-2, the procollagen C-terminal proteinase enhancer protein and the membrane-anchored glycoprotein, RECK. However, the mechanisms by which they inhibit MMPs are not well understood (Herman et al., 2001; Mott et al., 2000; Oh et al., 2001).

2.4. MMPs in insects and viruses

MMPs have been studied extensively in mammals, however; not much is known about MMPs in invertebrates and plants. Contrary to mammals which encode up to 24 MMP homologs, the maximum number of MMP homologs that have been characterized in an

insect species to date were found in *Tribolium castaneum* which has a total of three MMPs (Knorr et al., 2009).

In addition to having three MMP homologs, *Tribolium castaneum* also encodes one TIMP. *T*. *castaneum* MMPs have the typical propeptide domain, catalytic domain and hemopexin-like domain organization; they are involved in tracheal and gut development during embryogenesis and pupal morphogenesis. They also appear to play a role in insect innate immunity (Knorr et al., 2009).

Drosophila melanogaster has two MMPs (DmMMP1 and 2). The two *Drosophila* MMPs have a domain organization similar to that of mammalian MMPs (propeptide domain-catalytic domain-hemopexin-like domain). These MMPs are required for tissue remodeling during tracheal growth, pupal morphogenesis and metamorphosis. In addition, *D. melanogaster* encodes one TIMP that inhibits both MMP1 and 2 (Page-McCaw et al., 2003). A single MMP homolog is found in *Manduca sexta* (Vishnuvardhan et al., 2013) and *Galleria mellonella* (Altincicek and Vilcinskas, 2008). In both species, the single MMP present has the typical MMP domain organization and is involved in insect metamorphosis.

Very little is known about viral MMPs. The only study on virus MMPs to date was done in the baculovirus *Xestia c-nigrum* granulovirus (XcGV) (Ko et al., 2000). The protein encoded by ORF 40 in the XcGV genome (XcGV-MMP) shows significant homology to MMPs. XcGV-MMP has a conserved zinc binding motif within its catalytic domain. However, XcGV-MMP lacks a propeptide domain (cysteine switch motif) and a C-terminal hemopexin domain. In addition, XcGV-MMP does not have a signal peptide which is found in all MMPs characterized to date except human MMP23 (Velasco et al.,

1999). Recombinant XcGV-MMP expressed from *Bombyx mori* nucleopolyhedrovirus (BmNPV) exhibited protease activity when assayed with dye-impregnated collagen (azocoll). In insect studies, XcGV-MMP induced melanization in infected larvae (see section 3.2.1 for more information about insect melanization). The authors speculated that due to the lack of a signal peptide, XcGV-MMP might be released in the late stage of infection after cell lysis of infected cells. After release, XcGV-MMP might be involved in digestion of basement membranes (Ko et al., 2000).

3. CHITINASES AND CYSTEINE CATHEPSINS

3.1. Chitinases

Chitin is a polymer composed of *N*-acetylglucosamine residues linked by β-(1-4)-glycosidic bonds. It is the second most abundant polysaccharide in nature, after cellulose (Wills-Karp and Karp, 2004). Chitin is a major structural polymer of fungal and bacterial cell walls, crustacean eggshells, and arthropod exoskeletons (Fuhrman and Piessens, 1985; Vogan et al., 2002).

Chitinases are chitin-degrading enzymes which hydrolyze the β-(1, 4) linkages of chitin. Chitinases belong to glycohydrolase families 18 and 19 (Koga et al., 1999). Glycohydrolases are all involved in glycosidic bond hydrolysis in polysaccharides; they have been classified into over 50 families based on their amino acid sequences, substrate specificities and hydrolytic mechanisms (Henrissat and Bairoch, 1993). Chitinases are found in a wide range of organisms; family 19 chitinases are mostly found in plants while family 18 chitinases are found in bacteria, fungi, arthropods, vertebrates, and viruses (Koga et al., 1999). Chitinases usually play a role in

maintaining chitin homeostasis. In organisms that do not produce chitin, chitinases are involved in defense against chitinous pathogens (Burton and Zaccone, 2007). Viral encoded chitinases play a role in pathogenesis (Patil et al., 2000).

In insects, chitinases play an important role in postembryonic development, especially during larval molting and pupation. Insect chitinases have a molecular mass ranging between 40 and 85 kDa. On a structural level, insect chitinases are composed of three basic domains: a catalytic domain, a PEST-like region enriched in proline, glutamate, serine and threonine, and finally, a cysteine-rich region (Kramer and Muthukrishnan, 1997). The catalytic domain is located in the N-terminal region of the polypeptide sequence; it possesses a TIM-barrel structure which forms a groove on the protein's surface. The groove constitutes the active site where substrates are cleaved (Lasters et al., 1988). The catalytic domain is followed by a PEST-like region which is thought to play a role in enzyme turnover by increasing the enzyme's susceptibility to proteolysis (Rechsteiner and Rogers, 1996). The chitinase C-terminal cysteine-rich region consists of six conserved cysteines; it facilitates catalysis by targeting the enzyme to its substrates (Venegas et al., 1996). It is important to note that the PEST-like or the cysteine rich regions are not conserved in all insect chitinases. These two domains are not necessary for chitinase activity (Girard and Jouanin, 1999). All insect chitinases possess an N-terminal signal peptide which mediates enzyme secretion (Kramer et al., 1993).

3.1.1. Baculovirus chitinases

Baculovirus chitinases (v-chitinase) belong to glycohydrolase family 18. Baculovirus chitinase genes are found in the majority of alphabaculoviruses and betabaculoviruses. v-chitinase amino acid sequences are well conserved (54 to 91% identity) among all characterized baculovirus chitinases (Wang et al., 2005). Similar to insect chitinases, v-chitinases have an N-terminal signal peptide (Hawtin et al., 1997; Thomas et al., 1998). However; there are differences between insect chitinases and v-chitinases. First, insect chitinases have a narrow pH range (4-5) and they usually lose their activity above pH 6.5. v-chitinases, on the other hand, can be active in pH conditions as high as 10 (Daimon et al., 2006; Rogers et al., 1986). Second, v-chitinases have a C-terminal KDEL motif signaling their retention in the endoplasmic reticulum; this motif is not found in insect chitinases (de Jong et al., 2005; Hodgson et al., 2009; Saville et al., 2002). v-chitinases are therefore retained in the endoplasmic reticulum of infected cells throughout the infection process. Active v-chitinase accumulates in the endoplasmic reticulum and is only released after lysis of infected cells (Hom et al., 2002; Thomas et al., 1998).

3.2. Cysteine cathepsins

Cathepsins are a diverse group of proteases found in a wide variety of organisms (plants, animals, bacteria and viruses); they include cysteine, serine, and aspartic proteases. Cathepsin is derived from the Greek word “kathepsein” which means “digesting”. Cathepsins are mainly enzymes of the lysosomal/endosomal system and therefore play a central role in intracellular protein degradation (Wilson et al., 2009).

The majority of cathepsins are cysteine proteases and these cathepsins are referred to as cysteine cathepsins or papain-like thiol proteases. Eleven types of cysteine cathepsin have been identified in humans (B, C, F, H, K, L, O, S, V, X, and W) (Lecaille et al., 2002).

Cysteine cathepsins are usually 23 to 30 kDa; they have a two-domain structure composed of an N-terminal propeptide domain and a C-terminal catalytic domain. A catalytic cysteine residue in the active site acts as the nucleophile to initiate the cleavage of peptide bonds in the protein substrates. Cysteine cathepsins possess an N-terminal signal peptide which targets them to the endoplasmic reticulum (Drenth et al., 1968).

The propeptide domain plays three major roles. First, it acts as a scaffold allowing folding of the catalytic domain (Schilling et al., 2001); second, it operates as a chaperone for transport of the enzyme from the endoplasmic reticulum to the endosomal/lysosomal compartment (Chapman et al., 1997). Finally, the propetide domain is involved in maintaining cysteine cathepsins in a latent state by blocking access to the substrate-binding cleft within the catalytic domain (Carmona et al., 1996). Cysteine cathepsins are thus synthesized as inactive enzymes, and their activation occurs via proteolytic removal of the propeptide domain that blocks the active site. The removal of the propeptide domain which is initiated by proteases such as pepsin, neutrophil elastase or other cysteine proteases is triggered by a pH drop and therefore occurs in compartments such as lysosomes and endosomes (Taylor et al., 1995). Cysteine cathepsins activity was historically considered to be exclusively limited to the lysosomal/endosomal compartment because cysteine cathepsins require an acidic pH

for optimal functioning. However, numerous studies have shown that cysteine cathepsins are also involved in extracellular matrix degradation (ECM). It was shown that cysteine cathepsins such as cathepsin S don't have a strict acidic pH optimum (Shi et al., 1992). In addition, overexpression of cathepsins is usually accompanied by the secretion of pro-cathepsins (Gal et al., 1985). During disease conditions, the peri and extracellular space becomes acidified, which would allow cysteine cathepsin activation and ECM degradation (Konttinen et al., 2002). Acidification of the pericellular environment also seems to allow secretion of lysosomal proteases into the extracellular space (Rozhin et al., 1994). Finally, ECM degradation can occur intracellularly by endocytosis of ECM proteins (Everts et al., 1996).

3.2.1. Baculovirus cysteine cathepsins

Cysteine cathepsins are conserved in most alphabaculoviruses and betabaculoviruses (Hill et al., 1995; Kang et al., 1998; Ohkawa et al., 1994). Similar to cellular cathepsins, baculovirus cathepsins (v-cathepsins) have a signal peptide followed by a propeptide domain and a catalytic domain. A major difference between v-cathepsins and cellular cathepsins is that v-cathepsins are not trafficked to the endosomal/lysosomal compartment. They accumulate in the endoplasmic reticulum of infected cells (Hodgson et al., 2009; Hom and Volkman, 2000). AcMNPV v-cathepsin can be detected in the endoplasmic reticulum starting at 18 h.p.i. (Slack et al., 1995); it accumulates in the endoplasmic reticulum as an inactive proenzyme. A molecular interaction between v-chitinase and v-cathepsin within the endoplasmic reticulum enables cathepsin retention (Hodgson et al., 2011). v-chitinase is retained in endoplasmic reticulum because of its

C-terminal KDEL retention motif (Saville et al., 2002). During the late phase of infection, an unknown cellular factor triggers the proteolytic removal of the v-cathepsin propetide domain and virus-induced cell lysis ensues resulting in the release of active v-cathepsin and v-chitinase into the extracellular space (Hom et al., 2002). Therefore, contrary to cellular cathepsins, which are activated in the endosomal/lysosomal compartment, v-cathepsins are activated in the endoplasmic reticulum upon cell lysis. v-cathepsins are mainly involved in the degradation of host tissues. Together with v-chitinases which digest the larvae exoskeleton, they enable host liquefaction. In addition, v-cathepsins are involved in larval melanization which causes the insect cuticle to darken and turn completely black shortly after death. Larvae infected with an AcMNPV v-cathepsin mutant did not melanized, but instead maintained their regular greenish appearance (Slack et al., 1995). Similar findings were reported with BmNPV (Ohkawa et al., 1994) and CpGV (Kang et al., 1998).

Insect melanization is a component of the insect innate immune system. It consists of melanin deposition around the damaged tissue or intruding object (Soderhall and Cerenius, 1998). This process is controlled by the active form of the enzyme phenoloxidase, which is present in the hemolymph and the cuticle. Active phenoloxidase catalyzes the oxidation of monophenols which produce quinones. The presence of quinone molecules lead to formation of melanin which is deposited at the injury site or around the intruding microorganisms (Fujimoto et al., 1993). Melanin will then protect against the intruder by preventing or retarding its growth or help repair the damaged tissue. Activation of pro-phenoloxidase into phenoloxidase is controlled by a cascade of proteases (mainly serine proteases) found in the cuticle or hemolymph that

become active in the presence of microbial products associated with microorganisms (e.g., β-1,3-glucans, lipopolysaccharides, and peptidoglycans) or endogenous factors produced after tissue damage (Lee et al., 2002). Uncontrolled activation of pro-phenoloxidase and excessive production of melanin can have deadly consequences for the insect (Kan et al., 2008).

3.3. Role of v-chitinase and v-cathepsin in larvae liquefaction

v-cathepsin and v-chitinase are found in the majority of lepidopteran baculoviruses. All baculoviruses that encode v-cathepsin also encode v-chitinase, except for *Adoxophyes honmai* nucleopolyhedrovirus (AdhoNPV) which only encodes v-cathepsin (Nakai et al., 2003).

During larval liquefaction, the cuticle weakens and becomes brittle due to degradation of larvae tissues and the exoskeleton; finally the cuticle tears and liquefied tissues ooze out. Larvae liquefaction is dependent on the presence of both v-cathepsin and v-chitinase. Larvae infected with a virus lacking AcMNPV v-chitinase or v-cathepsin did not liquefy; however, when both mutants were used to co-infect larvae, liquefaction was restored (Hawtin et al., 1997). Similar results were observed with BmNPV (Ohkawa et al., 1994; Wang et al., 2005) and CpGV (Kang et al., 1998).

Deltabaculoviruses and gammabaculoviruses do not encode v-cathepsin and v-chitinase. As stated before, their infection is restricted to the midgut and they do not liquefy their hosts (Fisher et al., 1999; Moser et al., 2001).

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Chapter 2 - *Cydia pomonella* granulovirus matrix metalloprotease (MMP), an MMP lacking conserved MMP domains, plays a potential role in enhancing virus infection

ABSTRACT

The CpGV open reading frame 46 (*CpGV-ORF46*) predicts homology with matrix metalloproteases (MMPs), a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins. Bacterially purified CpGV-MMP exhibited MMP activity *in vitro*. To study the effects of CpGV-MMP on virus replication and dissemination, we constructed a recombinant *Autographa californica* M nucleopolyhedrovirus (AcMNPV) expressing *CpGV-ORF46*. The replication of this recombinant virus did not differ significantly from that of control AcMNPV in cultured cells. We also evaluated the effects of CpGV-MMP, cathepsin and chitinase on virus infection in *Trichoplusia ni* larvae, since these are also degradative enzymes that digest extracellular matrix components or the chitinous exoskeleton of insect hosts, respectively. Insect bioassays showed that the absence of cathepsin resulted in a significant delay in larval time of death; however, this delay could be compensated by expression of CpGV-MMP. The expression of both cathepsin and CpGV-MMP accelerated larvae lethality. Finally, CpGV-MMP was able to promote larvae melanization; however, CpGV-MMP was only able to promote larvae liquefaction when chitinase, which is usually retained in the endoplasmic reticulum of infected cells, was engineered to be secreted.

INTRODUCTION

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

Cydia pomonella granulovirus (CpGV) belongs to the *Baculoviridae* family of viruses. Baculoviruses are arthropod-specific viruses which primarily infect lepidopteran larvae. They contain large circular double-stranded DNA genomes (80 to 180 kbp) which are packaged into rod-shaped capsids (Rohrmann, 2013). Baculoviruses have been divided into four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* (Jehle et al., 2006). CpGV belongs to the *Betabaculovirus* genus; its 123,500 bp-genome contains 143 open reading frames (ORFs). At the sequence level, ORF46 in the CpGV genome (*CpGV-ORF46*) shows significant similarity to MMPs (Luque et al., 2001).

To date there has been only one study on viral MMPs, which was done in the baculovirus *Xestia c-nigrum* granulovirus (XcGV). XcGV-MMP is thought to be involved in degradation of host basement membranes during the late stages of infection (Ko et al., 2000).

Two types of degradative enzymes have been reported in baculoviruses. Cathepsin (v-cathepsin) is involved in the degradation of larval tissues. Host tissue degradation facilitates horizontal transmission of the virus to other hosts (Kang et al., 1998; Slack et al., 1995). Chitinase (v-chitinase) is another enzyme involved in virus horizontal transmission. v-chitinase digests chitin, the main component of the insect exoskeleton. 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., 1998).

In this study, we analyzed the phylogenetic relationships between CpGV-MMP and other baculovirus MMP homologs. We tested the enzymatic activity of CpGV-MMP *in vitro*. Finally, we determined the effects of *Autographa californica* nucleopolyhedrovirus (AcMNPV) expressing *CpGV-ORF46* on cultured cells and insect hosts in the presence or absence of *v-chitinase* and *v-cathepsin* to evaluate their role in insect mortality as well as liquefaction and melanization post death.

MATERIALS AND METHODS

Cells lines, bacmid maintenance, bacterial strain and insects

The Sf9 insect cell line (clonal isolate 9, derived from the *Spodoptera frugiperda* 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 *Escherichia coli* cells with helper plasmid pMON7124, encoding a transposase, were purchased from Invitrogen. Recombinant bacmids were maintained in DH10B cells (Invitrogen). *Trichoplusia ni* larvae were purchased from Benzon Research (Carlisle, PA) and larvae (4th instar) were reared in a 27°C chamber with a 12 h/12 h light/dark cycle.

Expression and purification of the CpGV-MMP protein in *E. coli*

CpGV-ORF46 was PCR-amplified from CpGV genomic DNA using primers NcoI-ORF46 CpGV (5'-CCATGGTAATGACGAGGCGTCTAATTTTCGTGGTGGTGA-3') and NotI-HA-ORF46 CpGV (5'-GCGGCCGCGGCGTAATCTGGGACGTCGTATGGGTAGCAAGTGTACATCAGCGGTTGTATA-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 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.

In order to construct a plasmid that expressed CpGV-ORF46 in *E. coli,* primers NcoI-CpGV46HAw/o-S-seq-F (5'-TAGCCCGACGCCATGGTAGAACGATTGAGTCTGC-3') and NotI-CpGV46HAw/o-S-seq-R (5'-ATATAATATTAACTGCGGCCGCGGCGTAATCTGGGACGTC-3') were used to generate the CpGV-ORF46-HA w/o-s-seq PCR product using pCRII-CpGV-46-HA as a

template. This PCR product does not include the first 54 nucleotides at the 5' end of *CpGV-ORF46* which encode its secretion signal sequence. The PCR product was digested with NcoI and NotI, and the resulting fragment was inserted into a pET32a vector (Novagen) to generate the expression vector pet32a-CpGV-46-HA w/o sig.seq. To express CpGV-MMP protein, BL21 (DE3)/pLysS competent cells were transformed with plasmid pet32a-CpGV-46-HA w/o sig.seq 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 (OD_{600}) of 0.6. Gene expression was induced by adding isopropyl-1-thio-b-D-galactoside (IPTG) to a final concentration of 1 mM, and cells were incubated at 18°C overnight. For protein purification, a 2-liter culture was harvested by centrifugation at 5000 × g for 15 min at 4°C. The pellet was resuspended in 50 ml of cold extraction buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 7.5), containing a protease inhibitor cocktail tablet (Roche), and cells were disrupted by twelve 10-second sonication bursts at maximum intensity. The lysate was clarified by centrifugation at 15,000 × g for 30 min at 4°C. The clarified lysate was mixed with 500 µl Talon metal affinity resin (Clontech) that had been pre-equilibrated with the extraction buffer. The mixture was incubated for 3 h at 4°C with gentle agitation, transferred to a Talon column, washed three times with extraction buffer, and eluted sequentially with 600 µl of extraction buffer containing 50, 100, 200, or 300 mM imidazole. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific).

MMP activity assays

MMP activity assays were measured using the SensoLyte 520 generic MMP assay kit (AnaSpec). The activity assays were performed according to the instructions of the manufacturer. Briefly, 30 ng of His-tagged purified CpGV-MMP or 30 ng of control human MMP9 (AnaSpec) were diluted in MMP assay buffer (200 mM NaCl, 5 mM $CaCl_2$, 20 µM $ZnS0_4$, 0.05% Brij 35, 50 mM Tris-HCl, pH 7.5) to a final volume of 50 µl. The protein samples were then mixed with 50 µl of generic fluorescent MMP substrate (5-FAM/QXL^{TM}520) in 96-well microtiter plates and incubated for 15 min at 37°C. To test for MMP specific activity, GM6001 (broad-spectrum MMP inhibitor) and EDTA (metal chelator) were used. Purified CpGV-MMP (30 ng) or control human MMP9 (30 ng) were mixed with 400 µM GM6001 (Millipore) or 50 µM EDTA and the final volume was brought to 50 µl by adding MMP assay buffer. The MMP/inhibitor mixture was incubated for 15 min at 37°C and mixed with 50 µl of generic MMP substrate in 96-well microtiter plates. To confirm that CpGV-MMP activity was not due to bacterial protein, bacterially purified pET32 vector (30ng) was incubated with the fluorescent substrate and used as a negative control. Fluorescence measurements were performed every 5 min for up to 2 h using a Wallac Victor3^{TM} (PerkinElmer) plate reader at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Construction of the Ac-CpGV-ORF46-PG and AcWT-PG bacmids

The bacmid Ac-CpGV-ORF46-PG was constructed by inserting *CpGV-ORF46*, the *polyhedrin* (*polh*) gene and the *enhanced green fluorescent protein* (*egfp*) gene 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'-TCGAGACCAGTTCCTAAGGATGACGAGGCGTCTAATTTTCGTGGTGGTGATTTTGTTGC-3') and CpGVorf46HA-STOP Bsu36I R (5'-CTGAAACTGGTCCCTAAGGTCAGGCGTAATCTGGGACGTCGTATGGGTAGCAAGTGTAC-3') were used to PCR-amplify *CpGV-ORF46-HA* 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 control of the *Drosophila* heat shock protein 70 (hsp70) promoter, and a polyadenylation signal derived from the *Orgyia pseudosugata* inhibitor of apoptosis gene (OpIAP-pA).The *egfp* was replaced with *CpGV-ORF46-HA* to generate pHS-CpGV-46-pA. Next, the hsp70 promoter-CpGV-ORF46-HA-OpIAP-pA cassette (2410 bp) was PCR-amplified from pHS-CpGV-46-pA using primers HSPromo-Insert-pA-F w/EcoRI (5'-ACGTACGTACGTGAATTCGGATCCTTAAATTGTATCCTATATTAAAACAGAAGAAAGT-3') and HSPromo-Insert-pA-R w/StuI (5'-ACGTACGTACGTAGGCCTCGAAAATCGGGCTAGATTTAAC-3'). The resulting PCR product was digested with EcoRI and StuI and cloned into the transposition vector pFB1-PH-GFP to generate pFB-PG-HS-CpGV-46-pA whose sequence was verified by nucleotide sequence analysis. pFB1-PH-GFP was constructed by modifying pFastBac1 (Invitrogen) to include two transfection markers: the *polh* gene from AcMNPV with its native promoter and the SV40 poly A signal; and the *egfp* gene under the AcMNPV *ie-1* promoter and the SV40 poly A signal (Wu et al., 2006).

DNA from bacmid bMON14272, hereafter referred to as AcBac, was electroporated into DH10B cells, containing the helper plasmid pMON7124. Electrocompetent DH10B cells containing helper plasmid pMON7124 and AcBac were transformed with pFB-PG-HS-CpGV-46-pA to generate the bacmid Ac-CpGV-ORF46-PG. Transformed cells were grown on kanamycin (50 µg/ml), gentamicin (7 µg/ml), tetracycline (10 µg/ml), 5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (X-Gal) (100 µg/ml), and IPTG (40 µg/ml) media according to the Bac-to-Bac Expression System Manual (Invitrogen). White colonies were selected, streaked onto fresh plates and the successful transposition was verified in individual colonies by PCR analysis. The correct recombinant bacmids were electroporated into *E. coli* DH10B cells and screened for tetracycline sensitivity to ensure that the isolated bacmids were free of helper plasmids. Bacmid DNA was extracted and purified with the Qiagen large-construct kit and quantified by optical density (OD) measurement.

The control bacmid AcWT-PG, which was used as a representative of the parental AcMNPV-based bacmid, was constructed similarly. Briefly, electrocompetent DH10B cells containing helper plasmid pMON7124 and AcBac were transformed with pFB1-PH-GFP to generate the bacmid AcWT-PG. Correct transposition was confirmed by PCR and a tetracycline screen was conducted to ensure that the correct bacmids were free of helper plasmids.

Generation of Ac-△cath-CpGV-ORF46-PG, a bacmid lacking *v-cathepsin*

The AcMNPV *v-cathepsin* knockout bacmid was generated through homologous recombination in *E. coli* where a large portion of the *v-cathepsin* gene (775 bp) in the

previously constructed Ac-CpGV-ORF46-PG bacmid was replaced with the *zeocin* resistance gene (*zeo*) under control of the EM7 promoter. For this, Ac-CpGV-ORF46-PG was electroporated into BJ5183 electrocompetent cells (Stratagene). Then, using the vector pIZ/V5-His (Invitrogen) as a template and primers Fwd-EM7-pIZ (5'-ATGAACAAAATTT TGTTTTATTTGTTTGTGTACGGCGTTGTAAACAGCGCGGCGTACGACCTTTTGAAA GATCTCTGCAGCACGTGTTGAC-3') and Rev Zeocin (5'-TTTGTTGTACCCTGAAAAA TCCGTCCTCTCCCCAATCCGTGCCCCAAGTGTTTTTAAAGGTCCAATATGGTAAAC AAGTTTCGAGGTCGACC-3'), a 669 bp fragment, containing the 529 bp *zeo* cassette and 70 bp of *v-cathepsin* flanking regions at each end, was amplified. Purified PCR fragment (1 µg) was used to transform BJ5183 cells containing Ac-CpGV-ORF46-PG. After incubation (4 h) in LB media at 37°C, the transformed cells were plated on LB agar plates supplemented with kanamycin (50 µg/ml) and zeocin (50 µg/ml). Colonies resistant to both antibiotics were selected for further confirmation by PCR analysis. The resulting Ac-△cath-CpGV-ORF46-PG bacmid was electroporated into *E. coli* DH10B cells and screened for tetracycline sensitivity. Bacmid DNA was extracted, purified and quantified by OD measurement.

Construction of Ac-△CC-CpGV-ORF46-PG and Ac-△CC-PG, two bacmids lacking *v-cathepsin* and *v-chitinase*

The AcBac *v-chitinase* and v-*cathepsin* deletion bacmid (AcBac△CC) was obtained from M. van Oers. It was generated through homologous recombination in *E. coli* in

which large portions of the *v-chitinase* and *v-cathepsin* genes and their promoters were replaced with the chloramphenicol resistance gene (Kaba et al., 2004). AcBac△CC was electroporated into DH10B cells containing the helper plasmid pMON7124. To generate Ac-△CC-CpGV-ORF46-PG, electrocompetent DH10B cells, containing helper plasmid pMON7124 and AcBac△CC, were transformed with pFB-PG-HS-CpGV-46-pA. Bacmid Ac-△CC-PG was produced by electroporating DH10B cells, containing helper plasmid pMON7124 and AcBac△CC, with pFB1-PH-GFP. Transformed cells were selected according to recommendation in the Bac-to-Bac Expression System Manual and successful transposition was verified by PCR analysis. The correct recombinant bacmids were transformed into *E. coli* DH10B cells and cured of helper plasmids as described above. Extracted bacmid DNA was purified and quantified by OD measurement.

Construction of Ac-△CC-ChiA△KDEL-PG and Ac-△CC-ChiA-PG bacmids

To generate Ac-△CC-ChiA△KDEL-PG and Ac-△CC-ChiA-PG, electrocompetent DH10B cells, containing helper plasmid pMON7124 and AcBac△CC (Kaba et al., 2004), were transformed with the transposition vectors pFB-HSP70-ChiA△KDEL-PG or pFB-HSP70-ChiA-PG to produce Ac-△CC-ChiA△KDEL-PG and or Ac-△CC-ChiA-PG, respectively. In both vectors, the *v-chitinase* gene is under the control of the *Drosophila* hsp70 promoter. The transposition vector pFB-HSP70-ChiA△KDEL-PG was generated by cloning the *Drosophila* hsp70 promoter with XbaI sites at both ends into the multiple cloning site of pFB-PG-pA (Wu and Passarelli, 2010). Then, the CHIA△KDEL (*v-chitinase* without the KDEL coding sequence) was amplified from the AcMNPV genome

using primers ChiAf-SacI (5'-AAAGAGCTCAAATGTTGTACAAATTGTTAAACG-3') and ChiA△KDEL rev-XbaI (5'-TTTTCTAGATTAAGGTTTAAACTGTGCGTTTATCG-3') and cloned into the SacI and XbaI sites of the multiple cloning site of pFB-PG-pA . The transposition vector, pFB-HSP70-ChiA-PG, used to generate Ac-△CC-ChiA-PG was constructed in a similar manner; however, the entire *v-chitinase* sequence (ChiA) was amplified from AcMNPV using primers ChiAfSacI (5'-AAAGAGCTCAAATGTTGTACAAATTGTTAAACG-3') and ChiAwithKDEL rev XbaI (5'-TTAATCTAGATTACAGTTCATCTTTAGGTTTAAAC-3').

Budded virus production from transfected bacmid DNA

Budded virus was produced by transfection of recombinant bacmid DNA into Sf9 cells by liposome-mediated transfection. 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 2 ml of fresh TC-100 medium supplemented with 10% fetal bovine serum. Budded virus in the media were harvested after 72 h and passaged twice to scale up before titers were determined by 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assays (O'Reilly et al., 1994).

Virus growth curve analyses

Sf9 cells were infected at a multiplicity of infection (MOI) of 5 PFU/cell. Supernatant aliquots containing budded virus were collected at different times post infection (p.i.)

and titers were determined by $TCID_{50}$ 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 the control Ac-WT-PG virus. At different time points, cells or extracellular media aliquots were collected and centrifuged at 1000 × g for 5 min at 4°C. After centrifugation, the cleared extracellular media was transferred to new tubes. Cell pellets were washed twice with phosphate buffered saline (PBS;1 mM $Na_2HPO_4 \cdot 7H_2O$, 10.5 mM KH_2PO_4, 140 mM NaCl, 40 mM KCl,), pH 6.2, and resuspended in PBS. Cell pellets or media were mixed with an equal volume of 2X 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.

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 collected at 24 h p.i. and centrifuged at 1000 × 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 were incubated for 10 min at 100°C with glycoprotein denaturing buffer (New England Biolabs). After incubation, G7 buffer (50 mM $NaPO_4$, pH 7.5; New England Biolabs), NP-40, and PNGase F (New

England Biolabs) were added to the mixture and incubated at 37°C for 4h. Proteins in the treated cell pellets were separated by SDS-PAGE, followed by immunoblotting.

Immunoblotting

Protein samples were combined with equal volumes of 2X Laemmli buffer and incubated at 100°C for 10 min. Proteins were resolved by 10% SDS-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 antiserum (Li et al., 2007) or a specific chitinase antibody (donated by Susumu Katsuma (Daimon et al., 2006)). The primary antibodies were followed by horseradish peroxidase-conjugated secondary antibodies (Sigma). SuperSignal West Pico chemiluminescent substrate (Pierce) was used for immunodetection and blots were visualized using X-ray films.

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.

Occlusion body purification and bioassays with *Trichoplusia ni*

T. ni larvae (4th instar) were transferred to artificial diet (Southland Products, Inc.) contaminated with occlusion bodies (OBs) purified from virus-infected Sf9 cells (O'Reilly et al., 1994). OBs were purified from insect cadavers, resuspended in distilled water, and quantified 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 OBs of the designated virus (Ac-WT-PG, Ac-CpGV-ORF46-PG, Ac-△CC-CpGV-ORF46-PG, Ac-△CC-PG, Ac-△cat-CpGV-ORF46-PG or Ac-p6.9-chiA. Ac-6.9-chiA expresses *v-chitinase* under the control of the late core protein *p6.9* promoter and does not express *v-cathepsin* due to a disruption of its native promoter (Hodgson et al., 2007). Mock-infected larvae were given uncontaminated food. Larvae that consumed all the diet after 12 h were then placed on 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 GraphPad Prism. The LT_{50} was determined using probit analysis in SAS 9.2 (SAS Institute, Cary NC).

Phylogenetic analysis

Sequence alignments and a phylogenetic tree were obtained using 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 (CrleGV: NP_891890.1), *Pieris rapae* granulovirus (PrGV: YP_003429361.1), *Choristoneura occidentalis* granulovirus (ChocGV: YP_654454.1), *Clostera anachoreta* granulovirus (ClanGV: YP_004376233.1), *Phthorimaea operculella* granulovirus (PhopGV: NP_663206.1), *Agrotis segetum* granulovirus (AgseGV: YP_006303.1), *Adoxophyes orana* granulovirus (AdorGV: NP_872491.1), *Pseudaletia unipuncta* granulovirus (PsunGV: YP_003422377.1), *Xestia c-nigrum*

granulovirus (XcGV: NP_059188.1), *Spodoptera litura* granulovirus (SpliGV: YP_001256988.1), *Plutella xylostella* granulovirus (PxGV: NP_068254.1), *Epinotia aporema* granulovirus (EpapGV: YP_006908552.1), *Clostera anastomosis* (L.) granulovirus (CaLGV: YP_008719974.1), *Drosophila melanogaster* (MMP1: NP_726473.2, MMP2: NP_995788.1), *Tribolium castaneum* (MMP1: NP_001157646.1, MMP2: EFA09264.1, MMP3: XP_972146.1), *Anopheles gambiae* (MMP1: XP_001688107.1, MMP2: XP_320653.4, MMP3: XP_554330.4), *Manduca sexta* (MMP1: AEQ27775.1), *Bombyx mori* (MMP1: NP_001116499.1, MMP2: XP_004932298.1, MMP3: XP_004921585.1), human MMP3 (NP_002413.1), and *Hydra vulgaris* (AAD45804.1).

RESULTS

MMPs in Betabaculoviruses

BLAST searches were performed to identify putative viral MMPs in the GeneBank™ virus protein database. Results revealed the presence of putative *mmps* in the *Baculoviridae, Ascoviridae*, *Iridoviridae*, *Hytrosaviridae*, *Entomopoxvirinae* and Nudiviruses. These six virus groups are diverse large double-stranded DNA viruses but all infect insects in several families.

In baculoviruses, MMP homologs are present in all species from the genus *Betabaculovirus* (granuloviruses) sequenced to date but absent in species from *Alphabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus*. Phylogenetic analyses suggest that all baculovirus MMPs have a common ancestor. Baculovirus MMPs cluster

more closely with the group of insect MMP3 than with the group of insect MMP1 or MMP2 (Fig. 2.1.A).

Most MMPs are secreted or membrane-associated proteins. Signal peptide prediction programs predicted the presence of a signal peptide in all baculovirus MMPs except in *Xestia c-nigrum* granulovirus (XcGV), *Pieris rapae* granulovirus (PrGV) and *Epinotia aporema* granulovirus (EpapGV) MMPs. Baculovirus MMPs and all other MMPs characterized to date carry a conserved zinc-binding motif (HEXGHXXGXXHS/T) within their catalytic domain (Fig. 2.1.B). The three underlined histidine residues act as ligands of a zinc ion (Zn^{2+}) cofactor necessary for catalysis. The serine or threonine residue following the third histidine differentiates MMPs from other metzincins (Hooper, 1994). The baculovirus MMPs predicted catalytic domains also contain a conserved methionine- turn (Met-turn) region, where the 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 contain a conserved catalytic domain, comparison of the predicted baculovirus MMP sequences with those of cellular MMPs revealed that they lack typical MMP structural features. First, baculovirus MMPs do not have a cysteine switch motif (PRCGV/NPD). This motif is conserved in the N-terminal propeptide domain of most cellular MMPs and is involved in maintaining MMP latency. Interaction between the cysteine residue in the conserved motif and Zn^{2+} in the catalytic domain renders the active-site cleft unavailable for substrate binding, thereby keeping the enzyme inactive. Disruption of the Cys-Zn^{2+} complex (*e.g.*, proteolytic cleavage, conformational perturbation, etc.) allows activation of latent MMPs (Van Wart and

Birkedal-Hansen, 1990). In addition, baculovirus MMPs lack a hemopexin-like domain at their C terminus. This domain is present in the C-terminal region of most cellular MMPs where it mediates substrate recognition, specificity, and binding to tissue inhibitors of MMPs (TIMPs) (Murphy et al., 1992). Baculovirus MMPs also possess a long C-terminal segment downstream of their catalytic domain; however, using the SMART (simple modular architecture research tool) online program (Letunic et al., 2012), no known functional domain could be identified in that region. Overall, these analyses indicate that even though baculovirus-encoded MMPs lack some cellular MMP conserved domains, baculovirus-MMPs belong to the MMP family of proteins.

Enzymatic activity of CpGV-MMP

Open reading frame 46 in the CpGV genome (1638 nucleotides) predicts a protein with a conserved MMP catalytic domain (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 to 303) catalytic domain.

To determine if CpGV-MMP was a functional MMP, *CpGV-ORF46* was cloned into the pET-32a bacterial expression vector and fused in frame to a polyhistidine tag. The recombinant protein was expressed in *E. coli*, purified using metal-affinity columns and analyzed by immunoblotting using an anti-His antibody (Fig.2.2.A). MMP activity was assayed using a synthetic quenched fluorescently-labeled generic MMP substrate (5-FAM/QXL^{TM}520). 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 increase in relative fluorescence units was measured in samples containing CpGV-MMP during the assay time course (Fig. 2.2.B). Bacterially purified pET32 vector was used as a negative control to confirm that CpGV-MMP activity was not due to bacterial protein. MMP activity of CpGV-MMP and the positive control, human MMP9, 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*.

Construction of Ac-CpGV-ORF46-PG and AcWT-PG recombinant bacmids

To investigate the effects of CpGV-MMP during virus replication in cell culture and its function in insect hosts, a recombinant AcMNPV containing *CpGV-ORF46*, Ac-CpGV-ORF46-PG, was generated (Fig. 2.3.A). Like other alphabaculoviruses, AcMNPV does not encode a native *mmp*. Briefly, *CpGV-ORF46* fused to an HA tag and expressed under control of the *Drosophila* hsp 70 promoter control, the *polh* gene driven by its promoter, and *egfp* under the baculovirus *ie-1* promoter were inserted at the *polh* locus of the AcMNPV bacmid bMON14272 by site-specific transposition. In addition, the corresponding control AcMNPV bacmid, AcWT-PG, with *polh* and *egfp* was constructed (Fig. 2.3.B).

Synthesis of CpGV-MMP during virus infection

To monitor CpGV-MMP synthesis during infection, Sf9 cells were infected with Ac-CpGV-ORF46-PG and production of HA-tagged CpGV-MMP over the course of infection was monitored by immunoblotting using an anti-HA antibody. An HA-

immunoreactive band was observed in the sample from pellet fraction as well as in that of the extracellular media from infected cells (Fig. 2.4.A) at all times tested (12-72 h p.i.). The presence of CpGV-MMP in the extracellular media collected from infected Sf9 cells confirmed secretion of CpGV-MMP. CpGV-MMP had a molecular mass of approximately 72 kDa, which was higher than the predicted mass of 65 kDa. The increased mass may be due to protein N-glycosylation. Thus, using the online tool NetNGlyc 1.0 (Chuang et al., 2012), we searched for predicted N-glycosylation sites. *In silico* results revealed the presence of eight potential N-glycosylation sites in CpGV-MMP. In order to confirm N-glycosylation of CpGV-MMP, we treated Ac-CpGV-ORF46-PG-infected cells (cell pellet and extracellular media) with PNGase F, a glycosidase that cleaves N-linked oligosaccharides from glycoproteins, and analyzed the results by immunobloting. We observed a significant reduction in molecular mass when CpGV-MMP was treated with PNGase F (Fig. 2.4.B). The molecular mass of PNGase-treated CpGV-MMP was approximately close to the predicted mass of 65 kDa. These findings indicate that CpGV-MMP is a secreted glycoprotein.

Effects of CpGV-MMP on virus replication

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 an 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, number, and gross morphology of occlusion body formation between the two viruses (Fig. 2.5.A). Budded virus from Ac-CpGV-ORF46-PG- or AcWT-PG-

infected cells (5 PFU/cell) was collected at selected time points and titers were determined by $TCID_{50}$ endpoint dilution assays. There were no significant differences in the budded virus production kinetics between Ac-CpGV-ORF46-PG and AcWT-PG (Fig. 2.5.B). Overall, CpGV-MMP had no obvious effects on virus replication in infected Sf9 cells.

Construction of Ac-△cath-CpGV-ORF46-PG, Ac-△CC-CpGV-ORF46-PG, and Ac-△CC-PG recombinant bacmids

Two degradative enzymes (v-cathepsin and v-chitinase) have previously been reported in baculoviruses. Similar to MMPs, cathepsins are proteases 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, the main component of the insect exoskeleton. These two proteins are necessary for larvae liquefaction which enables occlusion body release from the dead larval cadaver allowing efficient virus transmission to other hosts (Hawtin et al., 1997; Kang et al., 1998).

In order to determine the effects of CpGV-MMP on virus infection in insects in the absence of other proteins with potentially similar or overlapping functions, three additional recombinant viruses were generated. First, Ac-△cath-CpGV-ORF46-PG, a virus lacking *v-cathepsin* and expressing *CpGV-ORF46*, was constructed by removing a large portion of the v-*cathepsin* gene (775 bp) from Ac-CpGV-ORF46-PG by replacing it with the *zeocin* resistance gene under control of the EM7 promoter through homologous recombination (Fig. 2.3.C).

The AcMNPV *v-chitinase/v-cathepsin* double knockout bacmid (AcBac△CC) was generated by replacing v-*chitinase* and v-*cathepsin* by a chloramphenicol resistance gene (Kaba et al., 2004) (Fig. 2.3.D) and it was used as the backbone to construct two other bacmids (Ac-△CC-CpGV-ORF46-PG and Ac-△CC-PG). First, Ac-△CC-CpGV-ORF46-PG was constructed by inserting *CpGV-ORF46* under *Drosophila* hsp70 promoter control, *polh* and its promoter, and *egfp* under the *ie-1* promoter control at the *polh* locus of AcBac△CC by site-specific transposition (Fig. 2.3.E). The other bacmid, Ac-△CC-PG, lacks *v-cathepsin* and *v-chitinase* and was generated in a similar manner; however, it does not contain *CpGV-ORF46-PG* (Fig. 2.3.F). We analyzed and compared budded virus production kinetics of the recombinant viruses by infecting Sf9 cells (5 PFU/cell), collecting budded virus at different times p.i. and titering. All viruses exhibited a consistent increase in budded virus production comparable to that of AcWT-PG over the course of the infection (Fig. 2.6.A). Finally, we confirmed expression of CpGV-MMP in Sf9 cells infected with the recombinant viruses by immunoblotting analysis (Fig. 2.6.B).

Effects of CpGV-MMP on insect hosts

To evaluate the effects of CpGV-MMP on virus infection in insect larvae, we performed a bioassay in *Trichoplusia ni* using the five recombinant viruses, AcWT-PG, Ac-CpGV-ORF46-PG, Ac-△cath-CpGV-ORF46-PG, Ac-△CC-CpGV-ORF46-PG and Ac-△CC-PG, and an additional *v-cathepsin* knockout virus, Ac-p6.9-chiA. Ac-p6.9-chiA expresses *v-chitinase* under the control of the late *p6.9* promoter and does not express *v-cathepsin* due to a mutation within its native promoter (Hodgson et al., 2007). Early 4th instar

larvae were transferred into individual plastic cups and 55 larvae per virus were orally infected with 500 occlusion bodies of the designated virus or mock-infected. Larval mortality and appearance, namely, melanization and liquefaction, were scored at 12-h intervals.

A significant delay in LT_{50} (~ 10 h) was observed when *v-chitinase* and/or *v-cathepsin* were deleted (Ac-△CC-PG- and Ac-p6.9-chiA-infected larvae) compared to AcWT-PG (Table 2.1). However, the delay in time of death was compensated by the expression of CpGV-MMP (Ac-△cath-CpGV-ORF46-PG and Ac-△CC-CpGV-ORF46-PG-infected larvae) (Table 2.1). In addition, there was a significant decrease in LT_{50} when both CpGV-MMP and v-cathepsin were expressed (Ac-CpGV-ORF46-PG) compared to AcWT-PG. Taken together, the bioassay survival results suggest that expression of CpGV-MMP was able to enhance larvae mortality. Delays in time of death that occur in the absence of v-cathepsin can be compensated by CpGV-MMP, and viruses with CpGV-MMP and v-cathepsin accelerated death compared to Ac-WT-PG.

During bioassays, the appearance of dark spots on the cuticles of larvae infected with AcWT-PG, Ac-CpGV-ORF46-PG, Ac-△cath-CpGV-ORF46-PG and Ac-△CC-CpGV-ORF46-PG a few hours prior to death were observed. These dark spots are characteristic of early stages of melanization. The larval cuticles then turned brownish and became completely black a few hours after death (Fig. 2.7). Larvae infected with Ac-△CC-PG and Ac-p6.9-chiA, on the other hand, did not show as many signs of melanization on their cuticles, retaining their light green color for a longer period (more than 24 hours) after death (Fig. 2.7). 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 groups (Ac-△cath-CpGV-ORF46-PG, Ac-△CC-CpGV-ORF46-PG and Ac-△CC-PG and Ac-p6.9-chiA), on the other hand, retained a firm consistency after death and did not liquefy. Overall, larvae melanization and liquefaction results indicated that melanization occurred in the absence of v-cathepsin when CpGV-MMP was expressed and it was similar to that observed in the presence of v-cathepsin. However, CpGV-MMP did not appear to promote larvae liquefaction.

Effects of CpGV-MMP on v-chitinase release from infected cells

v-chitinase is required for larvae liquefaction which occurs by degradation of the chitinous exoskeleton (Hawtin et al., 1997). In AcMNPV, v-chitinase is trafficked to the endoplasmic reticulum where it is retained, because it contains a C-terminal KDEL retention motif (Hodgson et al., 2011; Saville et al., 2002). v-chitinase accumulates in the endoplasmic reticulum and is only released after lysis of infected cells (Thomas et al., 1998). v-chitinase release is therefore a prerequisite for larvae liquefaction. Surprisingly, Ac-△cath-CpGV-ORF46-PG-infected larvae did not liquefy even though this virus expressed its native v-chitinase in addition to CpGV-MMP. We hypothesized that no liquefaction might be due to a reduction or delay in release of v-chitinase from infected cells. To investigate the release of v-chitinase from infected cells, we infected Sf9 cells with AcWT-PG, Ac-CpGV-ORF46-PG, Ac-△cath-CpGV-ORF46-PG and Ac-△CC-PG at an MOI of 5 PFU/cell. In addition, *Cath* (-), a virus expressing *v-chitinase* under its native promoter but lacking *v-cathepsin* was used as an additional control. In the *Cath* (-) virus, the v-cathepsin gene was disrupted by insertion of a p10/β

galactosidase cassette (Slack et al., 1995). 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). There was less v-chitinase detected in the extracellular media of *Cath* (-)- and Ac-△cath-CpGV-ORF46-PG-infected cells compared to that of AcWT-PG- and Ac-CpGV-ORF46-PG-infected cells (Fig. 2.8). On the other hand, the amount of v-chitinase in cell pellets of *Cath* (-)- and Ac-△cath-CpGV-ORF46-PG-infected cells was greater than the quantities of v-chitinase in the pellets of AcWT-PG- and Ac-CpGV-ORF46-PG-infected cells (Fig. 2.9). Thus, it appeared that less v-chitinase was released from infected cells when *v-cathepsin* was deleted (*Cath* (-)-infected cells). Replacing v-cathepsin with CpGV-MMP did not seem to affect v-chitinase release (Ac-△cath-CpGV-ORF46-PG-infected cells). The smaller amounts of v-chitinase released in the extracellular media of Ac-△cath-CpGV-ORF46-PG- compared to AcWT-PG-infected cells may explain why no liquefaction occurred when larvae were infected with Ac-△cath-CpGV-ORF46-PG.

In view of these results, we tested if allowing v-chitinase to be secreted from infected cells by removing the KDEL retention motif could lead to larvae liquefaction. To this end, we generated a recombinant virus expressing *v-chitinase* without the C-terminal KDEL motif (Ac-△CC-CHIA△KDEL-PG). The Ac *v-chitinase/v-cathepsin* double knockout bacmid (AcBac△CC) (Kaba et al., 2004) was used as a backbone to generate Ac-△CC-ChiA△KDEL-PG. Ac-△CC-ChiA△KDEL-PG was constructed by inserting ChiA△KDEL (*v-chitinase* without the sequence coding for the C-terminal KDEL) under *Drosophila* hsp70 promoter control, *polh* driven by its promoter, and *egfp* under the *ie-1* promoter at the *polh* locus of AcBac△CC by site-specific transposition. Ac-△CC-ChiA-

PG, a control virus containing the entire *v-chitinase* sequence (v-chitinase with KDEL) was constructed in a similar manner.

To confirm synthesis and secretion of v-chitinase in these recombinant viruses, Sf9 cells were infected with Ac-△CC-ChiA△KDEL-PG or Ac-△CC-ChiA-PG and cell pellets and culture media were collected over the course of infection and analyzed for v-chitinase by immunoblotting. In both Ac-△CC-ChiA△KDEL-PG and Ac-△CC-ChiA-PG-infected cells, v-chitinase was detected in the cell pellet starting at 24 h p.i. (Fig. 2.9). In contrast to the cell pellet, the amount of v-chitinase in the extracellular media of Ac-△CC-ChiA△KDEL-PG-infected cells was significantly higher than that of cells infected with Ac-△CC-ChiA-PG. v-chitinase was detected in the media of Ac-△CC-ChiA△KDEL-PG-infected cells starting at 24 h p.i. In the media of Ac-△CC-ChiA-PG-infected cells, on the other hand, v-chitinase was detected starting at 72 h p.i. and in much lower amounts compared to those in Ac-△CC-ChiA△KDEL-PG-infected cell media (Fig. 2.9). These results confirm that deleting the v-chitinase C-terminal KDEL motif enables v-chitinase secretion.

Finally, we tested the ability of Ac-△CC-ChiA△KDEL-PG to liquefy *T. ni* larvae alone or in combination with Ac-△cath-CpGV-ORF46-PG. To this end, early 4th instar *T. ni* larvae were divided into groups (10 larvae per group). Larvae were then transferred into individual plastic cups and orally infected with 500 occlusion bodies of the designated virus or co-infected with 250 occlusions bodies of each virus (Table 2.2). Larval liquefaction was monitored throughout the course of the infection process. As previously observed, larvae infected with AcWT-PG liquefied. Larvae infected with Ac-△CC-CpGV-ORF46-PG, Ac-△CC-ChiA△KDEL-PG, Ac-△CC-ChiA-PG, or co-infected with

Ac-△CC-CpGV-ORF46-PG and Ac-△CC-ChiA-PG did not liquefy (Table 2.2). However, when Ac-△CC-CpGV-ORF46-PG and Ac-△CC-ChiA△KDEL-PG were used to co-infect *T. ni* larvae, liquefaction was observed (Table 2.2). Overall, these results indicate that v-chitinase secretion is necessary for larval liquefaction. However, v-chitinase alone cannot induce larvae melanization; it requires the presence of a protease such as v-cathepsin or CpGV-MMP.

DISCUSSION

Baculovirus MMPs have a unique nonconventional domain organization

Sequence alignments revealed that baculovirus MMPs have a conserved zinc-binding motif (HEXGHXXGXXHS/T) within their catalytic domain (Fig. 2.1.B). Computer analyses predicted the presence of a signal peptide in all baculovirus MMPs except in XcGV, PrGV and EpapGV-MMP. MMPs generally function in the extracellular compartment. All previously identified cellular MMPs have an N-terminal signal peptide with the exception of human MMP23. It was suggested that MMP23 might play a role in an intracellular compartment (Velasco et al., 1999). A stretch of hydrophobic amino acids near the N-terminal end of MMP23 serves as a signal anchor, allowing the protein to associate with cellular membranes (Ohnishi et al., 2001). XcGV, PrGV and EpapGV-MMP do not contain any signal anchor sequence.

Baculovirus MMPs lack two commonly conserved cellular MMP domains. The propeptide domain which contains the conserved cysteine switch motif (PRCGV/NPD) involved in maintaining MMP latency is missing in baculovirus MMPs. This motif is conserved in all cellular MMPs except human MMP23 (Velasco et al., 1999). The

absence of a cysteine switch suggests that baculovirus MMPs have evolved to overcome one of the MMP regulatory controls enabling them to be active immediately after synthesis. Unregulated MMP activity has been associated with pathological processes (Forget et al., 1999). A hemopexin-like domain is present in the C-terminal region of all cellular MMPs with the exception of human MMP23, 7 and 26. These MMPs have a shorter (MMP23) (Velasco et al., 1999) or truncated (MMP7, 26) (de Coignac et al., 2000) C-terminal region. Baculovirus MMPs, on the other hand, have a long C-terminal segment downstream of their catalytic domain which does not contain any known functional domain. Despite the lack of a number of conserved cellular MMP domains, the presence of a conserved MMP catalytic domain in baculovirus-encoded MMPs is sufficient to classify them into the MMP family.

Baculovirus MMP classification

Based on their amino acids composition and/or domain organization, MMPs have been traditionally subdivided into five major subclasses: gelatinases, collagenases, stromelysins, membrane-type MMPs and matrilysins.

CpGV-MMP could not be classified as a member of any of the defined MMP subclasses following amino acid sequence analysis. CpGV-MMP lacks the fibronectin repeats found in gelatinases that play a role in substrate binding (Collier et al., 1992). The deduced CpGV-MMP sequence does not contain three residues (Tyrosine, Aspartic acid, and Glycine) that are conserved within the catalytic domain of all collagenases and that are necessary for their enzymatic activity. In addition, CpGV-MMP lacks an insertion of nine mainly hydrophobic residues (XPLVPTXXV) at the C-terminal end of its catalytic

domain. These residues are characteristic of all stromelysins (Freije et al., 1994). Sequence analysis of the predicted CpGV-MMP peptide sequence does not predict a transmembrane domain or a glycosyl-phosphatidylinositol (GPI) anchor site which are determinant features of membrane-type MMPs (Fillmore et al., 2001). Finally, CpGV-MMP does not have the minimal domain organization (propeptide domain, C-terminal catalytic domain) distinctive of matrilysins (de Coignac et al., 2000; Marti et al., 1992). However; there are numerous MMPs that do not fit into those subclasses and have not been assigned to any subclass to date. They include a number of human MMPs (MMP12, 19, 20, 21, 23, 27, and 28) and several MMPs from insect species (*Drosophila melanogaster*, *Tribolium castaneum, Manduca sexta*). These MMPs are usually referred to as “other MMPs”. We suggest that CpGV-MMP and other baculovirus MMPs should be included into the “other MMPs" category.

CpGV-MMP is a secreted functional MMP which does not affect virus replication in cultured cells

MMP activity assays using bacterially purified CpGV-MMP demonstrated that CpGV-MMP has specific MMP activity *in vitro* which can be abolished by MMP inhibitors (Fig. 2.2.B). We constructed a recombinant AcMNPV expressing *CpGV-ORF46* (Ac-CpGV-ORF46-PG) to study the synthesis of CpGV-MMP in Sf9 cells and its effects on virus replication. CpGV-MMP predicts an 18-amino acid long signal peptide suggesting that the protein is secreted. We detected HA-tagged CpGV-MMP in the extracellular media of infected Sf9 cells (Fig. 2.4.A), confirming that CpGV-MMP was a secreted protein similar to most MMPs characterized to date. The molecular mass of CpGV-MMP was

however higher than the expected mass of 65 kDa. Online N-glycosylation prediction tools revealed the presence of 8 putative N-glycosylation sites on CpGV-MMP. PNGase F treatment confirmed that the high molecular mass was in part attributed to protein N-glycosylation. The larger bands observed in the untreated pellet fraction can be explained by the presence of both the glycosylated and non-glycosylated forms of the protein in the pellet (Fig. 2.4.A and B). Finally, CpGV-MMP does not appear to have any obvious effect on virus replication or occlusion body formation *in vitro* (Fig. 2.5.A and B).

Effects of CpGV-MMP on larval survival time

In order to determine the functional relationships between CpGV-MMP, v-chitinase, and v-cathepsin, and their effects on insects, we constructed recombinant viruses expressing *CpGV-ORF46* in the presence or absence of *v-chitinase* and/or *v-cathepsin*. v-chitinase and v-cathepsin are involved in degradation of larval tissues and exoskeleton allowing virus transmission to new hosts (Hawtin et al., 1997; Kang et al., 1998). *T. ni* bioassay survival results revealed that the absence of v-cathepsin led to a significant delay in time of death. However, the delay in time of death that occurred without v-cathepsin could be compensated by CpGV-MMP. In addition, when v-cathepsin and CpGV-MMP were both present, there was a decrease in LT_{50} compared to AcWT-PG (Table 2.1). These results imply that CpGV-MMP can rescue the delay in time of death observed when v-cathepsin was absent and could be potentially used in combination with v-cathepsin to enhance mortality of baculovirus-infected larvae. It is possible that CpGV-MMP helps virus spread by degrading larvae basement membranes and other matrix proteins. 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 capable of degrading 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 thought to be retained by an interaction with v-chitinase. v-cathepsin is only activated and released into the extracellular space after cell lysis of infected cells (Hodgson et al., 2011; Hom et al., 2002). This indicates that v-cathepsin is not present in the extracellular environment during the early stage of infection to degrade extracellular matrix components. CpGV-MMP, on the other hand, is secreted from infected cells (Fig. 2.4.A). The secretion and early availability of CpGV-MMP in the extracellular space might be beneficial for the virus by allowing it to spread faster to tissues beyond midgut epithelial cells. Another baculovirus MMP, the *Xestia c-nigrum* granulovirus MMP (XcGV-MMP) has been previously characterized (Ko et al., 2000). It is important to note that contrary to CpGV-MMP, XcGV-MMP does not have a signal peptide and as stated by Ko et al., it is likely that XcGV-MMP is released after lysis of infected cells. This implies that XcGV-MMP contrary to CpGV-MMP may function in the late stages of infection (after lysis of infected cells) or it might be playing a role intracellularly.

Effects of CpGV-MMP on larvae melanization and liquefaction

In addition to survival times, *T. ni* bioassay showed that CpGV-MMP can promote larval melanization. Melanization did not occur in the absence of v-cathepsin but was restored by CpGV-MMP. Melanization is triggered by the activation of prophenoloxidase (proPO)

into active phenoloxidase (PO). This activation is mainly carried out by serine proteases (Cerenius and Soderhall, 2004). Excessive and uncontrolled activation of PO 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). We speculate that CpGV-MMP can activate directly or indirectly the PO cascade which leads to melanization. Similar observations were seen with XcGV-MMP (Ko et al., 2000). A study revealed that a metalloprotease secreted by the gram-negative insect pathogen *Photorhabdus luminescens* was capable of inducing melanization (Held et al., 2007). In addition, we observed that when v-cathepsin and v-chitinase were present (AcWT-PG and Ac-CpGV-ORF46-PG), infected larvae liquefied after death. However, when v-cathepsin was absent, with or without CpGV-MMP (Ac-△cath-CpGV-ORF46-PG, Ac-p6.9-chiA), or when v-cathepsin and v-chitinase were lacking with or without CpGV-MMP (Ac-△CC- CpGV-ORF46-PG, Ac-△CC-PG), dead larvae did not liquefy (Fig. 2.7). From these findings it seems that CpGV-MMP does not promote larvae liquefaction. It is known that v-chitinase is required for degradation of the insect chitinous exoskeleton which leads to liquefaction (Hawtin et al., 1997). However, v-chitinase has an endoplasmic reticulum retention motif (KDEL) and is retained within the endoplasmic reticulum of infected cells. Liquefaction can only occur when v-chitinase is released after lysis of infected cells (Thomas et al., 1998). In our bioassay (Fig. 2.7), liquefaction only occurred when v-cathepsin and v-chitinase were both present. v-chitinase alone was not able to initiate larvae liquefaction. The same observations were reported previously (Hawtin et al., 1997). Our results also show that less v-chitinase appears to be released into the extracellular media of infected cells when v-cathepsin is absent

(Fig. 2.8). Replacing v-cathepsin with CpGV-MMP (Ac-△cath- CpGV-ORF46-PG) does not result in increased v-chitinase release (Fig. 2.8). This suggests that v-chitinase release from infected cells may be aided by v-cathepsin. Knowing that cell lysis is a prerequisite for v-chitinase release (Hawtin et al., 1997; Thomas et al., 1998), we suggest that v-cathepsin facilitates lysis of infected cells. CpGV-MMP, on the other hand, does not seem to be playing a similar role.

Finally, we wanted to know if engineering v-chitinase to be secreted from cells by removing the KDEL motif could lead to liquefaction of infected larvae in the absence of v-cathepsin. We constructed a recombinant virus (Ac-△CC-ChiA△KDEL-PG) expressing a mutant *v-chitinase* lacking the sequence coding for the KDEL retention motif. When the KDEL motif was removed, a large amount of v-chitinase could be detected in the extracellular media of Ac-△CC-ChiA△KDEL-PG-infected cells (Fig. 2.9). As previously shown in other studies (Hodgson et al., 2011; Saville et al., 2002), our result confirmed that removing the KDEL motif allows v-chitinase to be secreted. Nonetheless, Ac-△CC-ChiA△KDEL-PG-infected-larvae did not liquefy even though v-chitinase was secreted in large amount in the extracellular space. This again supports that v-chitinase alone cannot initiate larvae liquefaction. However, when larvae were co-infected with Ac-△CC-ChiA△KDEL-PG and Ac-△cath- CpGV-ORF46-PG, liquefaction occurred (Table 2.2). This indicates that CpGV-MMP is able to promote larvae liquefaction when v-chitinase is available in the extracellular space. In the insect cuticle, chitin, its main component, forms long chains that are linked to sclerotized proteins (Merzendorfer and Zimoch, 2003). Hawtin et al. suggested that proteases are required to “unmask” the insect cuticle in order to allow v-chitinase to degrade chitin (Hawtin et

al., 1997). Our results support that even when v-chitinase is present in the extracellular milieu; it still requires a protease such as cathepsin or CpGV-MMP to liquefy the insect exoskeleton.

Figure 2.1

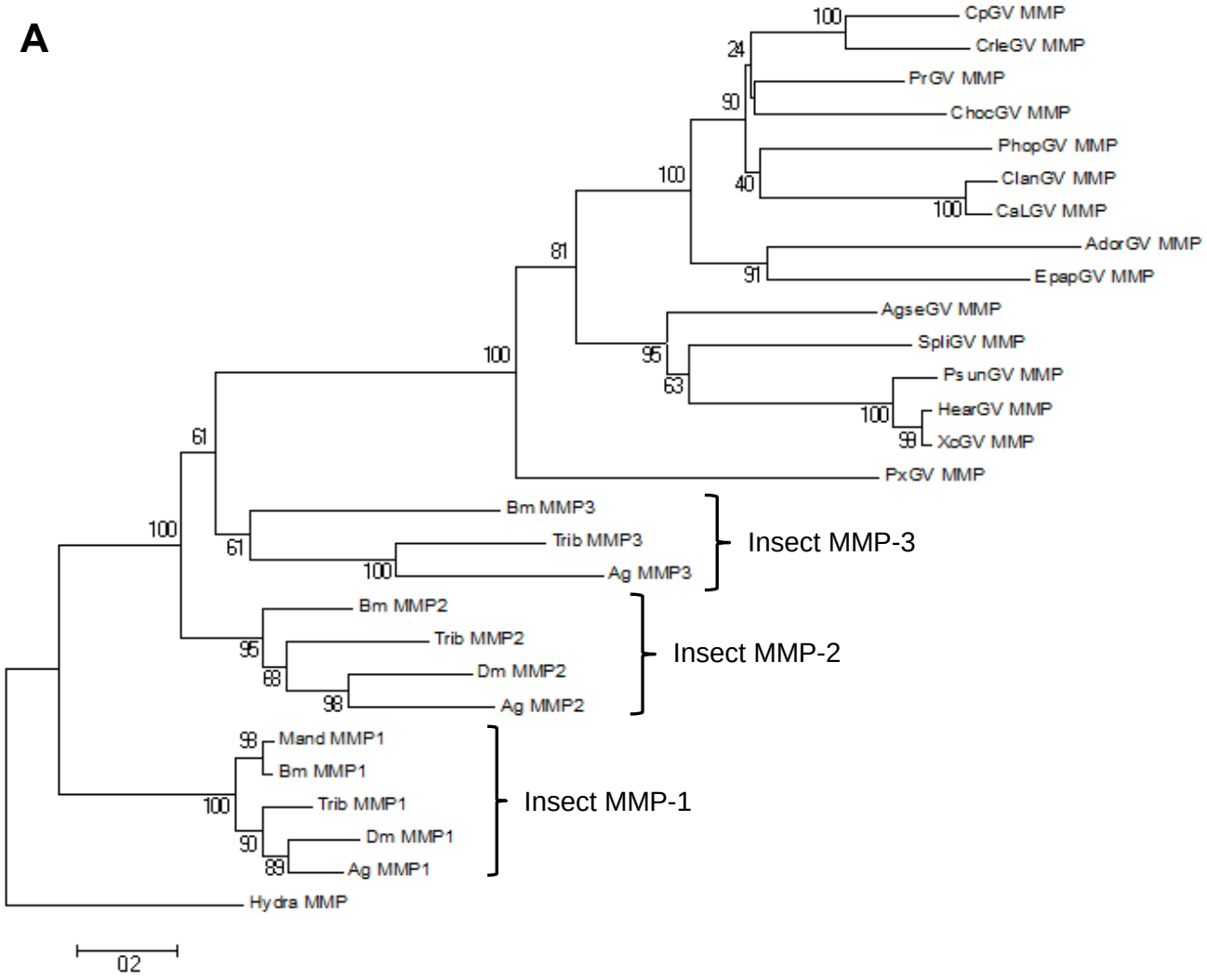

A
CpGV MMP
CrleGV MMP
PrGV MMP
ChocGV MMP
PhopGV MMP
ClanGV MMP
CaLGV MMP
AdorGV MMP
EpapGV MMP
AgseGV MMP
SpliGV MMP
PsunGV MMP
HearGV MMP
XcGV MMP
PxGV MMP
Bm MMP3
Trib MMP3
Ag MMP3
Insect MMP-3
Bm MMP2
Trib MMP2
Dm MMP2
Ag MMP2
Insect MMP-2
Mand MMP1
Bm MMP1
Trib MMP1
Dm MMP1
Ag MMP1
Insect MMP-1
Hydra MMP
0.2

Figure 2.1

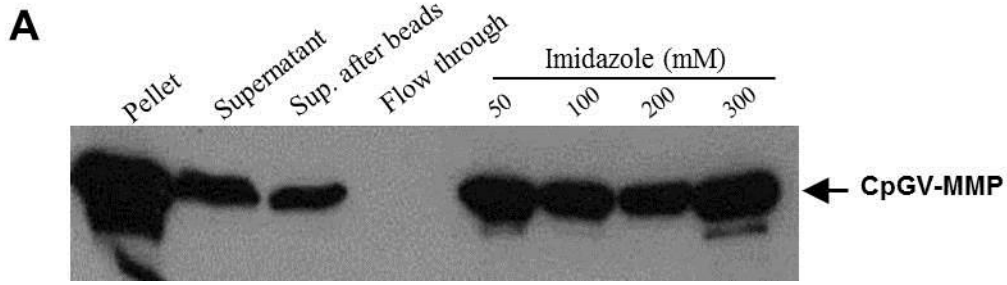
B


[CpGV-MMP] : VTHEIGHALGLYHSSVRNSIMYQLY
[CrleGV-MMP] : LVHEIGHALGLLHSSVKKSLMYSYY
[PrGV-MMP] : LTHEIGHAIGLSHSSVNTSIMYPWY
[ChocGV-MMP] : LVHEIGHALGLYHSSLEDSVMYPYY
[ClanGV-MMP] : LIHEIGHALGLYHSSDPKSIMFPVY
[PxGV-MMP] : TVHELGHILGLGHSSVKTSVMYSYY
[CaLGV-MMP] : LIHEIGHALGLYHSSDPRSIMYPVY
[AdorGV-MMP] : LLHEVGHALGLFHSHAENSVMNPNY
[AgseGV-MMP] : VAHEIGHTLGLQHSSVKRAMMYAWY
[HearGV-MMP] : AAHEIGHALGLHHTSVRDAIMYWYY
[XcGV-MMP] : AAHEIGHALGLHHTSVRDAIMYWYY
[EpapGV-MMP] : IVHEIGHALGLYHSSIKNALMHPSY
[PsunGV-MMP] : AAHEIGHALGLHHTSVRSAIMYWYY
[PhopGV-MMP] : LLHEIGHALGLFHSSNHQSIMYHLY
[SpliGV-MMP] : AAHEIGHTLGLMHSSVKEALMYALY
Human-MMP3 : AAHEIGHSLGLFHSANTEALMYPLY
Dm-MMP1 : AAHEFGHSLGLSHSDQSSALMAPFY
Zn-binding motif
Met-turn

Figure 2.1- Phylogenetic analysis of baculovirus MMPs

(A) Phylogenetic relationships between baculovirus MMPs and insect MMPs. A rooted neighbor-joining tree was built using MEGA6 with 1000 bootstrap replicates. Numbers at the nodes represent bootstrap scores. The tree was generated using aligned sequences of putative baculovirus MMPs and MMP sequences from *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Trib), *Anopheles gambiae* (Ag), *Manduca sexta* (Mand) and *Bombyx mori* (Bm). A *Hydra vulgaris* MMP was used as an outgroup. **(B)** Alignment of baculovirus MMP domains. Baculovirus MMPs have a conserved zinc-binding motif (HEXGHXXGXXHS/T) and a Met-turn sequence within their catalytic domain. Shading shows conserved amino acids. Virus full names and MMP accession numbers are listed in the materials and methods section.

Figure 2.2


A
Pellet
Supernatant
Sup. after beads
Flow through
Imidazole (mM)
50
100
200
300
CpGV-MMP

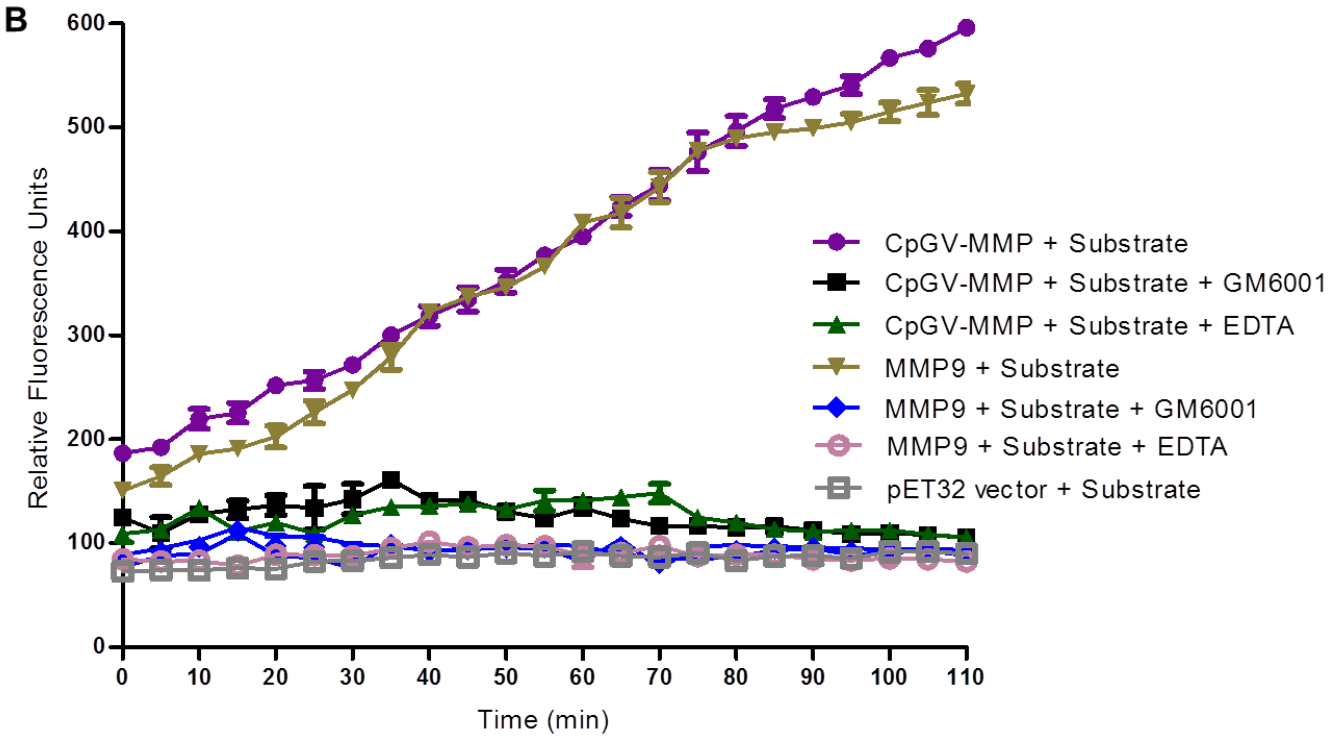

B
Relative Fluorescence Units
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
80
90
100
110
Time (min)
CpGV-MMP + Substrate
CpGV-MMP + Substrate + GM6001
CpGV-MMP + Substrate + EDTA
MMP9 + Substrate
MMP9 + Substrate + GM6001
MMP9 + Substrate + EDTA
pET32 vector + Substrate

Figure 2.2- CpGV-MMP expression, purification and enzymatic activity

(A) *E. coli* expression and purification of CpGV-MMP. His-tagged CpGV-MMP protein was expressed in *E. coli* and purified using a cobalt-charged resin. 20µl of the indicated fraction was analyzed by immunoblotting using anti-His antibody.

(B) MMP activity assay using *E. coli*-purified purified CpGV-MMP. CpGV-MMP (30 ng) or control human MMP9 (30 ng) were incubated at 37ºC with 50 µl of generic fluorescent MMP substrate. MMP inhibitor, 400 µM GM6001 or 50 µM EDTA, was added to the protein-substrate mixture. To confirm that CpGV-MMP activity was not due to bacterial protein, bacterially purified pET32 vector (30ng) was used as a negative control. Fluorescence measurements were performed every 5 min at λ_{ex}= 485 nm and λ_{em}= 535 nm. The error bars represent standard deviations for two independent repeats.

Figure 2.3

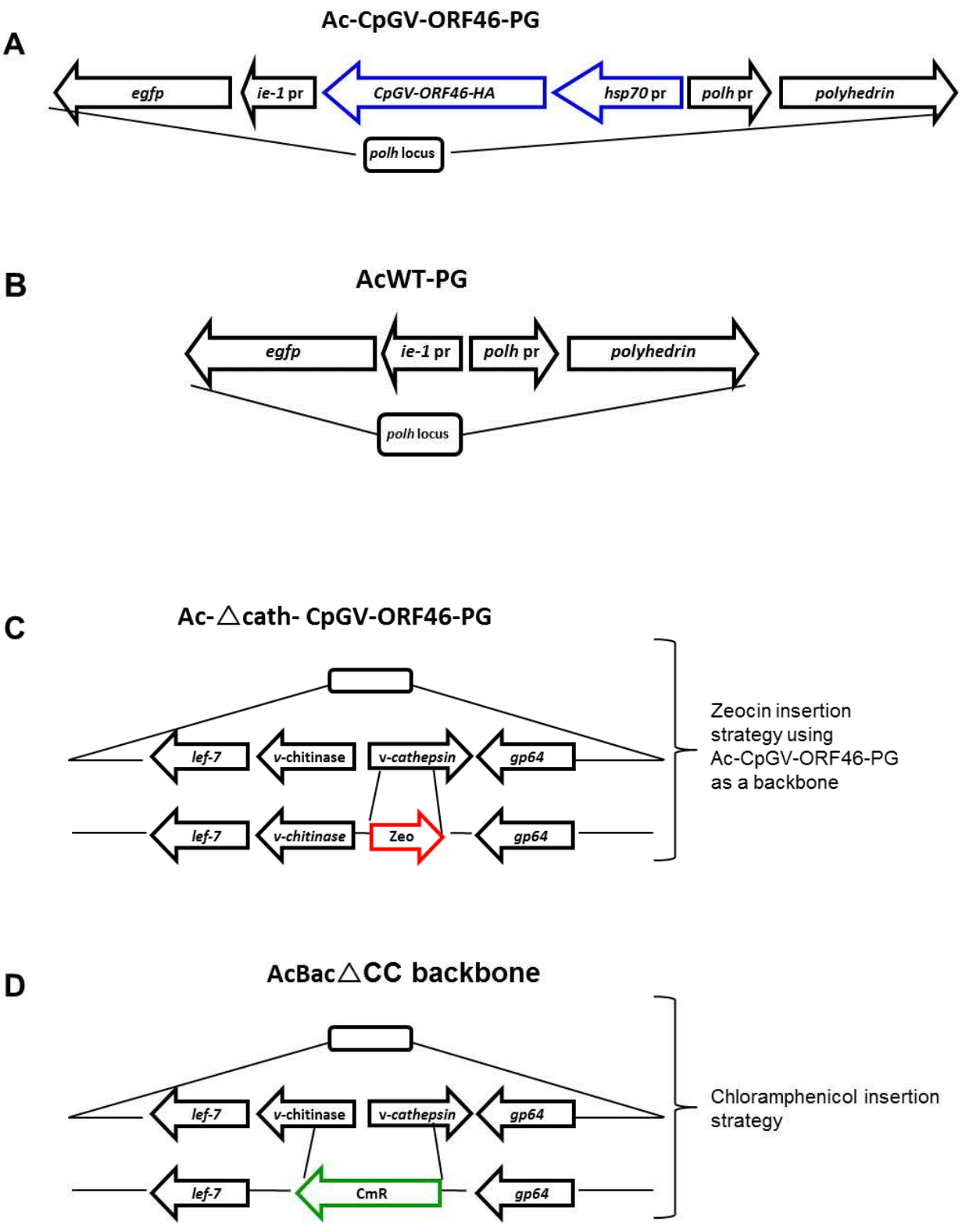

A
Ac-CpGV-ORF46-PG
egfp
ie-1 pr
CpGV-ORF46-HA
hsp70 pr
polh pr
polyhedrin
polh locus
B
AcWT-PG
egfp
ie-1 pr
polh pr
polyhedrin
polh locus
C
Ac-△cath- CpGV-ORF46-PG
lef-7
v-chitinase
v-cathepsin
gp64
lef-7
v-chitinase
Zeo
gp64
Zeocin insertion strategy using Ac-CpGV-ORF46-PG as a backbone
D
AcBac△CC backbone
lef-7
v-chitinase
v-cathepsin
gp64
lef-7
CmR
gp64
Chloramphenicol insertion strategy

Figure 2.3

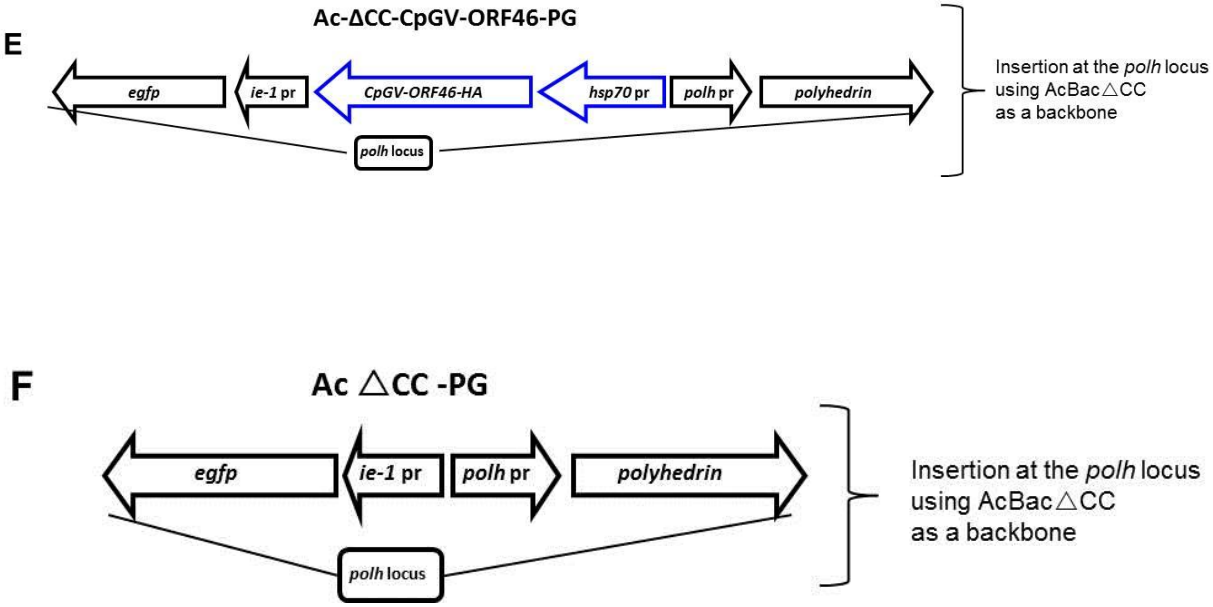

E
Ac-ΔCC-CpGV-ORF46-PG
egfp
ie-1 pr
CpGV-ORF46-HA
hsp70 pr
polh pr
polyhedrin
polh locus
Insertion at the polh locus
using AcBac△CC
as a backbone
F
Ac △CC -PG
egfp
ie-1 pr
polh pr
polyhedrin
polh locus
Insertion at the polh locus
using AcBac△CC
as a backbone

Figure 2.3- Strategy for construction of recombinant bacmids

(A) Construction of Ac-CpGV-ORF46-PG. HA epitope-tagged *CpGV-ORF46* under the *Drosophila* hsp70 promoter control, *polh* and its promoter, and *egfp* under the *ie-1* promoter were inserted into AcMNPV at the *polh* locus.

(B) Construction of Ac-WT-PG. *egfp* under the *ie-1* promoter and *polh* and its promoter were introduced into AcMNPV at the *polh* locus.

(C) Construction of Ac-△cat-CpGV-ORF46-PG. A fragment (775 bp) containing *v-cathepsin* in Ac-CpGV-ORF46-PG was replaced by the zeocin resistance gene (*zeo*) by homologous recombination.

(D) AcBac-△CC backbone used to generate △CC-CpGV-ORF46-PG and Ac-△CC-PG. *v-cathepsin*/*v-chitinase* deletion was obtained by replacing a region encompasing *v-cathepsin* and *v-chitinase* by the chloramphenicol resistance gene (*CmR*) (Kaba et al., 2004).

(E) Construction of Ac-△cath-CpGV-ORF46-PG. *CpGV-ORF46* under *Drosophila hsp70* promoter control, *polh* and its promoter, and *egfp* under the *ie-1* promoter were inserted at the *polh* locus of AcBac-△CC.

(F) Construction of Ac-△CC-PG. *polh* under its promoter, and *egfp* under the *ie-1* promoter were inserted at the *polh* locus of AcBac-△CC.

Figure 2.4

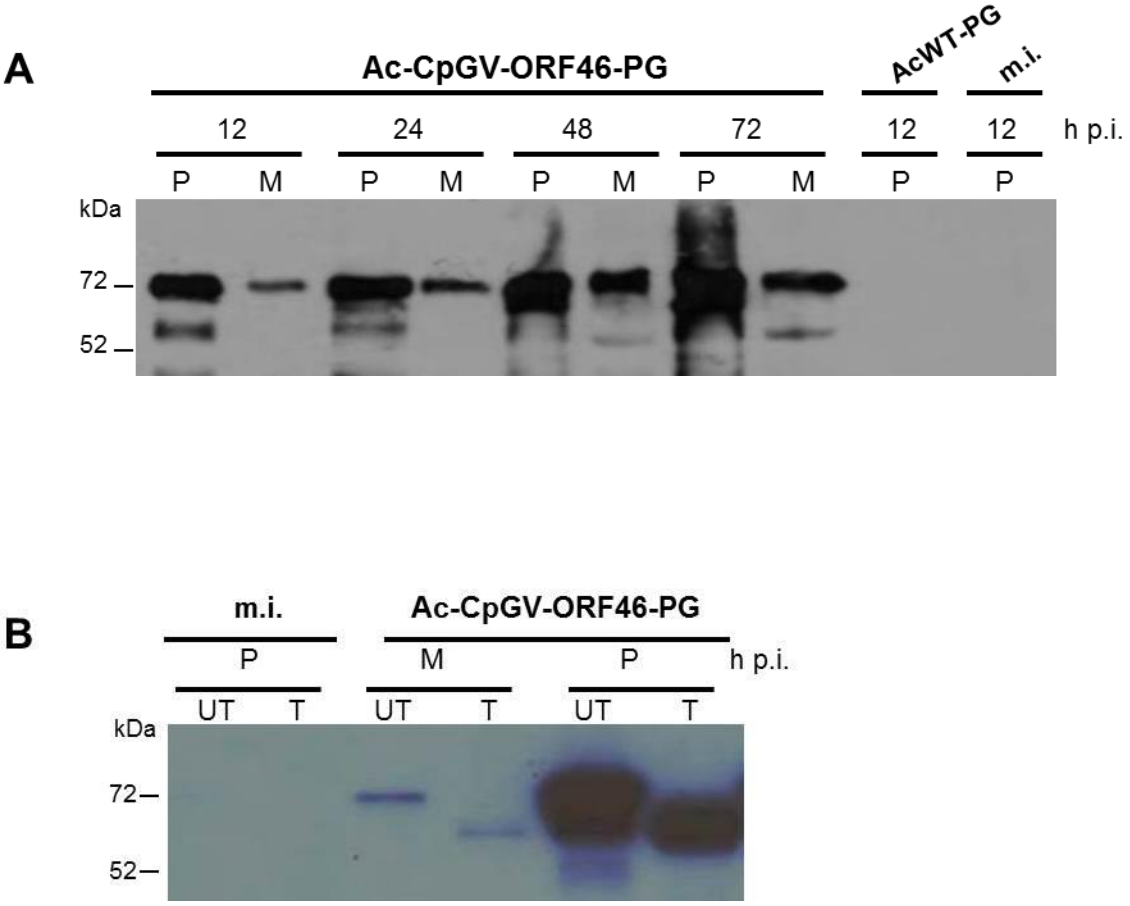

A
Ac-CpGV-ORF46-PG
AcWT-PG
m.i.
12
24
48
72
12
12
h p.i.
P
M
P
M
P
M
P
M
P
P
kDa
72
52
B
m.i.
Ac-CpGV-ORF46-PG
P
M
P
h p.i.
UT
T
UT
T
UT
T
kDa
72
52

Figure 2.4- Synthesis of CpGV-MMP during infection

(A) Synthesis of CpGV-MMP. Sf9 cells were infected at an MOI of 5 PFU/cell with Ac-CpGV-ORF46-PG, Ac-WT-PG, or mock-infected (m.i). At the indicated time points, cell pellet (P) and extracellular media (M) were collected and immunoblotted using anti-HA antibody to detect CpGV-MMP.

(B) Glycosylation of CpGV-MMP. Sf9 cells were infected at an MOI of 5 PFU/cell with Ac-CpGV-ORF46-PG or mock-infected. Cell pellets (P) and extracellular media (M) were collected at 24 h p.i. and incubated with PNGase F at 37°C for 4 h. PNGase F-treated (T) and untreated (UT) samples were then analyzed by immunoblotting using anti-HA antibody.

Figure 2.5

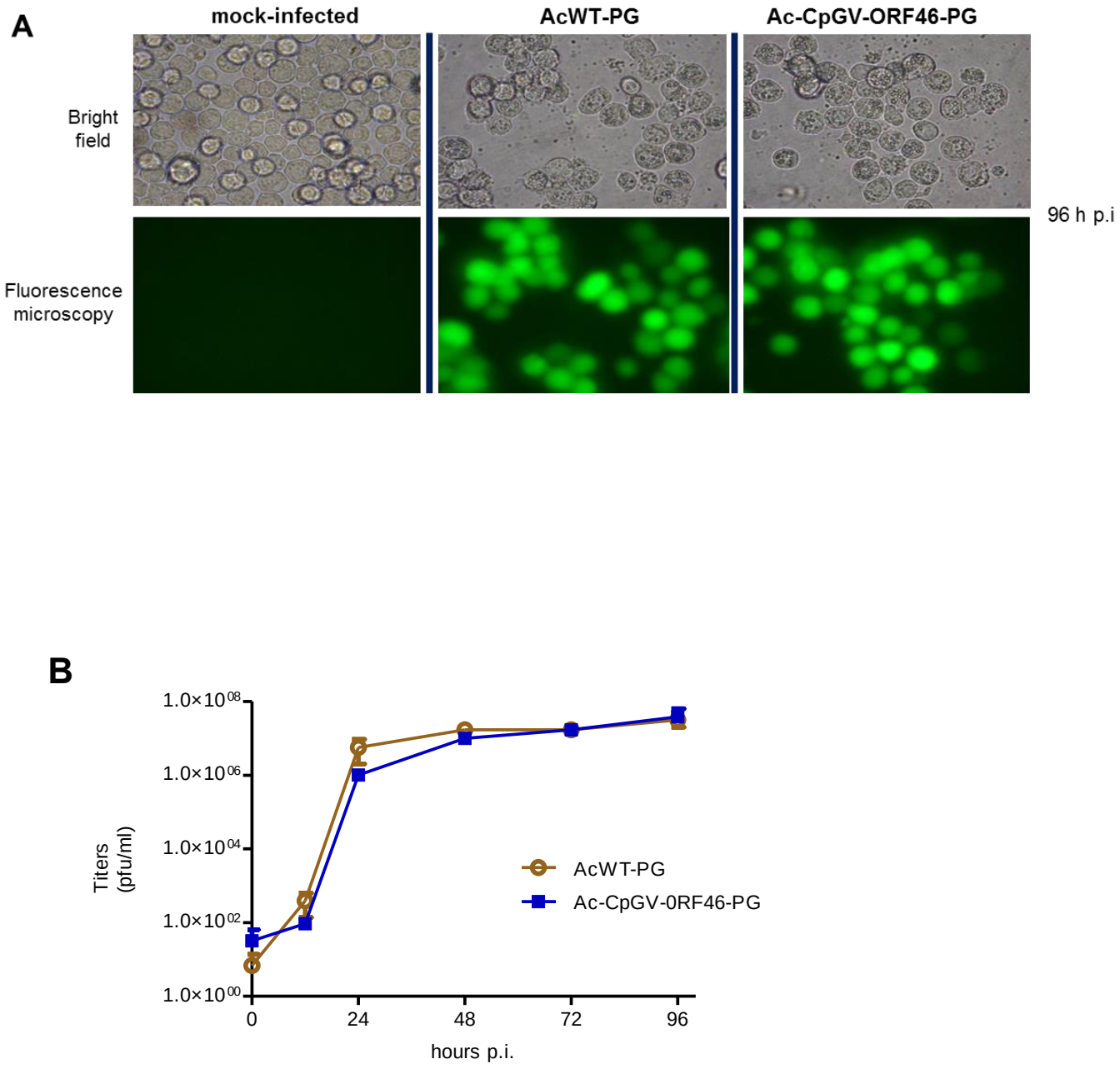
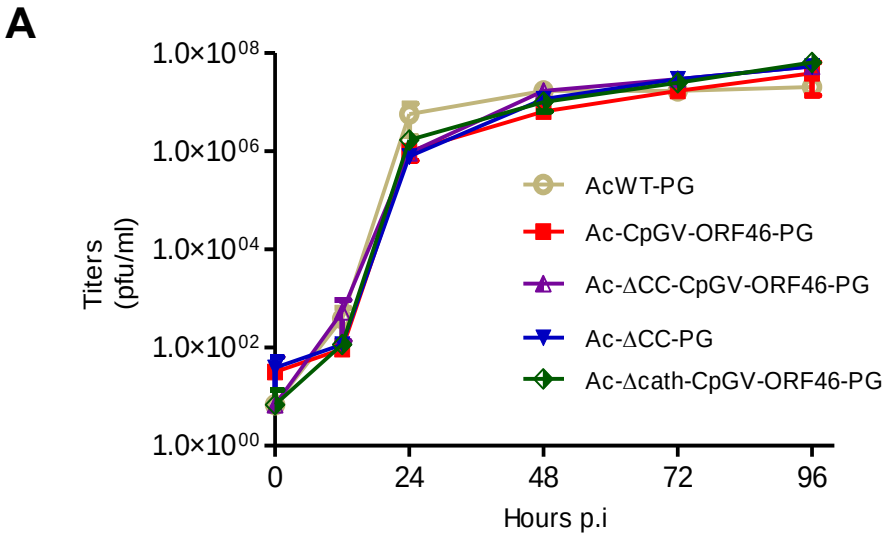

A
mock-infected
AcWT-PG
Ac-CpGV-ORF46-PG
Bright field
Fluorescence microscopy
96 h p.i
B
Titers (pfu/ml)
1.0×10^08
1.0×10^06
1.0×10^04
1.0×10^02
1.0×10^00
0
24
48
72
96
hours p.i.
AcWT-PG
Ac-CpGV-0RF46-PG

Figure 2.5- Effects of CpGV-MMP on virus replication

(A) Cells were infected with the indicated virus at an MOI of 5 PFU/cell. Cells were examined for presence of occlusion bodies (top panel) and eGFP fluorescence (lower panel) at 96 h p.i.

(B) Virus growth curves. Sf9 cells were infected with Ac-CpGV-ORF46-PG or AcWT-PG (5 PFU/cell); budded virus was collected at different times p.i. and titered by $TCID_{50}$ endpoint dilution assays. The error bars represent standard deviations for two independent repeats. Data shown here is the same as that in experiments in Fig. 2.6.A and presented here to show direct comparison between Ac-CpGV-ORF46-PG and AcWT-PG.

Figure 2.6


A
1.0×10^08
1.0×10^06
1.0×10^04
1.0×10^02
1.0×10^00
Titers (pfu/ml)
0
24
48
72
96
Hours p.i
AcWT-PG
Ac-CpGV-ORF46-PG
Ac-ΔCC-CpGV-ORF46-PG
Ac-ΔCC-PG
Ac-Δcath-CpGV-ORF46-PG

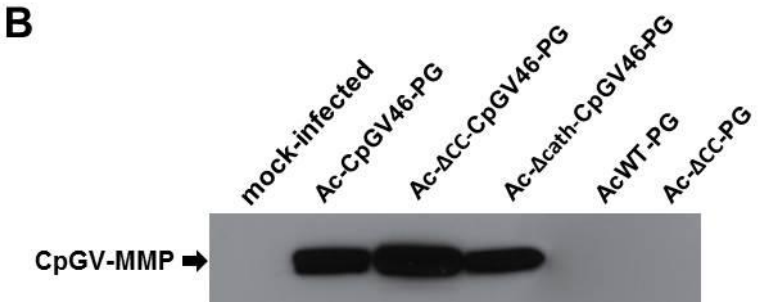

B
mock-infected
Ac-CpGV46-PG
Ac-ΔCC-CpGV46-PG
Ac-Δcath-CpGV46-PG
AcWT-PG
Ac-ΔCC-PG
CpGV-MMP

Figure 2.6- Virus growth curves and CpGV-MMP expression from Ac-△cath-CpGV-ORF46-PG, Ac-△CC-CpGV-ORF46-PG, Ac-△CC-PG, Ac-CpGV-ORF46-PG and AcWT-PG bacmids

(A) Sf9 cells were infected at an MOI of 5 PFU/cell with the indicated virus; budded virus was collected at different time p.i. and titered by TCID50 endpoint dilution assays.

(B) Cell pellets from Sf9 infected with the indicated virus (5 PFU/cell) were collected at 24 h p.i. and HA-tagged CpGV-MMP was detected with anti-HA antibody.

Figure 2.7

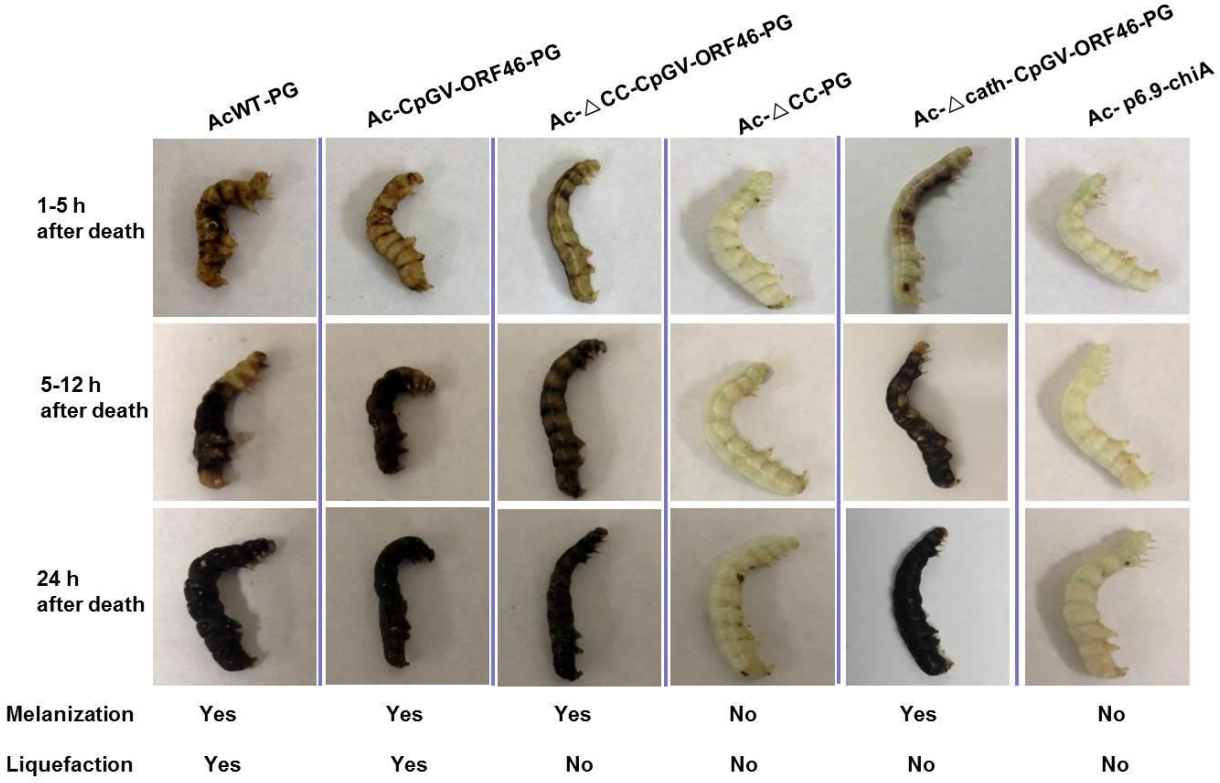
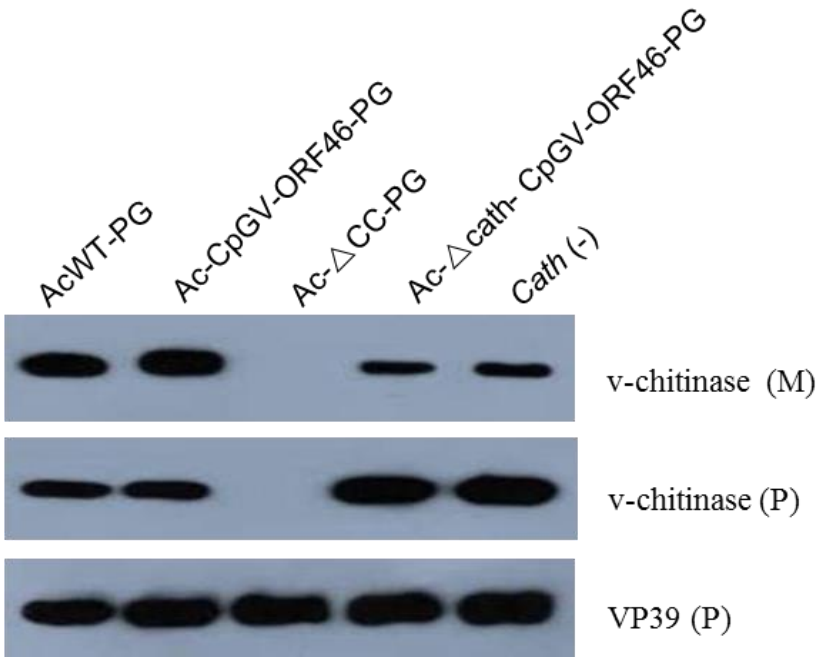

AcWT-PG
Ac-CpGV-ORF46-PG
Ac-△CC-CpGV-ORF46-PG
Ac-△CC-PG
Ac-△cath-CpGV-ORF46-PG
Ac- p6.9-chiA
1-5 h after death
5-12 h after death
24 h after death
Melanization Yes Yes Yes No Yes No
Liquefaction Yes Yes No No No No

Figure 2.7- Infected larva cuticle phenotypes

Early 4th instar *T. ni* larvae were orally infected with 500 occlusion bodies of the designated virus and photographed at the indicated times. Representative larvae are shown.

Figure 2.8


AcWT-PG
Ac-CpGV-ORF46-PG
Ac-△CC-PG
Ac-△cath- CpGV-ORF46-PG
Cath (-)
v-chitinase (M)
v-chitinase (P)
VP39 (P)

.

Figure 2.8- Effects of CpGV-MMP on v-chitinase release from infected cells

Sf9 cells were infected at an MOI of 5 PFU/cell with Ac-WT-PG, Ac-CpGV-ORF46-PG, Ac-△cath-CpGV-ORF46-PG, Ac-△CC-PG or *Cath* (-). Cell pellets (P) and extracellular media (M) from infected cells were collected at 72 h p.i. and analyzed by immunoblotting for the presence of v-chitinase (Cell pellets input: 2.5 µl /Extracellular media input: 5 µl). VP39, the major capsid protein is shown as a loading control.

Figure 2.9

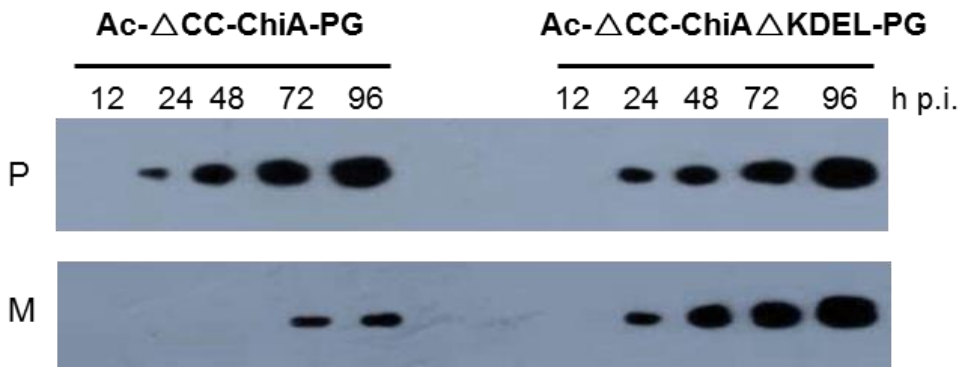


Figure 2.9- Confirmation of of v-chitinase secretion in Ac-△CC-ChiA△KDEL-PG or Ac-△CC-ChiA-PG- infected cells

Sf9 cells were infected at an MOI of 5 PFU/cell with Ac-△CC-ChiA△KDEL-PG or Ac-△CC-ChiA-PG and cell pellets (P) and extracellular media (M) were collected at the indicated times and analyzed for the presence of chitinase by immunoblotting.

Table 2.1- Time mortality response of virus infected-*T. ni* larvae

First bioassay

Virus	LT_{50} (h)	95% fiducial limits	Slope ± SE*
AcWT-PG	133.3 (a)	130.9-135.8	29.3881 ± 2.8223
Ac-CpGV-ORF46-PG	126.9 (b)	124.1-129.7	21.9528 ± 1.9243
Ac-△CC-CpGV-ORF46-PG	132.9 (a)	130.4-135.5	27.0176 ± 2.5520
Ac-△CC-PG	143.9 (c)	141.4-146.5	35.7926 ± 4.0845
Ac-△cath-CpGV-ORF46-PG	133.1 (a)	130.5-135.8	25.9665 ± 2.4547
Ac-p6.9-chiA	141.4 (c)	138.7-144.3	31.7008 ± 3.4388

*SE: standard error / (a) (b) (c) indicate groups with non-overlapping 95% fiducial limits

Second bioassay

Virus	LT_{50} (h)	95% fiducial limits	Slope ± SE*
AcWT-PG	132.8 (a)	130.7-134.9	26.4429 ± 2.4646
Ac-CpGV-ORF46-PG	127.0 (b)	124.3-129.8	20.9664 ± 1.9099
Ac-△CC-CpGV-ORF46-PG	132.6 (a)	130.3-134.9	24.7070 ± 2.2847
Ac-△CC-PG	142.9 (c)	140.1-145.2	34.3133 ± 3.8210
Ac-△cath-CpGV-ORF46-PG	134.3 (a)	131.9-137.1	21.8105 ± 2.0571
Ac-p6.9-chiA	142.8 (c)	140.4-145.4	36.9587 ± 4.1808

*SE: standard error / (a) (b) (c) indicate groups with non-overlapping 95% fiducial limits

Table 2.2- Effects of v-chitinase secretion on larvae liquefaction

Virus	Larvae liquefaction
AcWT-PG	Yes
Ac-△CC-CpGV-ORF46-PG + Ac-△CC-ChiA△KDEL-PG	Yes
Ac-△CC-CpGV-ORF46-PG + Ac-△CC-ChiA-PG	No
Ac-△CC-CpGV-ORF46-PG	No
Ac-△CC-ChiA△KDEL-PG	No
Ac-△CC-ChiA-PG	No

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Chapter 3 - Conclusions and Future Prospects

CONCLUSIONS AND FUTURE PROSPECTS

MMPs are important players in many physiological (embryonic development, morphogenesis, tissue resorption, etc.) and pathological (tumor progression, fibrosis, chronic inflammation, bacterial and viral infections, etc.) processes because of their ability to remodel the extracellular matrix. Considerable advancements have been made to understand the biochemical and structural properties of MMPs, including their activation mechanisms and their specific biological functions in mammals. However, little is known about MMPs in invertebrates. Knowledge on viral MMPs is also very limited. The only study on a viral MMP to date was done in the baculovirus *Xestia c-nigrum* granulovirus, which encodes an *mmp* that is thought to be involved in digestion of basement membranes in the late stages of virus infection.

The data presented in this thesis helped gain insight into baculovirus MMPs domain composition, the effects of CpGV-MMP on baculovirus replication and dissemination in cultured cells and insect larvae, and finally the functional relationships between CpGV-MMP and two other baculovirus degradative enzymes, v-chitinase and v-cathepsin, during larvae infection.

In silico analyses revealed that MMP homologs are only found in six virus groups (*Baculoviridae, Ascoviridae*, *Iridoviridae*, *Hytrosaviridae*, *Entomopoxvirinae* and Nudiviruses) which are all known to infect insects mainly in the order *Lepidoptera* and *Diptera*. As virus MMPs are only found in insect virus groups, it suggests a conserved function during viral pathogenesis specifically in these hosts.

Further analyses of the deduced baculovirus MMPs amino acid sequences revealed that despite having a conserved zinc binding domain, baculovirus MMPs lack the

conserved cysteine switch motif and the C-terminal hemopexin-like domain present in most cellular MMPs. MMPs have a long stretch of amino acids (~250 amino acids) located downstream of their catalytic domain in which no known functional domains were identified. While it could be understandable that baculovirus MMPs lost the cysteine switch motif as an evolutionary mechanism to overcome one of the MMP regulatory controls, it would be interesting to know the function, if any, of the region downstream of the catalytic domain. One could investigate the effects of deleting that region on MMP activity. A detailed structural analysis (e.g., X-ray crystallography) of baculovirus MMPs could also shed more light into the function of that region and its interaction, if any, with the catalytic domain.

We demonstrated that bacterially produced CpGV-MMP was able to cleave a generic MMP substrate and its activity was inhibited by a broad spectrum MMP inhibitor (GM6001) and the metal chelator EDTA. This is a clear indication that CpGV-MMP is a functional MMP *in vitro*; however, this result could be complemented by an analysis of the specific extracellular matrix proteins (collagen, laminin, fibronectin, etc.) that may be degraded by CpGV-MMP. Some extracellular matrix components are only found in specific locations (e.g. collagen IV in basement membranes); this analysis could therefore provide an indication of where CpGV-MMP activity is mainly concentrated. In addition, the analysis would indicate the substrate specificity of CpGV-MMP.

In Sf9 cells, recombinant CpGV-MMP expressed from AcMNPV had no obvious effects on virus replication, namely occlusion body formation and budded virus production. In addition, CpGV-MMP was secreted from infected cells confirming that it was a secreted protein like the majority of MMPs characterized to date.

The most significant findings of this thesis resulted from insect bioassays. Our results showed that the absence of v-cathepsin resulted in a significant delay in larval time of death; however, this delay was compensated by expression of CpGV-MMP. In addition, larval time of death was accelerated when v-cathepsin and CpGV-MMP were co-expressed. Baculoviruses are mainly studied for their use as biopesticides. Therefore, CpGV-MMP could potentially be used in combination with v-cathepsin to engineer faster killing biopesticides.

CpGV-MMP was able to promote larvae melanization similar to v-cathepsin. CpGV-MMP was only able to promote larvae liquefaction when chitinase which is usually retained in the endoplasmic reticulum of infected cells, was engineered to be secreted. Thus, contrary to v-cathepsin, CpGV-MMP does not appear to facilitate v-chitinase release from infected cells.

Overall, insect bioassays indicated that the absence of v-cathepsin can be compensated by the expression of CpGV-MMP except for v-chitinase cellular release.

In the *Baculoviridae*, MMP genes are only found in betabaculoviruses. v-cathepsin and v-chitinase genes, on the other hand, are found in most alphabaculoviruses and betabaculoviruses. Therefore, most betabaculoviruses such as CpGV encode *v-chitinase*, *v-cathepsin* and *mmp*. Studying these enzymes in their native viruses would provide additional evidence of the functions and relationship between v-chitinase, v-cathepsin and MMP. Unfortunately, studies in GVs have been hindered by the absence of cell lines that can successfully propagate and purify GVs. Establishing a cell line that would efficiently propagate GVs will be of great value not only to precisely elucidating

the functions of baculovirus MMPs in virus replication and pathogenesis but also to help understand the GV general replication process which is poorly understood.