STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE BRACOVIRUS PROTEIN GLC1.8

by

SHU ZHANG

(Under the Direction of Michael Strand)

ABSTRACT

Parasitoid wasps are insects that develop as parasites of other arthropods during their immature stages but are free living as adults. Some parasitoid wasps in the superfamily Ichneumonoidea rely on polydnaviruses (PDVs) to suppress the host immune system. *Microplitis demolitor* bracovirus (MdBV), which symbiotically associates with the braconid wasp *Microplitis demolitor*, has been studied with its genome sequenced and gene families identified. Among the identified genes, *glc1.8* encodes for a glycoprotein which plays an essential role in blocking adhesion and phagocytosis of host hemocytes. In this study, I first identified that in the MdBV genome, there is another member of the *glc* gene family. I also determined that a cell line stably transfected with *glc1.8* contains two forms of this gene: *glc1.8* and a larger form of this gene designated $glc1.8_2$. The larger gene likely arose by recombination. Characterization of the glycans associated with Glc1.8 identified Man3NAc2 and Man3NAc2F as major constituents while functional studies indicated that glycosylation is essential for immunosuppressive activity. A C-terminal deletion mutant which has no cytoplasmic tail showed reduced activity, suggesting that Glc1.8 may interact with cytoplasmic factors in host

cells. Lastly, I found that viral infection reduces transcript abundance of host integrins that are important for cell spreading.

INDEX WORDS: Polydnavirus, *Microplitis demolitor* bracovirus, glc1.8, N-glycosylation

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SHU ZHANG

B.S., Peking University, P. R. China, 2000

M.S., University of Georgia, USA, 2006

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SHU ZHANG

Major Professor: Michael Strand

Committee: Mark Brown
Don Champagne
Markus Beck

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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Chapter 1. Literature Review

1.1 Biology of parasitoid wasps

Parasitoids are insects that develop during their immature stages on the surface or inside the body of a host and are free living as adults. Parasitoids exist mainly in the orders of Hymenoptera and Diptera. Some species of parasitoids are also found in the orders Coleoptera, Lepidoptera, Trichoptera, Neuroptera and Stepsiptera and altogether some estimates suggest parasitoids make up 10% to 20% of all insect species (Pennacchio and Strand, 2006; Whitfield, 2003). Based on the affect of parasitism on the development of the hosts, parasitoids are divided into idiobionts and koinobionts. Idiobionts refer to parasitoids whose hosts stop development after parasitism while koinobionts refer to parasitoids whose hosts continue to develop after parasitism. According to the place where the parasitoid offspring grow, they are divided into ectoparasitoids which develop on the surface of hosts and endoparasitoids which develop inside the body of hosts.

The Hymenoptera arose at least 220 million years ago (MYA) and several parasitoid groups appeared about 160 MYA. The Hymenoptera is divided into two suborders, the Symphyta and the Apocrita. The Symphyta are phytophagous, whereas the ancesters of the Apocrita and Orussoidea are all idiobiont parasitoids of Coleoptera. Orussoidea lay their eggs near the host and use venom to paralyse hosts permanently or kill the host immediately (Pennacchio and Strand, 2006). The basal lineages of the Apocrita exhibit similar habits as orussoids. Parasitism in Hymenoptera likely evolved in the ancestors of Orussoidea and

Apocrita. After parasitism evolved, free-living, ectoparasitic and endoparasitic species arose independently many times within or between lineages (Pennacchio and Strand, 2006).

Besides the evolutionary relationships among different parasitoid species, many studies have also examined the interaction between parasitoids and hosts. Since parasitoids always spend their early developmental stages in association with a host, these insects have evolved many strategies for interacting with the host immune system and development. The strategies which parasitoids use to modulate host development include regulation and conformation. Regulation refers to the situation in which parasitoids evoke development disruption of the host via endocrine pathways. On the contrary, a conformer parasitoid does not change host development but coordinates its development with that of the hosts (Lawrence, 1986). Many parasitoids use these two strategies in sequence, with the early instar having no adverse effects on host development and the last instar parasitoids affecting feeding, molting and metamorphosis of the host.

Lepidoptera are common hosts of parasitoid wasps. The developmental stages of Lepidoptera include the egg, several larval instars, the pupa and finally the adult. Larval molting and metamorphosis to a pupa is regulated by the endocrine system. Juvenile hormone (JH) and ecdysteroids are the essential hormones controlling insect development (Riddiford, 1995). In the larval stage, as the body reaches certain a size, neurosecretory cells in brain release prothoracicotropic hormone (PTTH) which induces prothoracic gland to release ecdysone which stimulates molting. In all larva-larva molts, an elevated JH titer is maintained to ensure another larva stage is formed. In the final larval stage, the JH titer decline to an undetectable level and ecdysteroid release induces the formation of pupa. In the pupal stage, another release of ecdysteroid without JH triggers the adult formation (Riddiford, 1995). Many parasitoids

interfere with host development by altering the JH and/or ecdysteroid titer (Beckage and Gelman, 2004).

Besides modulating host development, parasitoids also need to avoid the host immune response. Insects lack an acquired immune system but possess a well-developed innate response (Fearon, 1997). The innate immune system of insects is further divided into humoral and cellular immunity. In humoral immune responses, antimicrobial peptides and proteins are synthesized by the fat body, midgut and hemocytes to kill the invading microorganisms. Cellular immunity refers to that aspect of the immune system in which certain types of hemocytes are responsible for recognizing and eliminating invaders, such as parasitoid eggs (Strand and Pech, 1995). In the cellular immune response, hemocytes usually phagocytose the targets or form a capsule around the targets and finally kill them by melanization. To evade attack by the host immune response, parasitoids use two main strategies. One is a passive mechanism, which means the parasitoid develops in a location inaccessible to host hemolymph or have surface features which prevent host hemocytes from recognizing them as non-self. The other strategy is active mechanism which refers to factors injected by female wasp at oviposition or secreted by parasitoid larva inside host hemocoel. These factors then induce physiological changes which block the host immune response (Carton and Nappi, 2001).

Factors injected into hosts by adult parasitoids include venom, teratocytes and polydnaviruses (PDVs). I discuss these three factors in more detail below.

1.2 Characteristics and functions of parasitoid venoms

Venoms are synthesized in the venom gland and stored in a reservoir, which is connected to the female reproductive tract. Venom is usually injected into the host at or just before oviposition. Most of the components in venoms are proteins and peptides, which start to be

produced during the pupal stage of the parasitoid wasp and continue to be produced during the adult stage (Jones and Wozniak, 1991). Some studies indicate that venom proteins range in size from less than 5 kDa to greater than 100 kDa , with the large proteins existing in higher abundance (Schmidt and Jones, 1989). Other studies indicate that most venom proteins are acidic (Leluk et al., 1989; Krishnanet al., 1994; Parkinson et al., 2002). Some venom proteins are also post-translationally modified by glycosylation or phosphorylation (Leluk et al., 1989; Parkinson et al., 2002; Labrosse et al., 2005). However, the functions of these post-translational modifications are still unclear. Another characteristic of venom proteins is that some have similar antigenic profiles to venom proteins from other closely related parasitoid species. For example, the antibody raised against a protein from *Euplectrus comstockii* recognizes three venom proteins from *Euplectrus plathypenae* (Coudron et al., 2000). It was also reported that there was cross-recognition of venom components from Formicidae, Vespidae and Apidae by antibodies against *Chelonus*. sp. near *curvimaculatus* venom (Leluk et al., 1989). In the parasitoid wasp *Campoletis sonorensis*, there are several venom proteins that share the same epitopes with *C. sonorensis* polydnavirus (CsIV) suggesting the possible evolutionary relationship between the CsIV and venom genes (Webb and Summers, 1990). Besides proteins and peptides, there are also some non-proteinic components in parasitoid venoms. For example, the venom from *Ampulex compressa*, an idiobiont that parasitizes cockroaches, contains dopamine and gamma-aminobutyric acid (Libersat, 2003; Moore et al., 2006).

The main function of parasitoid venoms is to paralyse hosts and interfere in the development and immunity of the host. Some venoms also cooperate with other factors to modulate host physiology (Schmidt, 1982; Visser et al, 1983; Moreau et al, 2002).

1. Venoms that paralyse and/or control host behaviors

Many idiobionts inject venoms that paralyse hosts either permanently or transiently. Some parasitoid wasps also use their venoms to modify host behavior to facilitate the survival of their offspring. As early as 1976, Visser and his colleagues identified two paralyzing peptide toxins from the wasp *Microbracon hebetor* (Visser et al., 1976). Some toxins in venoms affect neurotransmission. In the venom of parasitoid wasp *Philanthus triangulum*, a neurotoxin named philanthotoxin was found to interfere with presynaptic neurotransmission at the neuromuscular junctions (Rathmayer, 1962; Piek and Spanjer, 1986). Venom-mediated alterations in host behavior have been best studied in the wasp *Ampulex compressa*. This wasp injects venom twice into the cockroach, with the first time being into the thorax and the second time into the head (Haspel and Libersat, 2003). The first injection induces transient paralysis of the front leg by blocking post synaptic neurotransmission so that the cockroach cannot fight back for the second injection. A combination of gamma-aminobutyric acid, taurine and beta-alanine in venom is considered as the main cause of this paralysis and further studies indicated that octopaminergic modulation contributed to this paralysis (Moore et al., 2006; Rosenberg et al., 2007). During the second injection, the wasp uses dopamine to paralyse the cockroach so that it can move the host to the nest and lay eggs on it (Haspel and Libersat, 2003). Based on these studies, the venom of *A. compressa* functions directly on the central nerve system of the prey.

2. Venoms that alter host development

Several parasitoids use their venoms to control host development. The koinobiont ectoparasitoid *Euplectrus separatae* uses its venom to induce arrest development and prevent the host larva from molting (Nakamatsu et al., 2006). The ectoparasitoid *Euplectrus comstockii* venom has very similar effects (Coudron and Brandt, 1996). In *Lacanobia oleracea*

(Lepidoptera: Noctuidae) parasitized by the ectoparasitoid *Eulophus pennicornis*, parasitoid venom induces increased synthesis of JH in the corpora allata and reduces activity of JH esterase by host hemocytes. These activities increase the hemolymph titer of JH which prevents the host larva from pupating (Edwards et al., 2006). Some endoparasitoids also produce venoms that arrest development of host. For example, injection of venom from the braconid *Aphidius ervi* into the fourth stage nymphs of the aphid *Acyrthosiphon pisum* induced host developmental arrest and even death (Tremblay et al., 1998). There are also some cases indicating the same venom may function differently depending on the host system. Venom from *Microplitis croceipes* has no affect on the development of *Helicoverpa zea*, while this venom induces developmental arrest in *Galleria mellonella* (Ferkovich and Gupta, 1998). For PDV-carrying parasitoids, venom may also cooperate with PDV to modify host development. To induce the developmental arrest of *Heliothis virescens*, both venom from *Cardiochiles nigriceps* and PDV must be injected. The combination of *C. nigriceps* venom with two other PDVs, which have the same host, has no affect on host development, suggesting that venom proteins and PDVs have coevolved (Tanaka and Vinson, 1991).

3. Venoms which block host immune responses

Polydnaviruses are well known to have immunosuppressive activity. In addition to PDV, venom may also play an important role in host immune suppression in non-PDV parasitoids where venom seems to compensate for the absence of PDVs. For example, venom from the pupal endoparasitoid *Pteromalus puparum* affects host cellular immunity by reducing the percentage of spreading plasmatocytes and encapsulation of Sephadex beads (Cai et al., 2004). In *Lacanobia oleracea* (Lepidoptera: Noctuidae), venom from *Pimpla hypochondriaca* (Ichneumonidae) reduces the viability and spreading activity of host hemocytes (Richards and

Parkinson, 2000). *P. hypochondriaca* venom was also shown to block melanin formation (Parkinson and Weaver, 1999). This venom contains three phenoloxidases (POs), POI, POII and POIII, which were suspected to contribute to host immune suppression (Parkinson et al., 2001).

In the PDV-producing parasitoids, venom can contribute to suppression of cellular immunity by promoting PDV infection of host cells. For example, in the parasitoid *Cotesia melanoscela*, venom facilitates viral uncoating and increases viral persistence in the host (Stoltz et al., 1988). Although *Cotesia rubecula* venom is not essential for entry of CrBV into host hemocytes, it is required for expression of CrBV genes (Zhang et al., 2004b). Besides cooperating with PDV, the venom from some parasitoids also functions directly to suppress host immune responses. In *C. rubecula*, a venom protein Vn50 was shown to inhibit the PO pathway by mimicing serine proteinase homologs (SPHs) (Asgari et al., 2003). SPHs are the cofactors in activation of prophenoloxidase (proPO) into PO, which is catalysed by proPO-activating proteinases (PAPs). SPHs are cleaved into two domains and this cleavage is essential for proPO activation. The possible mechanism of Vn50 inhibition may be that Vn50 is not cleaved and interferes with the interaction between proPO and SPHs (Zhang et al., 2004a).

1.3 Characteristics and functions of teratocytes

Some parasitoids in the families Braconidae and Scelionidae produce cells during embryogenesis which form a serosal or extraembryonic membrane around the parasitoid embryo. These cells then dissociate and disperse into host hemolymph when the parasitoid larva hatches and are thereafter called teratocytes (Dahlman, 1990). Once in the host hemolymph, teratocytes do not divide but increase in size and become polyploid (Hotta et al., 2001). Teratocytes normally have microvilli around their surface which are thought to be important for absorbing host nutrients or secrete factors into the host hemolymph (Beckage and Gelman, 2001;

Nakamatsu et al., 2002). By altering trophic, immune and endocrinological conditions of the host, teratocytes create an environment beneficial to the parasitoid's development.

One important function of teratocytes is to interfere with host metabolism and development. For example, teratocyte-secreted proteins (TSPs) from *M. croceipes* were identified and those proteins reduce the protein titer in host hemolymph and inhibit fat body proliferation in the host *Heliothis virescens* (Järlfors et al., 1997). Among those TSPs, TSP14 was found to reduce protein synthesis in the host (Rana et al., 2002; Dahlman et al., 2003). Other experiments showed that TSPs reduce JH esterase production by the fat body in vitro, which further modifies host development (Zhang et al., 1998). The reduction of JH esterase is not accompanied by a reduction of JH esterase mRNA so that the JH esterase level is reduced by the post-transcriptional events (Dong et al., 1996). Besides secreting regulatory proteins, there are also some other mechanisms used by teratocytes to modify host development. *Cardiochiles nigriceps* teratocytes produce JH directly which modifies host development. Besides JH titer, *C. nigriceps* teratocytes also reduce the titer of 20-hydroecdysone in host hemolymph by converting it to an inactive form (Pfaff et al., 2002).

In addition to affecting host development, teratocytes from some parasitoids also show immunosuppressive activity. For example, in *Plutella xyostella*, teratocytes from the parasitoid *Cotesia plutellae* suppresses host cellular immune responses significantly. Injecting cultured teratocytes into the host larva induced the reduction of hemocyte nodulation by about 40 percent. Similarly, *Cotesia glomerata*'s teratocytes have PO inhibition activity, which may block the PO pathway in *Pieris rapae crucivora* (Kitano et al., 1990). The presence of *Apanteles kariyai*'s teratocytes also reduces PO activity by 75 percent in the host *Pseudaletia separate*, while calyx fluid and venom has no inhibitory effect on PO activity (Tanaka and Wago, 1990).

1.4 Polydnaviruses

Besides venom and teratocytes, in some species in the superfamily Ichneumonoidea, female wasps also inject polydnaviruses into hosts that are essential for successful parasitism.

Compared with most viruses, polydnaviruses have a different life cycle. These viruses exist as either inactive proviruses or active virions. The proviruses are integrated in the genome of parasitoid wasps thus the proviral genome exists in all individuals of the wasps and is passed vertically from mother wasps to the offspring. Although the viral genome is present in all wasp tissues, it only replicates in the oviducts of female wasps, where virions accumulate in the calyx fluid. During oviposition, the mature virions are injected into the lepidopteran host larva or eggs. The viral genome does not replicate in the lepidopteran host larva but virions infect some larval tissues, such as hemocytes and the fat body. This infection is beneficial for the wasp but is detrimental for its lepidopteran host. Expression of viral genes in these tissues results in several physiological alterations in the host larvae, including altered growth and immune suppression (Webb, 1998).

Based on associated wasp families, the polydnaviridae is divided into two genera, ichnoviruses (IVs) which are associated with Ichneumonidae wasps and bracoviruses (BVs) which are associated with Braconidae (Stoltz et al., 1995). Both the IVs and the BVs have segmented circular dsDNA genomes, which share several common characteristics, such as high AT bias and low coding densities (Dupuy et al., 2006; Webb et al., 2006). The number of segments ranges from less than 10 to more than 28. The length of each segment ranges from 2 to over 25 kb (Fleming, 1992). In spite of these similarities in viral genomes, the IVs and BVs contain many differences in evolutionary history, morphological characteristics and gene family components. The BVs and IVs are clearly separated by some evolutionary progenitors lacking

polydnaviruses suggesting that the two polydnavirus genera derive from independent evolutionary lineages (Webb, 1998). Both morphological and molecular evidence supports the hypothesis that the IVs evolved from ascoviruses which are target lepidopterans and are vectored by some parasitoid wasps (Bigot et al., 2008; Federici and Bigot, 2003). Phylogenetic studies suggest that BVs associate with a monophyletic group, microgastroid (Whitfield, 2002; Federici and Bigot, 2003). Recent studies on the BV structural genes located in associated parasitoid wasp genomes reveal similarities to some structural genes of nudiviruses, suggesting that the BVs may derive from nudiviruses (Bézier et al., 2009ab). IV and BV virions also have different morphologies. IVs have an oblong or globular nucleocapsid surrounded by two membrane envelopes with the outer layer obtained as the IVs bud out of the calyx cells (Webb et al., 2000). BVs always have cylindrical nucleocapsids with various lengths and envelop single or multiple nucleocapsids with a single envelope (Webb et al., 2000). Studies indicated that for *Chelonus inanitus* bracovirus (CiBV) and *Microplitis demolitor* bracovirus (MdBV), different segment molecules are packaged in different nucleocapsids which are of different abundance (Albrecht et al., 1994; Beck et al, 2007). Comparison of several sequenced IV and BV genomes indicates that there is no common gene family between IVs and BVs with the exception that ankyrin (IkB) is identified in both viral genomes (Dupuy et al., 2006). More information about the gene families of PDVs is discussed below.

1. Roles of PDVs in altering host development

As discussed above, parasitoids modify host development in ways that benefit to the growth of their own offspring. Besides venom and teratocytes, PDVs are also important in this process (Pennacchio and Strand, 2006). *Campoletis sonorensis* uses symbiotically associated PDV to target prothoracic glands (PTGs) in host *Heliothis virescens* and finally induce the

destruction of these glands (Dover et al., 1988). Since PTGs synthesize ecdysteroid hormones to promote metamorphosis, *C. sonorensis* controls host growth by this way. Similarly, *Cardiochiles nigriceps* PDVs reduce the biosynthetic activity of PTGs in infected host *Heliothis virescens*, which in turn induces host developmental arrest (Vinson et al., 1998). Another example is that injection of *Cotesia congregata* PDV into *M. sexta* larva induces abnormally high levels of JH in hemolymph so that host metamorphosis is suppressed (Beckage and Riddiford, 1982). Alternatively, MdBV infection prevents the host from reaching the critical weight so that the host endocrine events which trigger metamorphosis are inhibited (Pruijssers et al., 2009). Other parasitoids induce precocious metamorphosis in their hosts. PDVs in the genus of *Ascogaster* and *Chelonus* induce precocious metamorphosis in their lepidopteran hosts. Wandering begins an instar earlier due to a precocious increase in JH esterase so that the JH titer decreases and the onset of premature metamorphosis is triggered (Jones, 1996; Johner et al., 1999). PDVs have also been implicated in castrating hosts in some systems. In *Manduca sexta* parasitized by *Cotesia congregata* or *Pseudaletia separata* parasitized by *Cotesia kariyai*, PDV infection causes host testes to atrophy (Beckage and Reed, 1997; Tanaka and Tagashira, 1998).

2. Roles in host immunity suppression

Besides affecting host development, PDVs also exhibit immunosuppressive activities. It was found that the calyx fluid of parasitoid wasp *Campoletis sonorensis* interfered with the function of the host hemocytes (Davies and Vinson, 1988). PDV from *Microplitis demolitor* also suppresses the spreading of plasmatocytes which are essential in cellular immunity (Strand and Noda, 1991). Similarly, injection of *Hyposoter fugitivus* PDV induces the inactivation of the host *Malacosoma disstria* hemocytes which results in suppression of the cellular immunity (Stoltz and Guzo, 1986). Besides inhibiting host cellular immune responses, PDVs also interfere

with host humoral immunity. For example, the PO activity in host hemolymph is essential for humoral immune responses. Injection of MdBV or *Cotesia congregata* BV (CcBV) into *Pseudoplusia includens* or *Manduca sexta*, respectively, significantly decreased PO activity in the host hemolymph (Strand and Noda, 1991; Beckage et al., 1990). As many PDV gene families have been identified, the functions of these genes have been studied in depth.

3. PDV gene families

As discussed above, compared to other viruses, the PDVs have a very special life cycle, in which replication and infection are separated in two organisms. Once several PDV genomes have been sequenced, comparison of gene families in different PDVs provides more material for studying the evolution of these viruses. Among all PDV gene families identified, there is no gene involved in replication or assembly of the virions. Several studies indicated that some viral structural genes were located in the host wasp's genome and were not packaged into virions (Deng et al., 2000; Bézier et al., 2009ab). Under the hypothesis that the PDVs evolved from some free-living viruses, those structural genes may be transferred from viral segments to the wasp's genome during evolution and are no longer packaged in the virions (Dupuy et al., 2006). Despite different evolutionary origins, the IVs and the BVs both contain IkB genes that may have been acquired from the wasp's genome after the PDVs associated with the wasps (Dupuy et al., 2006).

From the *Campoletis sonorensis* IV (CsIV), *Hyposoter fugitivus* IV (HfIV) and *Tranosema rostrale* IV (TrIV), whose genomes were completely sequenced, six common gene families were identified, including IkB, cys-motif gene, *rep* gene, *vinnexin* gene, N-family and the polar-residue-rich proteins (PRRPs) (Tanaka et al., 2007). Although the gene families are similar in these IVs, the proportions of each gene family are significantly different among these

species. TrIV contains a unique gene family, TrVs, which is not found in CsIV or HfIV. Other species-specific IV gene families are

found in *Hyposoter didymator* IV (HdIV), which encode serine- and threonine-rich proteins. One member of this gene family, Hd*Corf*P30, contains a mucin-like motif which is similar to MdBV Glc proteins, suggesting the possible convergent evolution between these two viruses (Galibert et al., 2003).

In addition to the IkB gene family, protein tyrosine phosphatases (PTPs) are present in all BVs, which might also be acquired from the wasp before subfamily diversification (Dupuy et al., 2006). Species-specific BV gene families are identified from CcBV, MdBV, *Glyptapanteles indiensis* BV (GiBV), *Glyptapanteles flavicoxis* BV (GfBV) and *Cotesia plutellae* BV (CpBV). Besides PTP and IkB, CcBV encodes cysteine-rich proteins (CRPs) and cysteine protease inhibitors (cystatin), which are unique in viruses, and five other gene families whose functions have not been found, including early-expressed protein 1 (EP1) (Espagne et al., 2004). In the CpBV genome, PTP gene families and EP1-like protein, which is similar to the CcBV EP1 genes, were identified (Choi et al., 2005). The GiBV and the GfBV also contain several species-specific gene families, such as cystatins, C-type lectins and a sugar transporter (Desjardins et al., 2008). In the MdBV genome, five gene families, including IkB, PTP, egf, glc and tRNA were identified (Webb et al., 2006).

The characteristics and functions of PDV gene families were studied. The IkB gene family is another common gene family in PDVs which is significantly similar to *Drosophila* IkB factors. IkB factors are important in the NF-kB signaling pathway which plays an important role in both immune responses and development (Kroemer and Webb, 2005; Tian et al., 2007; Ghosh et al., 1998). NF-kB is inactive when bound to IkB and becomes free and active when IkB is

phosphorylated and then cleaved or degradated. After entering cell nuclei, the free NF-kB functions as a transcriptional factor. Most genes regulated by NF-kB signaling pathway are related to immune responses or development (Ghosh et al., 1998). Different than host IkB factors, the PDV IkBs lack the regulatory domain so that they are not able to be degradated. In this way, the host NF-kB signaling pathway is always blocked by the IkB gene products (Thoetkiattikul et al., 2005).

Other IV-specific gene families have also been investigated. CRP gene family was so named because the proteins encoded by this gene family have a conserved, cysteine-rich motif (Dib-Hajj et al., 1993). Their predicted amino acid sequences also contain conserved hydrophobic signal peptides and N-terminal glycosylation sites (Cui and Webb, 1996). This gene family is involved in both host development modification and cellular immune suppression (Fath-Goodin et al., 2006; Li and Webb, 1994). Further evidence suggests that these gene products may block host protein synthesis at the post-transcriptional level in hemocytes, fat body and testis (Kim, 2005). The *rep* genes were first identified from the CsIV genome and are composed of a highly conserved 540-bp repeat, but the function for this gene family remains unclear (Theilmann and Summers, 1987). Similar repeated element genes were identified from HdIV and expressed not only in lepidopteran host fat body and cuticle epithelium but also in female parasitoid wasps, suggesting that this gene family may not be involved in host immunosuppression but in some other physiological changes of the host (Galibert et al., 2006). *Vinnexin* family members share homology with invertebrate *innexin* family proteins and are thought to modify cell-cell communication during encapsulation responses based on this homology (Kroemer and Webb, 2004).

PTP is a common gene family identified from all BVs. Some of the encoded protein tyrosine phosphatases (PTPs) were found to disrupt phagocytosis in host larvae (Pruijssers and Strand, 2007). PTP-H2 in MdBV also shows apoptotic activity to some insect cells (Suderman et al., 2008). It was also found that CpBV PTP reduces the spreading and encapsulation ability of cells (Ibrahim and Kim, 2008). PTPs from *Toxoneuron nigriceps* BV (TnBV) were found expressed in inactivated prothoracic glands (Falabella et al., 2006). The lectin gene family from *Cr*BV genome encodes a protein which is speculated to compete with the host lectin to mask the hemocyte from immune challenges (Glatz et al., 2003). CcBV cystatins were found to have cysteine protease inhibitory activity against several cysteine proteases (Espagne et al. 2005). CcBV EPs were found to be expressed only from the infected permissive host while the function remains unclear (Beckage and Tan, 2002; Harwood et al., 1998). *Egf* gene family from MdBV encodes proteins with trypsin inhibitor-like domains and one member, egf1.0, was found to inhibit the melanization process in the host. The mechanism of this egf1.0 protein is proposed to be a Laskowski mechanism and its target enzyme is prophenoloxidase-activating proteinase 3 (PAP3) (Beck and Strand, 2007). Recent experimental results show that the egf1.0 also blocks the PO pathway by inhibiting the amidolytic activity of PAP1 and the processing of pro-PAP1 and pro-PAP3 (Lu et al., 2008). The *glc* gene family encodes a cell surface glycoprotein Glc1.8. In the extracellular domain of this protein, there are five identical tandem repeats (Trudeau et al., 2000). This protein is the main viral factor to block the cellular immune responses and expression of this protein induces the cells to lose adhesion and phagocytosis ability (Beck and Strand, 2003; Beck and Strand, 2005).

1.5 Summary

Parasitoid wasps use hosts as the food source for offspring with parasitism almost always resulting in the death of the host. Since many hosts of parasitoids are agricultural pests, many parasitoids are used as biocontrol agents. Parasitoid wasps often induce changes in host physiology that facilitate survival of the parasitoid's offspring. Disturbing normal host development and evading host immune responses are the two major targets for parasitoid wasps. The factors used by wasps, such as venom, teratocytes and PDV, are all injected into the hosts along with eggs. In this review, I discussed the influence of these three factors on host development and immunity in detail. Some parasitoids also use other factors to redirect host development and immunity. Those factors include virus like particles (VLPs) and parasitoid larval secretions. VLPs are capsid-like structures synthesized in the calyx gland of female wasps and coated around the parasitoid eggs after ovipositon. These VLPs lack a DNA core like PDVs but they help wasp eggs to evade recognition so that the host immune responses are not evoked (Reineke et al., 2006). Some parasitoid larvae secrete factors which affect host development. For example, some chelonine braconid larvae release ecdysteroids into host hemolymph to redirect host development. The salivary glands of some parasitoid larvae secrete factors that regulate and redirect host metabolism so that host hemolymph amino acid components change (Beckage and Gelman, 2004).

Different parasitoid factors cooperate to modulate host physiological conditions. For example, in *Cotesia kariyai* and *Microplitis croceipes*, venom appears to be required as a cofactor to invoke the metamorphic disturbances. In some BVs, venom enhances the action of PDV by promoting uptake of virions by host caterpillar cells and enhancing the uncoating of the

virons in host cells, speeding up the entry of viral DNA into the nucleus of host cells and enhancing the persistence of virus in the host (Asgari, 2006).

Among the factors used by parasitoid wasps to evade host immunity, this dissertation will focus on the *glc1.8* gene in the MdBV, which is symbiotically associated with *M. demolitor*. The main goal of this dissertation is to learn the structural and functional characteristics of the Glc1.8 protein. The *glc1.8* gene is the only identified *glc* gene from the MdBV genome. Firstly in Chapter 2, I provided the evidence that proves the existence of another *glc* gene from the viral genome. The protein encoded by *glc1.8* prevents cell adhesion and phagocytosis and is predicted to have multiple N-glycosylation sites. In Chapter 3, I first experimentally confirmed and characterized this N-glycosylation. Different mutants were constructed to further investigate functional characteristics of this protein. The data suggest that the extracellular N-glycosylation on Glc1.8 is essential for the immunosuppressive activity of this protein. Further more, the cytoplasmic tail of this protein was proved to be required for its function, which suggested that some cytoplasmic factors or signaling pathways may be involved in the anti-adhesion and anti-phagocytosis activity of this protein. Based on this hypothesis, in Chapter 4, I studied the influence of the MdBV infection on cell adhesion signaling. My results proved that integrin subunits are downregulated and MAPK pathway is activated after the viral infection. Glc1.8 expression also affects actin polymerization which is essential for cell adhesive process.

1.6 References

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Chapter 2. Identification of *glc* genes in MdBV genome and a *glc1.8* stably transfected cell line

2.1 Introduction

As discussed in the previous chapter, the parasitoid wasp *Microplitis demolitor* uses its associated bracovirus to suppress host immune defenses (Strand, 1994). *Microplitis demolitor* bracovirus (MdBV) infection, which takes place after oviposition, induces a strong suppression to the host immune system so that its offspring are able to develop successfully. Previous studies indicate that the MdBV genome consists of 15 circular DNA segments named from A to O based on their sizes (Webb et al., 2006). By screening cDNA and expression library and analyzing the sequence of the MdBV genome, several viral gene families have been identified. These families include the *IkB*, protein tyrosine phosphatase (*ptp*), *egf* and *glc* genes, which target different factors in the host immune system (Thoetkiattikul et al., Pruijssers and Strand, 2007; Strand et al., 1997; Trudeau et al., 2000).

The *glc* genes encode glycoproteins which prevent cells from adhering to foreign surfaces and phagocytizing foreign targets (Beck and Strand, 2003; Beck and Strand, 2005). Up to now, there is only one *glc* gene, *glc1.8* identified in the MdBV genome. This gene exists in two copies on segment O and codes for a 1.8kb mRNA (Trudeau et al., 2000). The corresponding protein Glc1.8 is a 55kDa transmembrane protein with a signal peptide on its N-terminus and a transmembrane domain plus a short cytoplasmic tail on its C-terminus. In the extracellular region of this protein, there are five identical repeats which are predicted to be highly N-glycosylated. Functional studies indicate that expression of this protein induces host immune

cells to lose their adhesion and phagocytosis ability. Two copies of glc1.8 were identified on the annotated segment O. However, Glc1.8 specific probes recognized two mRNA bands (1.8 & 3.6 kb) from MdBV infected *Pseudoplusia includens* hemocytes on Northern blots, suggesting that there is another *glc* gene present in the MdBV genome (Trudeau et al., 2000).

In this chapter, I used both Southern blotting and Western blotting to confirm that there is another *glc* gene in the MdBV genome which is much larger than *glc*1.8. To further characterize Glc1.8, we made a pIZT/Glc1.8 stably transfected High Five cell line, from which I observed two forms of Glc proteins expressed. By Southern blotting, I confirmed that there is a larger form of the *glc* gene existing specifically in this cell line.

2.2 Materials and Methods

Insects and cell culture *Pseudoplusia includens* and *Microplitis demolitor* were reared at 27˚C under a 16 hour light : 8 hour dark photoperiod as previously described (Strand et al., 1997). *P. includens* larvae were fed on artificial diet and *M. demolitor* female adults were allowed to parasitize fourth instar *P. includens* larvae. High Five cells were maintained in Corning 75 cm^2 tissue-culture flasks in TC100 medium containing 10% fetal bovine serum at 27˚C (Beck and Strand, 2005).

Transfection of High Five cells I used the expression vector pIZT/V5-His (Invitrogen) to produce a recombinant form of wild type Glc1.8 (pIZT/Glc1.8). For transient transfection, High Five cells were seeded in 12-well plates 12 hours before transfection. The cells were incubated in transfection reagent (6 μg/ml plasmid DNA and 6% Lipofectin (Invitrogen) in TC100 medium) for 6 hours and resuspended in TC100 insect cell culture medium containing 10% FBS. High Five cells were transfected with pIZT/Glc1.8 and the stably transfected cell lines were generated by Zeocin selection. Cells were passaged 20 times before use in experiments.

Immunofluorescence staining Living cells or cells fixed with paraformaldehyde were processed for immunofluorescence microscopy as previously outlined (Beck and Strand, 2003) using an anti-Glc1.8 primary antibody (55F2G7) and a murine Alexafluor 568-conjugated secondary antibody (Molecular Probes). Samples were examined using a Leica IRE2 inverted epifluorscent microscope fitted with a Hamamatsu digital camera and SimplePCI software (Compix, Cranberry, PA) for image acquisition. Final images were assembled using Adobe Photoshop.

Adhesion and phagocytosis assays. Adhesion and phagocytosis assays with stably transfected cells were conducted as previously outlined (Beck and Strand, 2005). Briefly, control and stably transfected cells were collected from culture flasks and seeded at a density of 1 x 10^4 cells per well in culture wells. Assays were stopped by placing culture plates on ice after 2 h. Although GFP served as a marker for transfected cells, I also labeled living cells with our anti-Glc1.8 antibody to unambiguously identify cells expressing Glc1.8 from cells that did not. The percentage of rounded, non-adherent cells versus adherent attached cells expressing Glc1.8 were then scored by counting 200 cells per well from 4 randomly selected fields of view using our inverted epifluorescent microscope. Phagocytosis assays were also conducted as previously described using heat-killed *Escherichia coli* labeled with fluorescein isothiocyanate (FITC) as the foreign target (Beck and Strand, 2005). Cells were collected and added to new 12-well culture plates in medium without serum at a density of 1 x 10^3 cells per well. After a 2 h preincubation period, bacteria were added to each culture well at a ratio of 15: 1 for an additional 2 h. Culture plates were then placed on ice, and cells were labeled with anti-Glc1.8 antibody. I then determined the percentage of cells that had phagocytosed bacteria by adding a drop of trypan blue (1 mg/ml in citrate buffer; pH 4.4). Intracellular particles continued to fluoresce

green after addition of trypan blue while the fluorescence of extracellular particles was quenched. I also note that the intensity of staining of the FITC-labeled particles made it easy to distinguish them from the much weaker GFP signal in the cytoplasm and/or nucleus of transfected cells.

Statistical analysis. The number of samples for each experimental condition is indicated in the figure legends. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison procedure using JMP statistical software (SAS, Cary, NC).

MdBV collection and cell infection Calyx fluid was collected from the female wasps by established methods (Beck et al., 2007). Briefly, reproductive tracts were dissected from *M. demolitor* in phosphate buffered saline. MdBV virions were collected by centrifugation of the calyx fluid at 4˚C for 20 minutes at 16,000 x g and resuspended in phosphate buffered saline. The MdBV purified from one wasp was referred to as one wasp equivalent. For infection, the High Five cells were seeded in 12-well plate for 12 hours. Purified MdBV was added to the cells at the concentration of 0.5 wasp equivalent per well.

Western blotting Protein samples were collected from MdBV transfected High Five cells and pIZT/Glc1.8 transfected High Five cells 48 hours after infection or transfection by lysing the cells in SDS loading buffer containing 2-mercaptoethanol or TCEP. For Western blotting, protein samples were run on 4-20% precast SDS-PAGE gels (Lonza), transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) and probed with murine anti-Glc1.8 monoclonal antibody (55F2G7) (1:10000) or anti-V5 antibody (1: 5000) (Invitrogen). Bands were visualized with a goat anti mouse horseradish peroxidase-conjugated

secondary antibody (Jackson labs) (1:20000) and chemiluminescence of the ECL advanced substrate (Amersham Biosciences) and a GeneGenome Bio-Imaging system (Syngene).

Genomic DNA extraction MdBV was resuspended in 100 µl of reaction buffer (0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5) containing 2 µl DNase I (Ambion) and incubated 30 minutes at room temperature to eliminate any non-viral DNA. The virions were then collected by recentrifugation and washed with Pringle's saline (154 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$ and 22 mM D-glucose). To extract the genomic DNA from MdBV, purified viral particles were resuspended in PBS with 2 % sarcosyl and 0.5 mg/ml Proteinase K at 62˚C for 1 hour. The MdBV genomic DNA was purified by phenol:chloroform extraction and isopropanol precipitation (Beck et al., 2007). Genomic DNA from High Five cells was extracted using the Qiamp DNA mini kit (Qiagen).

Southern blotting For Southern hybridizations, digoxigenin (DIG) labeled DNA probes were prepared by random priming using DIG-dUTP in DIG High Prime DNA Labeling and Detection Starter Kit (Roche). The probes were first synthesized by PCR using the forward primer: 5'-TTACTGGTCAATCCGAAATC -3'and reverse primer: 5'- CTTATGGCAGAACCTTCATC -3' and gel purified. In 20 µl labeling reactions, 1.0 µg probe was mixed with 4 µl of DIG High Prime and incubated at 37˚C for 20 hours. For Southern blotting, the MdBV genomic DNA was digested by *Nsi* I and *Msc* I and the genomic DNA from High Five cells was denatured at 65˚C for 10 minutes and digested by *EcoR*I. The digested DNAs were size fractionated on a 0.8% agarose gel (10 ng/lane for MdBV genomic DNA and 5 µg/lane for High Five cells genomic DNA) and transferred to Nylon positive membrane (Roche) in 20x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Blots were prehybridized for 1 hour at 40˚C in hybridization buffer and incubated with DIG-labeled DNA probes at 25 ng/ml for 16 hours at 40˚C. Blots

were washed under low stringency conditions (2x SSC, 0.1% SDS, and room temperature) and high stringency conditions (0.5x SSC, 0.1% SDS, and 65˚C) and then blocked for 1 hour in blocking solution. The alkaline phosphatase conjugated anti-digoxigenin antibody was added to blots at a 1:10000 (75 mU/ml) dilution and incubated for 2 hours. Blots were washed with washing buffer (0.1 M Maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5) and incubated with CSPD ready-to-use substrate for 4 hours and exposed in a GeneGenome Bio-Imaging system (Syngene).

2.3 Results

Two different *glc* genes are present in MdBV genome

Our anti-Glc1.8 monoclonal antibody recognized a 115 kDa band plus a weaker band of ca. 250 kDa in homogenates of the MdBV-infected High Five cells (Fig 2.1A). Based on the protein sequence, the predicted molecular mass of Glc1.8 is 56,182 Da (Trudeau et al., 2000). Sequence analysis of Glc1.8 predicted 34 N-glycosylation sites which significantly increase the molecular mass of this protein. Since the 115 kDa band was detected in cells transiently transfected with pIZT/Glc1.8, this protein band represents Glc1.8 protein. To assess whether the 250 kDa band represents a protein encoded by a second glc gene, I conducted a Southern blotting analysis of MdBV genomic DNA. The probe was made against the repeat sequence of *glc1.8* and MdBV genomic DNA was digested by *Msc* I which cleaves at the 5' end of *glc1.8* gene and *Nsi* I which cleaves near the 3' end of *glc1.8*. This *glc* repeat probe recognized two genomic DNA bands, which were 3.6 kb and 8.2 kb respectively (Fig. 2.1B). Since the *glc1.8* genomic DNA is 3.6 kb long, the 8.2 kb band represents another *glc* gene family in MdBV which corresponds to the 250 kDa Glc protein band. Combined with the previous Northern blotting

data (Trudeau et al., 2000), these results indicate that a second *glc* gene is present in the MdBV genome.

Stably transfected High Five cell line has low adherence and phagocytosis activity

High Five cells normally have a fibroblastic morphology and grow as a monolayer attached to the surface of culture plates (Fig. 2.2A). Cells stably transfected with Glc1.8 in contrast had a rounded morphology as previously reported for MdBV-infected cells (Fig. 2.2B). Immunostaining revealed that similar to virus-infected cells (Beck and Strand, 2003), Glc1.8 was uniformly distributed on the surface of most stably transfected cells (Fig. 2.2C). The anti-Glc1.8 monoclonal antibody 55F2G7 did not give any signal in the control High Five cells (data not shown). Bioassays also indicated that stably transfected cells were non-adhesive and exhibited little phagocytic activity toward bacteria (Fig. 2.2D). As a result, the stable line grew as a suspension culture with growth rates similar to wild-type High Five cells (data not presented).

There is a new *Glc* gene in stably transfected High Five cells

Although the predicted molecular mass of Glc1.8 is 56,182 Da (Trudeau et al., 2000), SDS-PAGE under reducing conditions using ß mercaptoethanol and immunoblotting detected Glc proteins of larger molecular mass in pIZT/Glc1.8 stably transfected High Five cell line, which may be due to the predicted high level of N-glycosylation. This antibody detected 115 and 140 kDa bands, a ca. 250 kDa band, and several other bands of greater molecular mass in homogenates of cells stably transfected with pIZT/Glc1.8 (Fig. 2.3A). Anti-Glc1.8 detected no proteins in homogenates of mock-transfected High Five cells (data not presented). These results were corroborated using an anti-V5 antibody that recognized one of the epitope tags incorporated into recombinant Glc1.8. This antibody also detected the 115, 140, and ca. 250 kDa bands in transiently and stably transfected cells but only weakly recognized the higher molecular mass

proteins detected by anti-Glc1.8 (Fig. 2.3B). Glc1.8 contains three cysteine residues residing at amino acid position 17 in the extracelluar N-terminal domain, and positions 470 and 496 in the transmembrane domain and cytoplasmic tail respectively. When samples were processed in the presence of a stronger reducing agent (TCEP), anti-Glc1.8 detected the 115 kDa and 140 kDa proteins, but the ca. 250 kDa band and other higher molecular mass proteins were near absent (Fig. 2.3C). These results overall suggested the 115 and 140 kDa bands represent different forms of Glc1.8, whereas the higher molecular mass bands detected by anti-Glc1.8 likely reflect oxido-reductive interactions between Glc1.8 with itself or other unknown cellular proteins.

Some glycosylated cell surface proteins like mucins are polymorphic due to allelic variation in the number of tandem repeats in their extracellular domains (Fowler et al., 2001). Given the similar domain structure of Glc1.8, I considered the possibility that the two forms of Glc1.8 detected in the stably transfected cell line (115 and 140 kDa) could have arisen from selection of a parent cell that contained an integrated copy of wild-type *glc1.8* plus a mutant form of the gene with a larger number of tandem repeats in the extracellular domain. To assess whether such a recombinantion occurred, I conducted Southern blotting studies using genomic DNA from stably transfected High Five cells probed with the *glc* repeat probe. The genomic DNA was first digested by *EcoR*I which cuts once on the 5' end the *glc1.8* gene and once on the end of the fifth repeat. The probe which is specific to *glc* repeat region recognized two bands in the digested genomic DNA from the stable line (Fig. 2.3D). One band was about 3.6 kb which is the genomic DNA of *glc1.8*, and the other one was 5.0 kb which corresponds to a larger form of the *gl*c gene.

2.4 Discussion

Among the gene families identified from MdBV genome, *glc* is the major one which blocks the adhesion and phagocytosis ability of the cells. As the MdBV genome was sequenced, we found that there were two copies of *glc* genes located on segment O. However, the structure of tandem repeats of this family made it impossible to annotate the sequence accurately. Although we observed two transcripts of *glc* in Northern blot, we only cloned one *glc* gene out of the MdBV genome, which is *glc1.8*. Since we had no direct evidence supporting the presence of two different *glc* genes in MdBV genome, we assembled the O segment containing two identical *glc* genes. In the MdBV infected cells, two Glc proteins were identified which corresponds to the fact that there are two *glc* transcripts. Our Southern blotting analysis using a *Glc* specific probe indicated that in MdBV genome, there are two *glc* genes with different sizes. In the Southern blotting, the 3.6 kb band represents the genomic DNA of *glc1.8* and the 8.2 kb band is the larger form of the *glc* gene.

In the mammals and insects, mucins are another family of glycoproteins which have multiple tandem repeats. The mucins are characterized by a variable number of tandem repeats (VNTR) domain. The VNTR polymorphism of mucins was detected at the genomic DNA level and causes the mucin proteins of different length (Kramerov et al., 1996; Vinall et al., 1998). Similar with mucins, Glc1.8 has five tandem repeats in the the extracellular region which are highly glycosylated. Since we cannot detect the genomic DNA of the larger *glc* gene from the sequencing data, it is very possible that the new *glc* gene has similar structure as *glc*1.8 except the number of tandem repeats. Given that the size of one tandem repeat is 647 base pair long, the larger form of *glc* gene should contain about twelve tandem repeats while the *glc*1.8 only has

five. As we already know, Glc1.8 expression reduces about 70% of adhesion and phagocytosis activity of cells, however, the function of the larger form of *glc* gene remains unclear.

In the pIZT/Glc1.8 stably transfected High Five cell line, I also detected two Glc proteins, Glc1.8 and a larger form Glc. This larger Glc protein was observed exclusively in this stable cell line. Expansions of tandem repeats have been reported for several genes from *Drosophila* and yeast (Thompson-Stewart et al., 1994; Pâques and Wegnez, 1993; Welch et al., 1990). These rearrangements of tandem repeats were proved to be induced by double-strand break repair (Thompson-Stewart et al., 1994; Pâques et al., 1998). Given that the tandem repeats are the major component of *glc* gene, it is possible that some rearrangement of these repeats took place during the mitosis of the cells. If those two forms of *glc* genes only differ in the number of tandem repeats, from the size of the two bands of Southern blot, I can deduce that the larger form of *glc* gene should contain 7 repeats.

2.6 References

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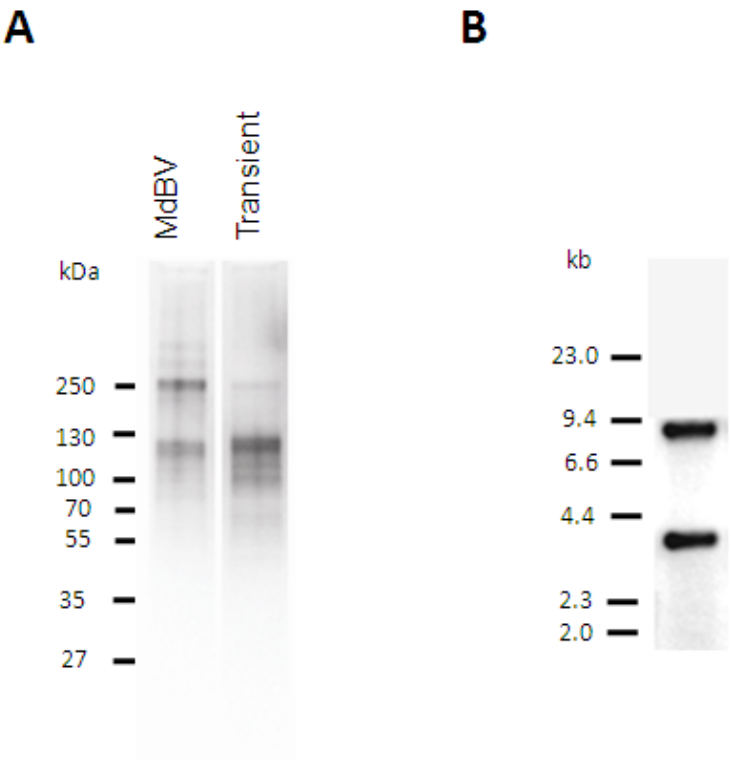
Figure 2.1. Glc proteins in MdBV infected High Five cells and *Msc* I/*Nsi* I MdBV genomic fragments recognized by a *glc1.8* probe. (A) Two Glc proteins were detected in High Five cells infected by MdBV (lane 1) by anti-Glc1.8 monoclonal antibody. The molecular weights of these two Glc proteins are 115 kDa and 250 kDa, respectively. In High Five cells transiently transfected by pIZT/Glc1.8, this antibody only recognized the 115 kDa bands (lane 2), which corresponded to Glc1.8. (B) Southern blot result of Msi I and Nsi I digested MdBV genome. The glc-containing fragments were recognized by a DIG labeled repeat-specific probe and visualized by AP-conjugated DIG antibody and a chemiluminescence substrate. Two fragments were detected at 3.6 kb and 8.2 kb. The genomic DNA of *glc1.8* is the 3.6 kb band and the 8.2 kb band is a new *glc* gene which is much larger than *glc1.8*.


A
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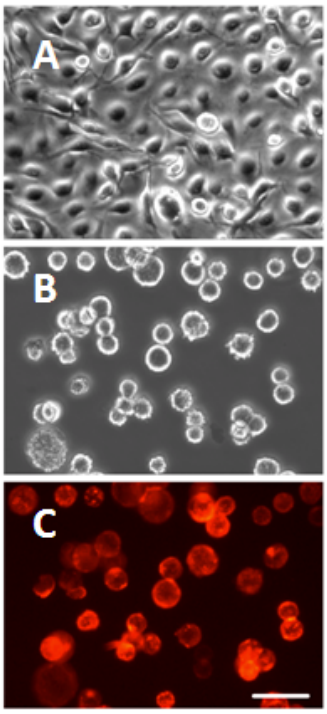
2.3
2.0

Figure 2.2. Stably transfected High Five cells exhibit a loss of adhesion and phagocytosis. (A) Normal High Five cells are always adhesive and have a fibroblastic morphology. (B). High Five cells stably transfected with pIZT/Glc1.8 are non-adhesive and rounded. (C) Epifluorescent micrograph of the same image as (B) visualized by labeling living cells with anti-Glc1.8 and an Alexa 568-conjugated secondary antibody. Scale bar in (C) is 40 μM with cells in (A-C) at equal magnification. (D) Adhesion and phagocytosis of FITC-conjugated *E. coli* by High Five cells stably transfected with pIZT/Glc1.8 (Glc1.8 stable) and normal High Five cells. Adhesion and phagocytosis rates were determined 2 h after transfer to new culture plates or addition of *E. coli*. A cell was scored as phagocytic if one or more bacteria had been internalized. Results are given as mean percentages ± standard error (SE) of adhesive cells relative to the total number of cells (200) counted per sample.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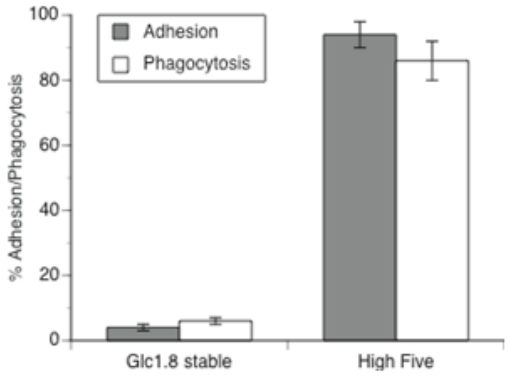
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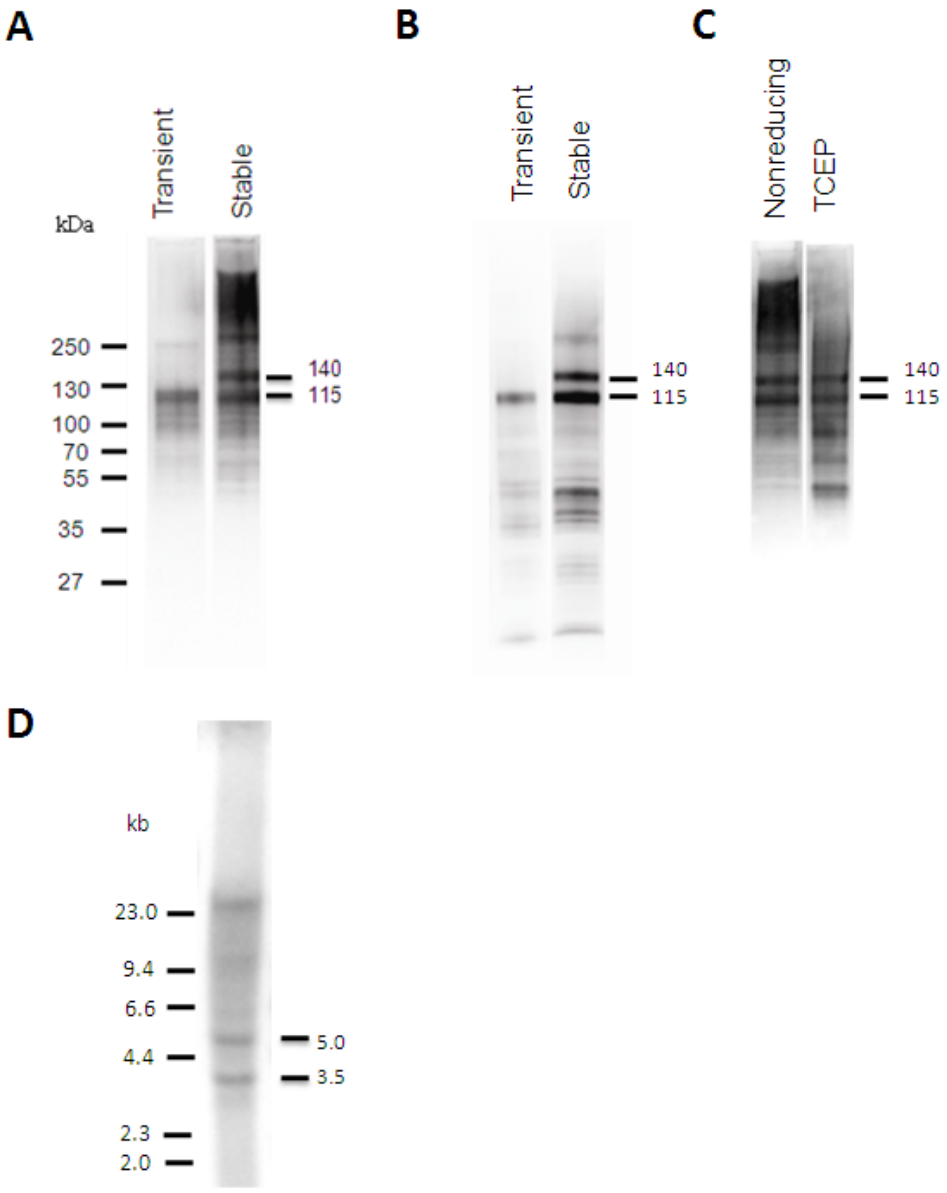
Phagocytosis
Glc1.8 stable
High Five

Figure 2.3. Two forms of Glc proteins are expressed in High Five cells stably transfected with *glc1.8*. (A) High Five cells stably transfected with pIZT/Glc1.8, or transiently transfected with pIZT/Glc1.8 were solublized in sample buffer containing ß mercaptoethanol followed by SDS-PAGE and immunoblotting using anti-Glc1.8. Sizes of the molecular mass markers are indicated to the left. Note that the predominant band in lanes loaded with transient transfected cell extracts is 115 kDa, whereas 115 and 140 kDa bands of equal intensity plus several additional bands of larger mass are detected in the stably transfected cell extract. (B) Transiently and stably transfected cells processed as in (A) using an anti-V5 antibody. (C) Stably transfected cells solublized in sample buffer with no reducing agent or sample buffer containing TCEP followed by SDS-PAGE and immunoblotting using anti-Glc1.8. (D) Southern blot result of EcoRI digested genomic DNA from stably transfected High Five cells were probed with the glc repeat specific probe. A 3.5 kb band and a 5.0 kb band were detected which corresponded to the Glc proteins observed in Western blot (A and B). These results indicated that in the stable line, there was a recombination event which resulted in expression of a larger form of the *glc* gene.


A
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C
Nonreducing
TCEP
140
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kb
23.0
9.4
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Chapter 3. Structural and functional characterization of the cytoplamic tail and N-glycosylation of the MdBV protein Glc1.8

3.1 Introduction

The Polydnaviridae (PDVs) is a large family of enveloped DNA viruses symbiotically associated with parasitoid wasps (Dupuy et al., 2006; Kroemer & Webb, 2004; Webb & Strand, 2005). The family is divided into two genera, bracoviruses (BVs) and ichnoviruses (IVs), carried by wasps in the families Braconidae and Ichneumonidae respectively. Each PDV from a given wasp species is genetically unique and persists as a stably integrated provirus in both sexes. Transmission to offspring is strictly vertical through the germ line, while replication occurs only in female wasps in a region of the reproductive tract called the calyx. Virions accumulate to high density in the lumen of the calyx and wasps inject a quantity of this virus into hosts when laying their eggs. Most PDV-carrying wasp species parasitize the egg or larval stage of insects in the order Lepidoptera (moths and butterflies), and virus particles infect several host tissues including immune cells (hemocytes) and the fat body. PDVs do not replicate in the wasp's host but do express genes whose products cause severe physiological alterations including suppression of the immune response toward the parasitoid's progeny (Pennacchio and Strand, 2006; Schmidt et al., 2001; Strand, 2008b; Webb and Strand, 2005).

The braconid wasp *Microplitis demolitor* carries *Microplitis demolitor* bracovirus (MdBV) and parasitizes the larval stage of *Pseudoplusia includens*, *Trichoplusia ni,* and other

moth species in the family Noctuidae. Cellular immune responses by insects toward parasites include encapsulation, which involves the adhesion of multiple immune cells (hemocytes) to the invader, and phagocytosis (Irving et al., 2005; Pennacchio and Strand, 2006; Strand, 2008a; Webb and Strand, 2005). Genome analysis and functional studies identify several MdBV genes with immunosuppressive activities including a 514-amino acid type I transmembrane protein named Glc1.8 (Trudeau et al., 2000; Webb et al.; 2006). Glc1.8 is characterized by a signal peptide at its N-terminus, a 390 amino acid extracellular domain, a C-terminal transmembrane domain, and a short cytoplasmic tail (Trudeau et al., 2000). This protein is targeted to the surface of virus-infected hemocytes and several permissive cell lines including hemocyte-like BTI-TN-5B1-4 (High Five) cells from *T. ni* (Beck and Strand, 2003; Beck and Strand, 2005; Trudeau et al., 2000; Webb et al., 2006). Transient expression of wild-type Glc1.8 inhibits High Five and other cells from adhering to foreign surfaces or phagocytizing small targets, whereas knockdown of *glc1.8* expression by RNA interference (RNAi) rescues adhesion and phagocytic activity of MdBV-infected cells (Beck and Strand, 2003; Beck and Strand, 2005). In contrast, Glc1.8 mutants with a truncated extracellular domain or deleted cytoplasmic region exhibit little or no anti-adhesive and anti-phagocytic activity (Beck and Strand, 2005). Taken together, these results identify Glc1.8 as a key determinant in suppressing the function of host insect immune cells.

The activity of transmembrane proteins can be significantly affected by glycosylation of extracellular regions as well as by cytoplasmic domains that interact with intracellular proteins. Sequence analysis predicts 34 *N*-linked glycosylation sites in the extracellular domain of Glc1.8 (Trudeau et al., 2000), but the identity of the glycans occupying these sites or whether they are required for immunosuppressive activity is unknown. More broadly, while the major *N*-linked

glycans associated with insect-produced glycoproteins appear to be of the high mannose type (Aoki et al., 2007; Harrison and Jarvis, 2006; Shi and Jarvis, 2007), no studies have characterized the glycan composition of polydnavirus-encoded proteins or their importance for function. Here I report that mono- and difucosylated *N*-glycans predominate the surface of Glc1.8 but I also detected several minor complex glycans. Functional assays further indicate that *N*-glycosylation of the extracellular domain is essential for biological activity.

3.2 Materials and methods

Cell culture and MdBV isolation. High Five cells were cultured in TC-100 modified medium (Sigma) supplemented with 10% fetal calf serum (Atlanta Biologicals) (Beck and Strand, 2003; Beck and Strand, 2005). Cells were maintained and passaged as adherent cells in Corning 75 cm^2 tissue-culture flasks. Most experiments were conducted in 12-well culture plates (Corning). Cells were seeded into wells at specific densities, allowed to settle, attach, and spread for 6-12 h, and then used for particular assays. MdBV was collected from adult *M. demolitor* females and High Five cells were infected as previously outlined (Beck and Strand, 2003).

Recombinant proteins and purification. We previously used the vector pIZT/V5-His (Invitrogen) to produce expression constructs for wild-type Glc1.8 (pIZT/Glc1.8), the deletion mutants pIZT/Glc1.8Δ1-4 that lacked 1-4 tandem repeats in the extracellular domain, and pIZT/Glc1.8ΔC that lacked both the transmembrane domain and cytoplasmic tail of the protein (Beck and Strand, 2005). This vector uses the OpIE2 promoter from the *Orgyia pseudotsugata* baculovirus for constitutive expression of the gene of interest, adds V5 and 6xHis epitope tags to the C-terminus of the recombinant protein, and encodes a Zeocin-green fluorescent protein (GFP) gene fusion under control of the OpIE1 promoter. During the current study, I used Zeocin

selection as outlined by the manufacturer to produce a clonal line of High Five cells stably transfected with pIZT/Glc1.8. After 20 passages over five months in the presence and absence of antibiotic selection pressure, recombinant Glc1.8 was affinity purified by placing cells in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole and 1% Triton, pH 8.0) and incubating overnight with Ni-NTA superflow beads (Qiagen). After washing in buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole and 1 % Triton, pH 8.0), recombinant proteins were eluted from beads using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM Imidazole and 1% Triton, pH 8.0) and concentrated.

I also produced three new mutants for the current study. Two mutants lacked either all of the extracellular domain (Glc1.8Δ5) or had an intact C-terminal transmembrane domain but a deleted cytoplasmic tail (Glc1.8Δcyt). Both were generated by PCR using pIZT/Glc1.8 as template. For Glc1.8Δ5, I first amplified a 5' region using the primers 5'-AATCTAGAATGGCGCAAATTACTT-3' (forward) and 5'- AGTAGTATTTAAGT TAGAATTGGTACATGAGAAGC -3' (reverse); and a 3' region using the primers 5'-TGGC TTCTCATGTACCAATTCTAACTTAAATACTACTA-3' (forward) and 5'- ATACCGCGGT AACTCGTGAGAAC -3' (reverse). *Xba*I and *Sac*II sites (underlined) were incorporated into one of the forward and reverse primers for directional cloning. I then used overlapping PCR to generate a mutant sequence in which all of the tandem repeats of the extracellular domain were deleted. After subcloning into pCR2.1-TOPO (Invitrogen) and digestion with *Xba*I and *Sac*II to release the insert, Glc1.8Δ5 was cloned into pIZT/His-V5. For Glc1.8Δcyt, I used the primers 5'-AATCTAGAATGGCGCAAATTACTT-3' (forward) and 5'-ATACCGCGGCTA CGCATGGTACGAAAAAC-3' (reverse). The reverse primer created a deletion from position 1480 to 1548 (stop codon) that resulted in retention of the transmembrane membrane domain

plus four downstream amino acids and loss of the remainder of the cytoplasmic tail. This product was then subcloned and moved into pIZT/V5-His as described above. Sequences for each mutant were confirmed by cycle sequencing (Applied Biosystems). Expression plasmids were then transiently expressed in High Five cells by cationic lipid-mediated transfection with transfection efficiencies averaging 70% as measured by GFP expression (Beck and Strand, 2005).

Another mutant, Glc1.8ΔNglyc, which lacked predicted *N*-glycosylation sites in the extracellular domain, was produced using a novel approach founded on the controlled and ordered oligonucleotide ligation (COOL) procedure of Blachinsky et al. (2004). Briefly, each tandem repeat (78 amino acids, 234 nucleotides) in the extracellular domain contains 6 predicted *N*-glycosylation sites (N-X-S/T/C where X is any amino acid) (Fig. 3.1A). I first had two sequences named Rep 1-4 and Rep 5 commercially synthesized (GenScript Corporation, Piscataway, NJ) (Fig. 3.1A). Rep 1-4 corresponded to the Glc1.8 cDNA sequence (GenBank accession no. AF267175) from position 147 (*Afl*II site) to 416 with a *Sbf*I site added to the 3' end. Each asparagine codon within the N-X-S/T/C sites of the Rep 1-4 sequence was mutated to a glutamine by single nucleotide replacement. In addition, an *Nsi*I site was introduced by point mutation at position 184 and the *Afl*II site was removed at position 381 without altering the amino acid sequence. Rep 5 matched the Glc1.8 sequence from position 1083 (*Afl*II site) to 1340 and included an additional *Sbf*I site at the 3' end. In addition to changing all asparagine codons to glutamines, an *Nsi*I site was generated at position 1120. Note that the *Nsi*I and *Sbf*I sites within the two synthesized sequences were located at the same distance in relation to the wild-type *Afl*II sites, creating an overlapping region between the 3' end of Rep 1-4 and the 5' end of Rep 5. Next, I digested pIZT/Glc1.8 DNA with *Afl*II and *Nsi*I, which removed the extracellular

domain, and then ligated Rep 5 into the opened construct using T4 ligase (Fig. 3.1B). At the 3' end of the insert, the compatible ends of *Sbf*I and *Nsi*I form a sequence that cannot be cut by either enzyme; thus locking Rep 5 into place during the next round of *Afl*II/*Nsi*I digestion used to open the vector and insert the next repeat. Another copy of Rep 1-4 was then ligated and locked into the 3' end by the compatible ends of *Sbf*I and *Nsi*I. After three more rounds of digestion and insertion of repeat 1-4, the cDNA sequence was rebuilt such that the *N*-glycosylation sites in the extracellular domain were eliminated (Fig. 3.1B). I then reverted the nucleotide changes at the *SbfI/NsiI* junctions introduced to perform the COOL procedure to the wild-type sequence using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) and the oligonucleotide 5'-CTAACAGAACCTGCACAAACCACTTATGCTACGCAATCAACGCTT ACAAG -3' (mutagenized nucleotides underlined).

SDS-PAGE, immunoblotting, and immunofluorescence staining. Protein amounts for cell extracts and purified recombinant Glc1.8 were determined using the Micro BCA Protein Assay Kit (Pierce). Equal quantities of sample were then placed in sample buffer (8 mM Tris-HCl, 2% SDS, 10% glycerol, 0.2 mg/ml bromophenol blue) with (reducing) or without (non-reducing) 8% ß mercaptoethanol. Samples were boiled for 3 min, run on 4-20% precast SDS-PAGE gels (Lonza) and stained with Coomassie blue or silver (Biorad). For immunoblotting, proteins were transferred to polyvinylidene difluoride PVDF membranes (Immobilon-P, Millipore), blocked with 5% bovine serum albumen, and incubated with either a murine anti-Glc1.8 monoclonal antibody (55F2G7) (1: 10000) (Bangham et al., 2006) or anti-V5 antibody (1: 5000) (Invitrogen). Bands were visualized with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson Labs) (1: 50,000) plus chemiluminescence using the ECL Advance Kit (Amersham Biosciences) and a GeneGenome Bio-Imaging System (Syngene). Living cells or

cells fixed with paraformaldehyde were processed for immunofluorescence microscopy as previously outlined (Beck and Strand, 2005) using anti-Glc1.8 and a murine Alexafluor 568-conjugated secondary antibody (Molecular Probes). Samples were examined using a Leica IRE2 inverted epifluorscent microscope fitted with a Hamamatsu digital camera and SimplePCI software (Compix, Cranberry, PA) for image acquisition. Final images were assembled using Adobe Photoshop.

Glycosidase digestions, tunicamycin treatment, and lectin blotting. For glycosidase digestions, purified recombinant Glc1.8 was dialysed into phosphate-citric buffer (pH 5.0) or phosphate buffer (pH 6.8) containing 2% Triton X-100 followed by digestion with N-glycosidase (PNGaseA) at 37˚C for 24 h as outlined in the protocol provided by the supplier. In other experiments, High Five cells transiently transfected with pIZT/Glc1.8 or pIZT/Glc1.8ΔNglyc were treated for 48 h with tunicamycin (Calbiochem) (1 μg/ml) dissolved in dimethylsulfoxide. Samples were analyzed by SDS-PAGE and immunoblotting as described above and compared to control samples maintained in medium without tunicamycin. For detection of lectin-binding sites, Glc1.8 purified from stably transfected cells was run on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% bovine serum albumen, blots were incubated with a panel of biotinylated lectins (5 μg/ml) (ConA, DBA, GS-I, LPA, PNA, SBA, SJA, UEA-I and WGA) that have binding preferences for different oligosaccharides (EY Laboratories). Lectin binding was then visualized by incubation with horseradish peroxidase-conjugated avidin and diaminobenzyl.

Preparation of glycopeptides and release of N-linked glycans. Recombinant Glc1.8 bands eluted from Ni-NTA beads were cut from Coomassie Blue-stained gels. In-gel tryptic digestion and release of N-linked glycans were performed as outlined by Chill et al. (2009). Briefly, gel

pieces were destained alternately with 40 mM ammonium bicarbonate and 100% acetonitrile until clear. The gel fragments were then reswelled in 10 mM dithiothreitol (DTT) in 40 mM ammonium bicarbonate at 55º C for 1 h. After cooling, the DTT solution was replaced with 55 mM iodoacetamide (IDA) and the gel pieces were incubated in the dark for 45 min followed by washing alternately with 40mM ammonium bicarbonate and 100% acetonitrile twice. The dehydrated gel fragments were then re-swelled with trypsin solution (trypsin in 40 mM Ambic) on ice for 45 min followed by overnight digestion at 37º C. The supernatant was transferred into another tube. Peptides and the glycopeptides were extracted from the gel in series with 20% acetonitrile in 5% formic acid, 50% acetonitrile in 5% formic acid and then 80% acetonitrile in 5% formic acid. The sample solutions were dried, combined into one tube, and passed through a C18 sep-pak cartridge followed by washing in 5% acetic acid. Peptides and glycopeptides were then eluted in 20% isopropanol in 5% acetic acid, 40% iso-propanol in 5% acetic acid, and 100% isopropanol. The isopropanol fractions were dried and then combined in one tube with water and heating at 100^{o} C for 5 min to inactivate trypsin and evaporated to dryness. The dried glycopeptides were then resuspended in 50 μl of 20 mM sodium phosphate buffer, pH 7.5, for digestion with PNGaseF, or in 50μl of 0.2 M citrate phosphate buffer, pH 5.0, for digestion with PNGaseA. Digestions were performed with both enzymes because glycan release by PNGaseF is inhibited for *N*-linked oligosaccharides with fucose linked α1-3 to the internal GlcNAc of the chitobiose core, whereas PNGaseA is not restricted by core fucosylation.

Following PNGase digestion for 18 h at 37° C, samples were passed through a C18 Sep-pak cartridge and the carbohydrate fraction was eluted with 5% acetic acid and dried by lyophilization. Released *N*-linked oligosaccharides were permethylated based on the method of Anumula and Taylor (1992) and analyzed by Matrix-assisted laser-desorption ionization time-of-

flight mass spectrometry (MALDI/TOF-MS) and nanospray ionization spectrometry (NSI-MS) (Aoki et al., 2007). MALDI/TOF-MS was performed in the reflector positive ion mode using a-dihyroxybenzoic acid (DHBA, 20 mg/mL solution in 50% methanol/water) as a matrix. Spectra were obtained by using a 4700 Proteomics analyzer (Applied Biosystems, Foster City, CA). Permethylated glycan solutions were mixed 1:1 with the matrix and 1 ml of the mixture was spotted onto the sample plate. Acquisition and processing methods were set as follows: operating mode, MSreflector positive; shots per sub-spectrum, 50; subspectra to accumulate, 20; laser intensity, 5,000--6,000; processing method, reflector default. Mass analysis was determined by using NSI-LTQ/MS^n according to the method of Aoki et al. (Aoki et al., 2007). Permethylated glycans were dissolved in 1 mM NaOH in 50% methanol and infused directly into the linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, Waltham, MA) at a constant flow rate of 0.4 mL/min. The capillary temperature was set at 210° C and MS analysis was performed in the positive ion mode. The collision energy was set at 28 for MS/MS fragmentation. For total ion mapping, automated MS/MS analysis (at 28 collision energy), m/z range from 500 to 2,000 was scanned in successive 2 mass unit windows that overlapped the preceding window by 2.8 mass units. Proposed glycan structures were assigned based on parent ion masses and MS^n fragmentations with the systematic nomenclature of Domon and Costello (1988) used to guide the depiction of fragmentation derived from MS spectra.

The total ion mapping (TIM) functionality of the XCalibur software package (2.0) was utilized to detect and quantify the prevalence of individual glycans in the total glycan profile (Aoki et al., 2007). Through TIM, automated MS/MS analysis (at 28 collision energy), *m/z* range from 500 to 2000 was scanned in successive 2.8 mass unit windows that overlapped the preceding window by 2 mass units. The 2.8 mass unit window allowed signals from the

naturally occurring isotopes of individual glycans to be summed into a single response, increasing detection sensitivity for minor structures, while the 2 mass unit overlap ensured that minor glycans, whose masses placed them at the edge of an individual window, would be sampled in representative fashion. Most permethylated oligosaccharides were identified both as singly and doubly charged species by NSI-MS. Peaks in TIM scans were quantified if 3-fold or greater above background. Glycan prevalence was calculated as the percent total profile where the total profile was taken as the sum of the peak intensities for all quantified glycans.

Adhesion and phagocytosis assays. Adhesion and phagocytosis assays with MdBV-infected, transiently transfected, or stably transfected cells were conducted as previously outlined (Beck and Strand, 2005). Briefly, transiently transfected cells were collected 48 h post-transfection, washed 2x with TC-100 medium (Sigma), and then seeded at a density of 1 x 10^4 cells per well in new culture plates containing 500 µl of TC-100 plus serum. Control and stably transfected cells were collected from culture flasks and seeded at the same density in culture wells. Assays were stopped by placing culture plates on ice after 2 h. Although GFP served as a marker for transfected cells, I also labeled living cells with our anti-Glc1.8 antibody (see above) to unambiguously identify cells expressing Glc1.8 from cells that did not. The percentage of rounded, non-adherent cells versus adherent attached cells expressing Glc1.8 were then scored by counting 200 cells per well from 4 randomly selected fields of view using our inverted epifluorescent microscope. Phagocytosis assays were also conducted as previously described using heat-killed *Escherichia coli* labeled with fluorescein isothiocyanate (FITC) as the foreign target (Beck and Strand, 2005; Pruijssers and Strand, 2007). Cells were collected and added to new 12-well culture plates in medium without serum at a density of 1 x 10^3 cells per well. After a 2 h preincubation period, bacteria were added to each culture well at a ratio of 15: 1 for an

additional 2 h. Culture plates were then transferred to ice with cells and labeled with anti-Glc1.8 antibody. I then determined the percentage of cells that had phagocytosed bacteria by adding a drop of trypan blue (1 mg/ml in citrate buffer; pH 4.4). Intracellular particles continued to fluoresce green after addition of trypan blue while the fluorescence of extracellular particles was quenched. I also note that the intensity of staining of the FITC-labeled particles made it easy to distinguish them from the much weaker GFP signal in the cytoplasm and/or nucleus of transfected cells.

Statistical analysis. The number of samples for each experimental condition is indicated in the figure legends. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison procedure using JMP statistical software (SAS, Cary, NC).

3.3 Results

N-linked glycans occupy predicted glycosylation sites in the extracellular domain.

Affinity chromatography using Ni-NTA beads resulted in significant purification of the 115 and 140 kDa forms of Glc1.8 from the stably transfected cell line (Fig. 3.2). PNGaseA treatment reduced the molecular mass of the 140 and 115 kDa forms to ca. 110 and 85 kDa (Fig. 3.3A). I also transiently transfected cells with pIZT-Glc1.8 in the presence of tunicamycin, which blocks N-glycan synthesis. Analysis of these samples by SDS-PAGE and immunoblotting indicated that tunicamycin treatment reduced the mass of the 115 kDa form of Glc1.8 to 70 kDa, which was very similar to the mass of the protein detected in cells transiently transfected with the non-glycosylated mutant construct pIZT/Glc1.8ΔNglyc (Fig. 3.3B). In contrast, tunicamycin had no effect on the mass of Glc1.8ΔNglyc itself (Fig. 3.3B). I also noted an absence of the ca. 250

kDa protein or other higher molecular mass bands in tunicamycin-treated cells expressing Glc1.8 or cells expressing Glc1.8ΔNglyc (Fig. 3.3B). I tested eight biotin-labeled lectins with different oligosaccharide binding preferences against the 115 and 140 kDa forms of Glc1.8 purified from the stably transfected cell line. Only two of these lectins bound to the proteins. Conconavalin A (Con-A), which preferentially binds high mannose type oligosaccharides, strongly recognized both forms of Glc1.8, while soybean agglutinin lectin (SBA), which binds weakly galactosamine residues, slightly recognized both forms of the protein (Fig. 3.3C). Taken together, these results indicated the estimated mass of Glc1.8 on SDS-PAGE gels relative to its predicted size is due in large part to high mannose *N*-linked glycans occupying glycosylation sites in the extracellular domain.

The *N*-linked glycan profile of Glc1.8 is dominated by high mannose oligosaccarides.

Following enzymatic release and permethylation, NSI/MS of the unfractionaled glycans revealed an overwhelming predominance of high mannose structures associated with the 115 and 140 kDa forms of Glc (Fig. 3.4). I also observed a significant increase in signals for fucosylated *N*-glycans released by PNGaseA when compared to PNGaseF indicating the presence of α1-3 linked fucose with both forms of Glc1.8 (Fig. 3.4A, B). The profiles detected by MALDI-TOF/MS for major glycans were near identical to those detected by NSI/MS (data not presented). For identification of minor glycans, permethylated, unfractionated, glycans were analyzed by TIM. For each collection window (2.8 mass units wide) in the TIM profile, MS2 spectra were manually inspected for loss of characteristic masses. Loss of the reducing terminal monosaccharide as GlcNAc (dm/z-277) or substituted GlcNAc (dm/z—451 for monofucosylated GlcNAc, dm/z=-625 for difucosylated GlcNAc), or loss of nonreducing terminal GlcNAc from

complex glycans (dm/z=-259) generated strong daughter ions that were diagnostic for the presence of a glycan within the mass window.

The total glycan profile for the 115 and 140 kDa forms of Glc protein included a series of non-, mono-, and difucosylated glycans that ranged in size from M2N2 to Glc3M9N2, and that included paucimannose, hybrid, and complex structures (Fig. 3.4C). The two most abundant structures for both forms of the protein were M3N2 and M3N2F that together accounted for more than 70% of the total glycan profile (Fig. 3.4C). Less abundant glycans included nine that carried Fuc in α1-3 linkage to core GlnNAc. These ranged from difucosylated, paucimannose glycans like M3N2F2^{3,6}, to complex and hybrid difucosylated species like NM3N2F2^{3,6}, and GalNM3N2F2^{3,6}. The hybrid glycan GalNM3N2 was also detected in non- and monofucosylated forms but no sialylated forms were detected (Fig. 3.4C). Given the low abundance of some of the hybrid and complex structures detected, I also analyzed control samples prepared with the 115 and 140 kDa forms of Glc protein without trypsin/PNGase treatment, or trypsin/PNGase samples without Glc1.8. Analysis by NSI-MS^n and TIM of permethylated control material yielded no peaks or parent or daughter ions attributable to the structures reported above for the experimental samples (data not presented).

The epitope for anti-Glc1.8 resides within a tandem repeat of the extracellular domain while glycosylation is required for biological activity.

We previously determined that the mutants Glc1.8Δ1-4, which lack 1-4 of the tandem repeats that make the extracellular domain, exhibit a progressive reduction in anti-adhesive and anti-phagocytic activity, while Glc1.8ΔC, which lacks both the transmembrane and cytoplasmic domain, has no activity (Beck and Strand, 2005). Loss of activity with truncation of the

extracellular domain could reflect either a requirement for the protein backbone or the *N*-glycans associated with this domain. In contrast, the inactivity of Glc1.8ΔC most likely reflects loss of the transmembrane domain, since this protein is secreted and the extracellular domain alone cannot bind to immune cells. In addition, our anti-Glc1.8 monoclonal antibody, 55F2G7, recognizes all of these mutants (Beck and Strand, 2005). Given that 55F2G7 also recognized Glc1.8ΔNglyc (see Fig 3.3), these results overall suggest this antibody recognizes a non-glycosylated epitope that resides within a single tandem repeat.

To further characterize the determinants required for antibody recognition and activity, I transiently expressed Glc1.8Δ5, that lacked all five tandem repeats of the extracellular domain, and Glc1.8Δcyt, that only lacked the cytoplasmic tail, in High Five cells. Immunoblots of the resulting cell extracts indicated that 55F2G7 recognized Glc1.8Δcyt but did not recognize Glc1.8Δ5, whereas the anti-V5 antibody to the epitope tag present in all of the Glc1.8 mutants we produced recognized both (Fig. 3.5A). This result confirmed that 55F2G7 binds an epitope within a single tandem repeat of the extracellular domain. I then asked whether anti-adhesion and anti-phagocytic activity depended on glycosylation of the extracellular domain by comparing Glc1.8ΔNglyc to Glc1.8Δ5 and other forms of Glc1.8 in functional assays. As expected, Glc1.8Δ5, had no anti-adhesion or anti-phagocytic activity, but neither did Glc1.8ΔNglyc even though its distribution on the surface of cells appeared identical to wild-type Glc1.8 (Fig. 3.5B-G). Cells transfected with Glc1.8Δcyt in contrast exhibited an intermediate response with anti-adhesion and anti-phagocytic activity that was weaker than cells expressing wild-type Glc1.8 but stronger than cells transfected with Glc1.8ΔC or the empty vector (Fig. 3.5B, C). Unlike

Glc1.8ΔC (Beck and Strand, 2005), Glc1.8Δcyt also localized to the surface of transfected cells with a distribution that was indistinguishable from the wild-type protein (Fig. 3.5F, G).

3.4 Discussion

MdBV encodes multiple genes whose combined activity suppresses both humoral and cellular defense responses of insects (Beck and Strand, 2003; Beck and Strand, 2005; Beck and Strand, 2007; Lu et al., 2008; Pruijssers and Strand, 2007; Strand et al., 1997; Suderman et al., 2008; Thoetkiattikul et al., 2005). Prior results identified Glc1.8 as the most important factor for disruption of adhesion and phagocytosis activity of immune cells, and indicated that both the extracellular and C-terminal domains of the protein are essential for activity (Beck and Strand, 2003; Beck and Strand, 2005). In the current study, I determined that the major glycans associated with Glc1.8 are high mannose and fucosylated paucimannose oligosaccharides while minor glycans include hybrid and complex *N*-linked species. Our results also indicate that glycosylation of the extracellular domain is essential for anti-adhesive and anti-phagocytic activity, whereas loss of the cytoplasmic tail of the protein only partially reduces activity.

Mammals produce an abundance of complex glycans through high-level activity of glycotransferases and other essential enzymes. The identification of glycotransferases and other glycan biosysnthetic enzymes in *Drosophila* and lepidopterans like *T. ni* indicate that insects are also capable of synthesizing complex glycans (Abdul-Rahman et al., 2002; Hollister et al., 2003; North et al., 2006; Palomares et al., 2003; Sarkar et al., 2006; Tomiya et al., 2004; van Die et al., 1996). Yet similar to Glc1.8, *N*-glycan analysis of a few endogenous proteins from Lepidoptera and recombinant proteins produced using baculovirus vectors and lepidopteran cell lines indicate associated major glycans have simple oligomannose or paucimannose structures. In contrast,

complex glycans containing galactose or sialic acids are minor components or absent (Aoki et al., 2007; Gagneux and Varki, 1999; Harrison & Jarvis, 2006; Shi and Jarvis, 2007). Several of the hybrid and complex structures I detected in Glc1.8 are to our knowledge novel for insect viruses and Lepidoptera but some of these structures have been detected in total glycan pools from *Drosophila* embryos (Aoki et al., 2007). One factor suggested to contribute to the low abundance of complex glycans in insect-produced glycoproteins is low-level expression of glycotransferases and other key enzymes in most insect cells (Harrison and Jarvis, 2006; Shi and Jarvis, 2007). Another potentially contributing factor is the presence of active hexosaminidase in the secretory pathway of insects that results in removal of terminal GlcNAc (Aumiller et al., 2006; Geisler et al., 2008; Léonard et al., 2006; Tomiya et al., 2006). In contrast, a higher level of complex glycan synthesis may occur in specialized cell populations or in restricted domains during insect embryogenesis suggesting a role for more complex glycoproteins in specific developmental processes (Aoki et al., 2007; Koles et al., 2004).

Whether the major glycans associated with Glc1.8 from High Five cells are similar to those from MdBV-infected primary host cells like hemocytes is unknown. However, the molecular mass of Glc1.8 expressed in host hemocytes is similar to the mass of the protein in High Five cells, which circumstantially suggests *N*-glycosylation patterns are also similar (Beck and Strand, 2005; Trudeau et al., 2000). From the result of the previous chapter, I know that the two forms of Glc proteins (115 and 140 kDa) in the stable line are due to some recombination on the genomic DNA level and the 115 kDa band corresponds to Glc1.8. Although I did detect some minor differences in the glycan profiles of the 140 and and 115 kDa forms, the majority of glycan moieties of the two bands are almost same.

We previously determined that a soluble form of Glc1.8 (Glc1.8ΔC) has no biological activity and that the protein must be anchored in the cell membrane for immunosuppressive function (Beck and Strand, 2005). This finding precluded a traditional approach for studying the functional importance of glycosylation like bioassaying a non-glycosylated, soluble form of the protein produced in bacteria. Drug treatment offered a second approach for conducting functional studies given that tunicamycin treatment blocks *N*-glycosylation of Glc1.8. I did conduct adhesion and phagocytosis assays using tunicamycin-treated cells expressing Glc1.8 and observed differences in adhesion and phagocytosis relative to controls (data not presented). However, I was not confident about interpretation of these data because tunicamycin treatment adversely affects cell viability due to disruption of *N*-glycosylation of other essential proteins, which in turn likely indirectly contributed to the reductions in adhesion and phagocytosis we observed. I thus took advantage of the modular structure of the extracellular domain to design a novel strategy for producing a mutant in which the extracellular domain was selectively non-glycosylated. The similarity in molecular mass of Glc1.8ΔNglyc and wild-type Glc1.8 from tunicamycin-treated cells on immunoblots, and the lack of effect of tunicamycin treatment on Glc1.8ΔNglyc itself is fully consistent with loss of the *N*-glycans that are normally incorporated into the extracellular domain. Moreover, our bioassay data with Glc1.8ΔNglyc lend strong support for the essential role of *N*-glycosylation for immunopathogenic activity.

Since cell surface glycoproteins form structures that extend above the cell surface, disruption of adhesion and phagocytosis could be due to this glycoprotein physically preventing adhesion and/or pattern recognition receptors of insect immune cells from binding ligands associated with different foreign entities (Bangham et al., 2006; Irving et al., 2005; Lavine and Strand, 2003; Strand, 2008a). Alternatively, *N*-glycans also influence protein structure and

mediate the specificity of protein-protein interactions (Harrison & Jarvis, 2006; Haltiwanger & Lowe, 2004) that in the case of Glc1.8 could be important for anti-adhesion and anti-phagocytic activity. Although wild-type Glc1.8 and Glc1.8ΔNglyc appear to be similarly distributed on the surface of cells, our immunoblotting results suggest Glc1.8 covalently interacts with other, currently unknown, proteins, whereas Glc1.8ΔNglyc does not.

Another virus-encoded transmembrane glycoprotein with anti-adhesive and immunopathogenic activity is the envelope glycoprotein (GP) from Ebola virus (Simmons et al., 2002; Sullivan et al., 2003; Zampieri et al., 2007). GP normally mediates binding and fusion of the virus to host cells (Chan et al., 2000), yet transient expression of GP alone induces loss of adhesion and varying degrees of cytotoxicity in several cell lines (Sullivan et al., 2005; Zampieri et al., 2007). The immunosuppressive activity of GP depends on its glycosylated extracellular domain, which is implicated in down regulating expression of surface proteins essential for adhesion (Shi and Jarvis, 2007; Sullivan et al., 2005; Takada et al., 2000). GP also adversely affects specific signaling factors involved in cell growth and survival (Zampieri et al., 2007). We do not observe any Glc1.8-induced cytotoxicity in any of the insect cell lines we have examined but recent studies do indicate that Glc1.8 down-regulates expression of specific integrin subunits implicated in regulating adhesion of insect hemocytes (Lavine and Strand, 2003; Levin et al., 2005; Moita et al., 2005; Zhuang et al., 2007; Lavine & Strand, unpublished). The reduction in biological activity of Glc1.8Δcyt compared to wild-type Glc1.8 further suggests this domain interacts with intracellular factors including possibly signaling proteins that mediate the activity of integrins or other adhesion receptors.

An estimated 30,000 species of parasitoids carry polydnaviruses and biological studies indicate that several isolates disable the insect immune response in a manner similar to MdBV (Asgari et al., 1997; Huw Davies et al., 1987; Ibrahim and Kim, 2008; Kwon and Kim, 2008; Strand, 2008b; Webb and Strand, 2005). However, it is unclear to what extent these immunopathogenic effects involve Glc1.8 homologs. Comparative genomic data on BVs is currently restricted to less than a dozen isolates from wasps in only a few genera (*Microplitis, Cotesia, Glyptapanteles, Toxoneuron,* and *Chelonis*) relative to the total number of genera and species (>17,000) of braconids that carry BVs (Dupuy et al., 2006; Kroemer and Webb, 2004; Murphy et al., 2008). Our own recent results suggest that Glc1.8 homologs are encoded by other wasp species in the genus *Microplitis* but thus far no Glc1.8 homologs have been identified in BVs from wasps in other genera. More extensive taxon sampling and comparative biological studies are needed to determine whether this virulence gene is only encoded by PDVs associated with wasps in the genus *Microplitis* or is more extensively distributed.

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Figure 3.1. Structural domains of *glc1.8* and schematics for the *N*-glycosylation mutant Glc1.8ΔNglyc and deletion mutants Glc1.8Δ5 and Glc1.8Δcyt. (A) Major domains in the previously published sequence for wild-type *glc1.8* (GenBank accession no. AF267175). A solid box represents the signal peptide, gray boxes represent the five tandemly arrayed direct repeats, and hatched boxes represent the transmembrane and cytoplasmic domains. *Afl*II and *Nsi*I sites are indicated. Below the schematic is shown the location of the synthetic Rep1-4 and Rep5 sequences used to generate Glc1.8ΔNglyc. (B) Summary of the procedure used to produce Glc1.8ΔNglyc (see text for details). (C) Schematics of the deletion mutant Glc1.8Δ5 that lacks the extracellular domain and Glc1.8Δcyt that lacks the cytoplasmic tail (see text for details).

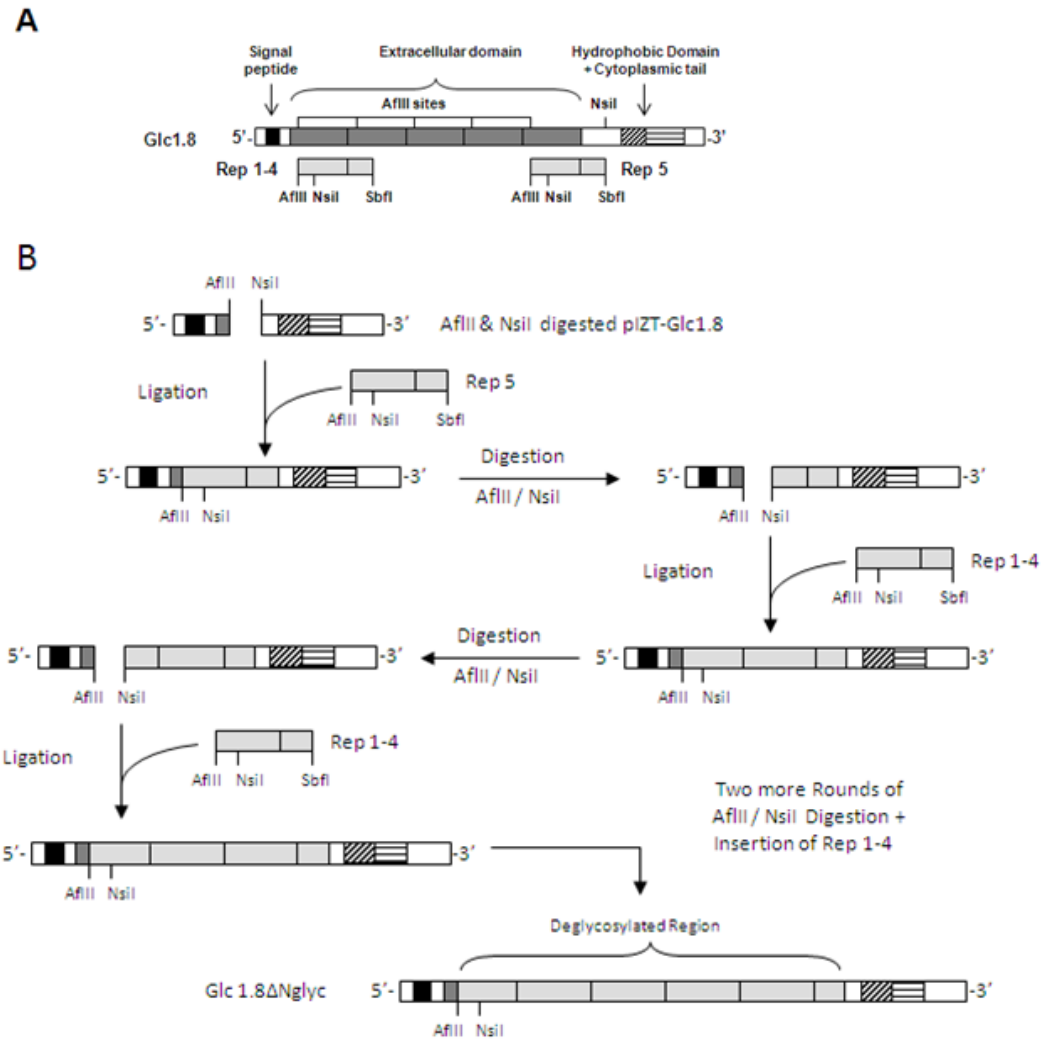

A
Signal peptide
Extracellular domain
Hydrophobic Domain + Cytoplasmic tail
AflII sites
NsiI
Glc1.8
5'-
-3'
Rep 1-4
AflII NsiI SbfI
Rep 5
AflII NsiI SbfI
B
AflII NsiI
5'-
-3'
AflII & NsiI digested pIZT-Glc1.8
Ligation
Rep 5
AflII NsiI SbfI
Digestion
AflII / NsiI
Ligation
Rep 1-4
AflII NsiI SbfI
Digestion
AflII / NsiI
Ligation
Rep 1-4
AflII NsiI SbfI
Two more Rounds of AflII / NsiI Digestion + Insertion of Rep 1-4
Deglycosylated Region
Glc 1.8ΔNglyc
AflII NsiI
C
Glc1.8Δ5 5'-
-3'
Glc1.8Δcyt 5'-
-3'

Figure 3.2. Affinity purification of Glc1.8 from stably transfected High Five cells. Glc1.8 proteins were Ni-NTA column purified with eluted proteins subjected to SDS-PAGE under reducing conditions followed by silver staining (A) and immunoblotting using anti-Glc1.8 (B). The lanes marked C show the crude cell extract prior to addition of Ni-NTA beads. Lanes 1-3 show the proteins eluted from Ni-NTA beads after the first, second and third step elution respectively.

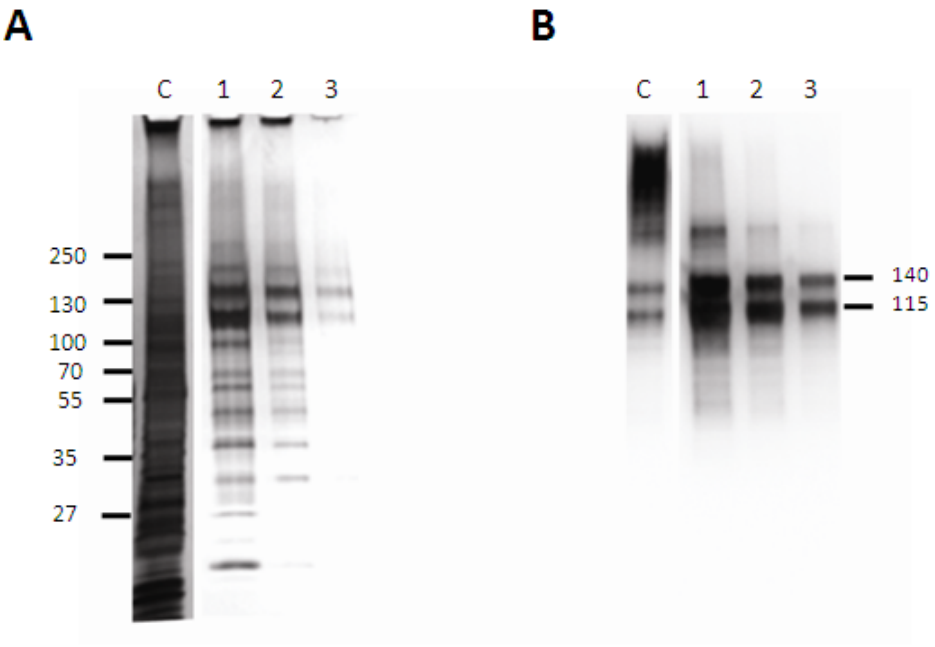

A
C
1
2
3
250
130
100
70
55
35
27
B
C
1
2
3
140
115

Figure 3.3. Glycosidase and tunicamycin treatment reduces the molecular mass of Glc1.8. (A) Affinity purified Glc1.8 from stably transfected cells was either untreated (Glc1.8 stable), treated with PNGaseA followed by SDS-PAGE and immunoblotting. PNGaseA treatment reduced the mass of the 115 and 140 kDa forms of Glc1.8. (B) Cells transiently transfected with pIZT/Glc1.8 or pIZT/Glc1.8ΔNglyc were cultured for 48 h in medium only or medium containing tunicamycin, followed by SDS-PAGE and immunoblotting. Note that transiently transfected cells only express the 115 kDa form of Glc1.8 which was reduced in mass by tunicamycin treatment to a similar size as Glc1.8ΔNglyc. (C) Affinity purified Glc1.8 from stably transfected cells was subjected to SDS-PAGE and lectin blotting using ConconavalinA (ConA) or soybean (*Glycin max*) agglutinin lectin (SBA). Sizes of the molecular mass markers are indicated to the left of A-C.

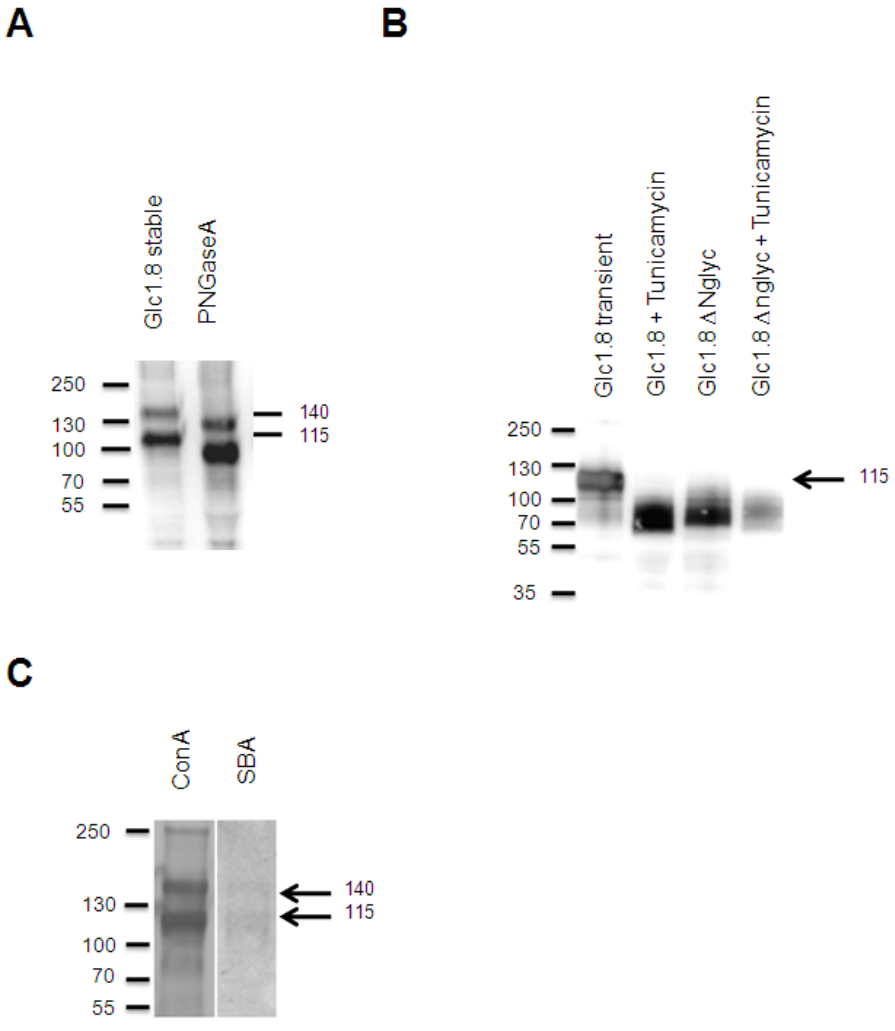
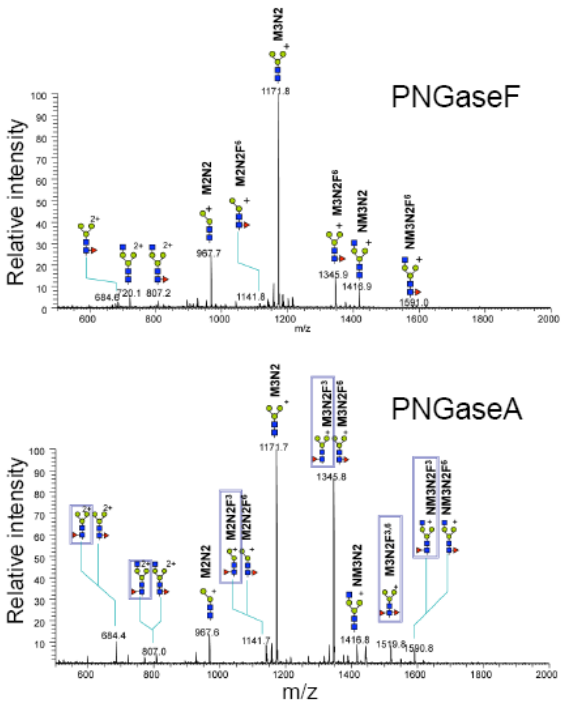
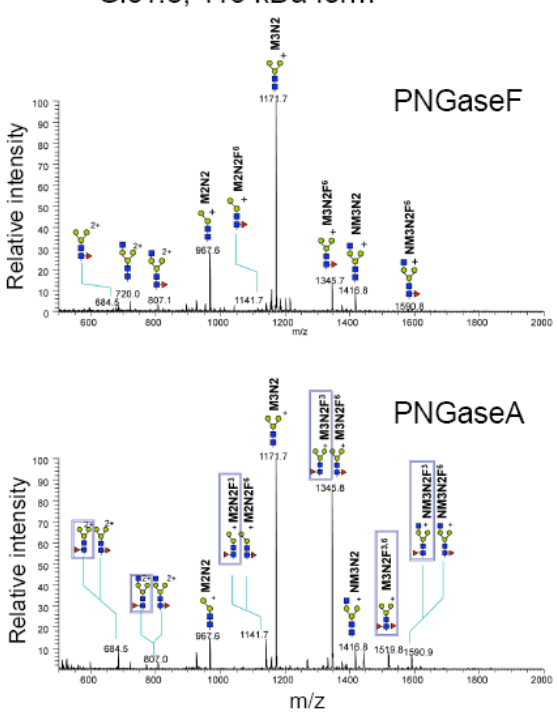

A
Glc1.8 stable
PNGaseA
250
130
100
70
55
140
115
B
Glc1.8 transient
Glc1.8 + Tunicamycin
Glc1.8 ΔNglyc
Glc1.8 Δnglyc + Tunicamycin
250
130
100
70
55
35
115
C
ConA
SBA
250
130
100
70
55
140
115

Figure 3.4. Detection and identification by NSI-MS of *N*-linked glycans from the 140 (A) and 115 kDa (B) forms of Glc proteins. Glycans were released from the 140 and 115 kDa forms of Glc proteins using PNGaseF or PNGaseA, followed by permethylation, and analysis. Glycans detected as singly and doubly charged (2+) species are indicated, with structural assignments based on parent ion masses and MS^n fragmentation pattern. Glycans with core α1-3 fucose released by PNGaseA but not PNGaseF are boxed. α1-3 fucosylation of core GlcNAc is indicated by a Fuc extending to the left while α1-6-fucosylation is indicated by a Fuc extending to the right. (C) The total *N*-linked glycan profiles for the 140 and 115 kDa forms of Glc proteins. Following permethylation, glycan profiles were quantified by TIM. Results are expressed as a percent of the total pool of detected glycans. Glycan structures were assigned as described above.

A



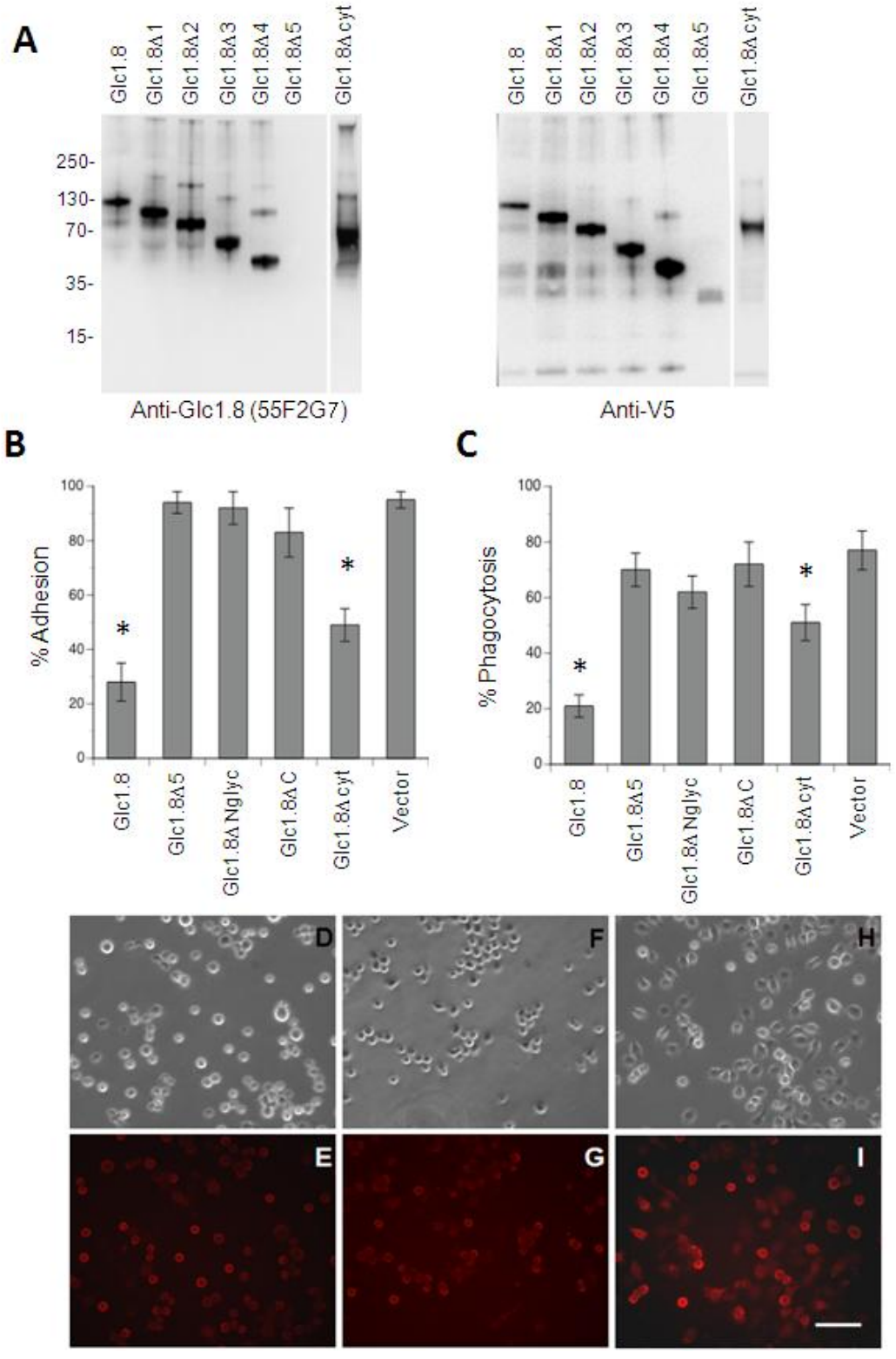
B



C

	140 kDa	115 kDa
M2N2	4.878%	5.328%
M3N2	36.616%	36.411%
M4N2	1.477%	1.323%
M5N2	0.358%	0.333%
M6N2	0.426%	0.501%
M7N2	0.157%	0.155%
M8N2	0.013%	0.062%
M9N2	0.124%	0.130%
Glc3M9N2	0.009%	0.011%
M2N2F	3.189%	5.231%
M3N2F	35.305%	34.014%
M4N2F	0.990%	0.910%
M5N2F	0.154%	0.125%
M2N2F2	0.702%	0.258%
M3N2F2	3.871%	3.726%
M4N2F2	0.059%	0.053%
M5N2F2	0.066%	0.026%
NM2N2	0.132%	0.153%
NM3N2	4.654%	4.624%
NM4N2	0.537%	0.457%
NM5N2	0.262%	0.243%
GalNM3N2	0.056%	0.059%
GalNM4N2	0.008%	0.011%
N2M3N2	0.298%	0.349%
NM2N2F	0.335%	0.349%
NM3N2F	3.853%	3.813%
NM4N2F	0.228%	0.189%
NM5N2F	0.266%	0.195%
GalNM3N2F	0.027%	0.021%
GalNM4N2F	0.030%	0.017%
N2M3N2F	0.656%	0.699%
NM2N2F2	0.054%	0.045%
NM3N2F2	0.177%	0.159%
NM4N2F2	0.024%	0.013%
GalNM3N2F2	0.006%	0.004%
	100.000%	100.000%

Figure 3.5. Glc1.8Δ5 and Glc1.8ΔNglyc lack immunopathogenic activity while Glc1.8Δcyt exhibits reduced activity. (A) The anti-Glc1.8 antibody 55F2G7 recognizes an epitope present within a tandem repeat of the extracellular domain. High Five cells were transiently transfected with pIZT-Glc1.8Δ5 or pIZT-Glc1.8Δcyt. Cells transfected with pIZT-Glc1.8 or the deletion mutants pIZT-Glc1.8Δ1-4 served as controls. Cell lysates were then subjected to SDS-PAGE and immunoblotting using anti-Glc1.8 (55F2G7) or anti-V5 antibodies. Molecular mass markers are indicated to the left. Note that Glc1.8Δ5 is only recognized by the anti-V5 antibody. (B and C) Anti-adhesion and anti-phagocytic activity of High Five cells expressing Glc1.8Δ5, pIZT-Glc1.8ΔNglyc, or pIZT-Glc1.8Δcyt. Cells transfected with pIZT-Glc1.8 served as a positive control while cells transfected with pIZT-Glc1.8ΔC or the empty pIZT/V5-His vector (Vector) served as negative controls. Adhesion and phagocytosis rates were determined 2 h after transfer to new culture plates or addition of *E. coli*. A cell was scored as phagocytic if one or more bacteria had been internalized. Results are given as mean percentages ± standard error (SE) of adhesive cells relative to the total number of cells (200) counted per sample. Asterisks indicate treatments that significantly differed from the empty vector control ($P<0.05$) (Dunnett's multiple comparison procedure; n=3 independent replicates per treatment). Phase-contrast (H) and epifluorescent (I) micrographs of High Five cells 48 h post-transfection with pIZT-Glc1.8ΔNglyc compared to High Five cells transfected with pIZT-Glc1.8 (D, E) or pIZT-Glc1.8Δcyt (F, G). Cells were fixed followed by labeling with anti-Glc1.8 and an Alexa-fluor 568-conjugated secondary antibody. Scale bar, 80 μm.


A
Glc1.8
Glc1.8Δ1
Glc1.8Δ2
Glc1.8Δ3
Glc1.8Δ4
Glc1.8Δ5
Glc1.8Δcyt
250-
130-
70-
35-
15-
Anti-Glc1.8 (55F2G7)
Anti-V5
B
% Adhesion
Glc1.8Δ Nglyc
Glc1.8Δ C
Vector
C
% Phagocytosis
D
F
H
E
G
I

Chapter 4. Influences of *Microplitis demolitor* bracovirus on cell adhesion regulation

4.1 Introduction

From the previous chapters, we know that MdBV-infected cells exhibit low adhesion activity and Glc1.8 is responsible for this activity. Generally, cell adhesion is regulated by several transmembrane receptors, including integrins. Integrins are αβ heterodimers with each subunit crossing the cell membrane once. After activation by a given signal, integrins activate a wide range of signal transduction pathways in cells through different cytoplasmic factors such as vinculin, paxillin and focal adhesion kinase (FAK). Integrin signaling also regulates a variety of functions that include cell proliferation, motility and cytoskeletal organization (Hynes, 2002). One signaling pathway that interacts with integrins is the mitogen-activated protein kinase (MAPK) pathway, which controls many physiological processes in response to mitogens, growth factors, cytokines, virus infection and environmental stress (Johnson and Lapadat, 2002). MAPKs are composed of three groups of kinases, MAPK kinase kinases (MKKKs), MAPK kinases (MKKs) and MAPKs, which form a phosphorylation cascade. Phosphorylated MAPKs activate downstream factors, including other kinases, phospholipases, transcription factors and cytoskeleton proteins (Boutros et al., 2002; Foukas et al., 1998; Garrington and Johnson, 1999; Sluss et al., 1996; Soldatos et al., 2003). Currently, three groups of MAPKs are known: extracellular signal regulated kinases (ERKs), c-Jun NH_3-terminal kinases (JNKs) and p38 enzymes (Johnson and Lapadat, 2002). These three groups of MAPKs are important for cell adhesion and migration triggered by integrins and other cytokines. In mammalian cells, integrin engagement activates the ERKs and p38 proteins by Ras-Raf-MEK cascade or activates the

JNKs by CAS/CRK-Rac-PAK cascade (Desban et al., 2006; Guo and Giancotti, 2004). It is also known that integrins activate the MAPK pathway through FAK (Wu et al., 2008).

In the lepidopteran host of *Microplitis demolitor*, *Pseudoplusia includens*, we have identified four integrin subunits from hemocytes (Lavine and Strand, 2003). The transcriptional level of some of these integrins increases as the cells attach to a foreign surface or make capsules around foreign targets, which suggests that some of these integrins play a role in these responses. Given that MdBV infection induces host hemocytes to lose their adhesion and phagocytosis abilities, I first tested the influence of MdBV infection on the transcriptional level of these four integrins in *P. includens*. Since the MAPK pathway seems to be involved in integrin-mediated signal transduction, I tested the influence of MdBV infection on MAPK activity. It is well known that MAPK pathways respond rapidly to H_2O_2 through integrins (Fischer et al., 2005; Wu et al., 2008). Given that the MdBV infection in High Five cells changes the transcriptional level of some integrins, I also tested whether Glc1.8 expression has any influence on MAPK pathways with H_2O_2 challenge. Since Glc1.8 expressed cells are nonadhesive and immunostaining indicates that the distribution of F-actin in these cells is different than that of the normal cells (Beck and Strand, 2005), I also measured the F/G-actin ratio in these cells.

4.2 Materials and methods

Insects and cell culture *Pseudoplusia includens* and *Microplitis demolitor* were reared at 27˚C and under a 16 hour light : 8 hour dark photoperiod as previous described (Strand et al., 1997). *P. includens* larvae were fed by artificial diet and *M. demolitor* female adults were allowed to parasitize the 4th instar *P. includens* larvae. Wild type High Five cells and pIZT/Glc1.8 stably transfected cells were maintained in Corning 75 cm^2 tissue-culture flasks in TC100 insect cell

culture medium containing 10% fetal bovine serum at 27˚C. The medium for the stable line also contained Zeocin at 500 μg/ml.

MdBV collection and infection Calyx fluid was collected from the female wasps by the established method (Beck et al., 2007). Briefly, the reproductive tracts were dissected out by pulling the ovipositor from the abdomen and calyces were collected in phosphate buffered saline. The calyx fluid was sterilized by passing through a 0.22 μm filer. The MdBV virions were purified by centrifugation at 16,000 x g for 20 minutes at 4C and resuspended in phosphate buffered saline. The MdBV purified from one wasp was refered as one wasp equivalent. For cell infection, the High Five cells were seeded in a 12-well plate for 12 hours. Purified MdBV was added to the cells at the concentration of 0.5 equivalent per well. For host infection, I injected 0.1 wasp equivalents of MdBV suspended into CO_2-anesthetized *P. includens* fifth instars using a glass needle mounted on a micromanipulator.

Hemocytes collection Hemocytes were collected from wild type or one day later MdBV infected fifth instars *P. includens* by anesthetizing larvae with CO_2 and bleeding them from an incision across the last abdominal segment. Hemolymph was collected in a microfuge tube containing anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na_2EDTA, and 41 mM citric acid, pH adjusted to 4.5) (Clark et al., 2004). Hemocytes were pelleted by 1 min centrifugation at 200g and the supernatant was removed. Hemocytes were then resuspended in 1 ml of fresh anticoagulant. After a 40-min incubation at 4°C, hemocytes were washed twice by centrifugation in Excell 400 medium (JRH Biosciences). Hemocytes from MdBV-infected *P. includens* were collected 24 hours after infection by the same methods.

RNA sample collection and Realtime-PCR Hemocytes collected from wild type or MdBV-infected *P. includens* were seeded in 12-well plates in Excell 400 medium. Total RNA was

extracted from the seeded cells by the High Pure RNA Isolation kit (Roche) at 0, 8, 16, 24 and 48 hours after seeding. 600 ng RNA for each treatment was used for synthesizing cDNA by SuperScript III reverse transcriptase (Invitrogen) with random primers in a 20 µl reaction volume. Realtime PCR was conducted using Piα1, Piα2, Piα3 and Piβ specific primers:

Piα1 forward 5'-AACGGGCAAACAAGAATCAC-3',

reverse 5'-GTATGCCCCGACTAACAAATC-3';

Piα2 forward 5'-GGGAAATCCTATGGCCAGTGAGCA-3',

reverse 5'-CAGTTGGGGTCCGAGTTTTGTGTC-3';

Piα3 forward 5'-GGGATGTCCGCAGCTGTCACCAA-3',

reverse 5'-CCACGCTGTACCCAAAATAATCAT-3';

Piβ forward 5'- CGGCGGAGCAGATCAGCGTGTA-3',

reverse 5'-CGGTCCTTGGGGCATTCCTTCAG-3' (Lavine and Strand, 2003).

In a 10 µl PCR reaction, there was 1 µl cDNA, 500 nM primers and 5 µl iQ SYBR Green Supermix (Bio-rad). 18S ribosomal RNA was used for endogenous control. Reactions were run on a Rotor Gene 2000 Real-Time PCR Cycler (Corbett Research) for 45 cycles with denaturation at 94˚C 20 seconds, annealing at 55˚C 20 seconds and extension at 72˚C 20 seconds. Data were analyzed by the ΔΔCt method. The transcriptional level of integrins in wild type *P. includens* hemocytes at time point 0 was standardized as 1.

Protein sample collection and western blotting To detect the influence of MdBV infection on MAPK pathway activation, the protein samples from the wild type High Five cells or MdBV-infected High Five cells were collected by lysing the cells in 1 x SDS sample buffer at 0, 0.5, 1, 3, 6, 12, 24 and 48 hours post-infection. To study the affect of Glc1.8 expression on MAPK pathway activity, protein samples were collected from wild type High Five cells or pIZT/Glc1.8

transiently transfected or stably transfected High Five cells after 20 minutes incubation with 2 mM H_2O_2. For the *P. includens* hemocyte samples, the hemocytes were collected and settled in wells 12 hours before challenge. Protein samples were collected after 20 minutes incubation with H_2O_2 or PBS. The protein samples were separated on precast 4-20% tris-glycine SDS PAGE and transferred to a PVDF membrane. Total ERK and JNK were detected using an anti-ERK and anti-JNK polyclonal antibody (1:10000) (Cell Signaling) while activated ERK and JNK were detected using an anti-phospho-ERK and anti-phospho-JNK monoclonal antibody (1: 2000) (Cell Signaling). Glc1.8 was detected using the monoclonal antibody 55F2G7 (1: 10000). Blots were incubated with HRP conjugated secondary antibodies (HRP-Goat anti Rabit or HRP-Goat anti mouse) (1: 20000) and visualized using ECL plus chemiluminscence substrate (Amersham, GE Healthcare).

F/G-actin ratio assay To analyze actin polymerization in the pIZT/Glc1.8 stably transfected High Five cell line, I collected filamentous and globular actin using the F/G-actin in vivo assay kit (Cytoskeleton). Briefly, wild type and pIZT/Glc1.8 High Five cells were lysed 12 hours after seeding in a culture well using the lysis and F-actin stabilization buffer from the kit. F-actin in the cells was pelleted by ultracentrifugation at 100,000 x g for 1 hour and depolymerized using the F-actin depolymerizing solution. F-actin and G-actin were then analyzed by immunoblotting using an anti-actin antibody. Positive and negative controls were included in the analysis by adding F-actin enhancing solution or F-actin depolymerization solution to the sample.

4.3 Results

MdBV infection reduces the transcription of integrins in hemocytes of *P. includence*.

Previous study identified four integrin subunits expressed in *P. includens* hemocytes, named Piα1, Piα2, Piα3 and Piβ (Lavine and Strand, 2003). The transcriptional levels of these

integrins were measured by real time-PCR at different time points after seeding in culture wells. Normal hemocytes spread rapidly on a foreign surface and I observed an increase in the transcription of Piα2 and Piβ as early as 8 hours after the cells were seeded (Fig. 4.1B & D, black bars) while the transcriptional level of Piα1 and Piα3 remains almost same (Fig. 4.1A & C, black bars). These data suggest that Piα2 and Piβ are important in cell adhesion and spreading, with Piα2 increasing about 80 fold at 8 hours after the cells were seeded on the foreign surface.

As we already know, hemocytes from MdBV-infected hosts have little adhesion or phagocytosis activity (Strand, 1994; Strand et al., 2006). By realtime-PCR, I found that transcript abundance of all four integrin subunits in the virus infected hemocytes was lower than normal. For Piα1 and Piα3, although transcript levels are stable during cell spreading, MdBV infection induces a significant decrease in transcript abundance (Fig. 4.1A & C, gray bars). For Piα2 and Piβ, which exhibit significant increase in transcript abundance as the cells spread, transcript levels in MdBV-infected samples were reduced significantly (Fig. 4.1B & D, gray bars). These results suggest that MdBV infection reduces integrin transcript levels, which may contribute to the anti-adhesion and anti-phagocytosis activity of this virus infection.

MdBV infection induces ERK and JNK phosphorylation

Many studies indicate that virus infection induces activation of the MAPK pathway. The activation of MAPK pathway after MdBV infection was determined by testing the phosphorylation of the ERKs or JNKs. Protein samples were collected at different time points after infection and probed with anti-pERK or anti-pJNK antibodies. Through the time points, the total amount of ERK or JNK exhibited little change (Fig. 4.2). Phosphorylated ERK and JNK were first detected at 12 hours post-infection and maintained afterwards (Fig. 4.2). The MdBV

protein Glc1.8 was first detected 6 hours after infection (Fig. 4.2E). These results indicated that the MAPK pathway in the cells is activated at about 12 hours after MdBV infection.

JNK activation by H_2O_2 is blocked in pIZT/Glc1.8 stably transfected High Five cell line

Previous studies have found that in mammalian cells, H_2O_2 activates MAPK signaling which results in an increase in intracellular calcium (Fischer et al., 2005). In wild type High Five cells, H_2O_2 challenge increased the phosphorylation of JNK (Fig. 4.3, lane 2). To study whether the Glc1.8 expression has any influence on the MAPK activation by H_2O_2, I tested the phospho-JNKs in both pIZT/Glc1.8 transiently transfected High Five cells and the pIZT/Glc1.8 stably transfected High Five cells after the challenge. In the transiently transfected High Five cells, there was still a significant amount of phospho-JNK detected after H_2O_2 challenge (fig. 4.3, lane 8), but I detected almost no phospho-JNKs in the stable line after the H_2O_2 challenge (Fig. 4.3, lane 4). In constrast, I did not observe any activation of ERKs by H_2O_2 in control wild type High Five cells (data not shown).

I also tested the phosphorylation of JNKs in MdBV infected *P. includens* hemocytes. In the wild type hemocytes without H_2O_2 challenge, the JNKs were activated which may be due to the stimulation of the environment as the cells were collected (Fig. 4.3, lane 9). The MdBV infected hemocytes behave similar as the wild type hemocytes with a high phospho-JNK level with or without the H_2O_2 challenge (Fig. 4.3, lane 11 & 12).

F/G actin ratio is increased in pIZT/Glc1.8 stably transfected High Five cell line

Actin polymerization is a key response to integrin activation and downstream signaling. The pIZT/Glc1.8 stably transfected High Five cells exhibit a round morphology which is different from the wild type High Five cells. The F/G actin ratio in this stable cell line was measured and shown in Figure 4.4. In the wild type High Five cells, which were spreading on

the foreign surface, the filamentous actin amount was significantly lower than the globular actin (lane 1 & 2). On the contrary, the filamentous actin amount in the stable High Five line was similar as the globular actin amount in these cells (lane 7 & 8), which indicated that the F/G actin ratio in the pIZT/Glc1.8 stably transfected High Five cells is significantly higher than that in the wild type High Five cells. The high level of filamentous actin may form a cortex around the cells and inhibit cell adhesion to foreign surfaces.

4.4 Discussion

Integrins play a key role in responding to extracellular signals and are regulated either by conformational or localization changes or by transcriptional regulation (González-Amaro and Sánchez-Madrid, 1999; Kim and Yamada, 1997). Some viral infection downregulates integrins. For example, Ebola virus glycoprotein (GP) expression induces cell rounding and reduces many cell surface receptors, including integrins (Simmons et al., 2002). Experiments also suggest that those downregulated integrins facilitate Ebola virus entrance (Takada et al., 2000). Similarly, MdBV infection induces cell rounding and our data suggest the transcript of integrin subunits Piα2 and Piβ is downregulated by the infection. We know that the MdBV-infected cells cannot be reinfected by any other viruses including MdBV itself (data not shown). The downregulation of integrins on the infected cell surface might be a possible mechanism.

Once integrins are activated, several signal transduction pathways are activated, among which MAPK pathway is important in responses to many extracellular signals (Wu et al., 2008). These signals include growth factors, cytokines, virus infection and stress (Johnson and Lapadat, 2002). Many viruses modulate MAPK pathways after infection and thus turn the cells into a synthesis machine for viral replication (Pleschka, 2008). Although MdBV does not replicate in infected cells, the viral infection also activated MAPK pathway. Ebola virus GP expression

activates MAPKs which further regulate cell proliferation and survival (Zampieri et al., 2007). Although Glc1.8 is structurally and functionally similar to GP, expression of Glc1.8 in cell lines does not active MAPKs. On the contrary, in Glc1.8 stable High Five cells, activation of JNK after the H2O2 challenge is blocked.

As cell morphology is regulated by signaling pathways, the final step is to alter the actin polymerization. Previous study indicates that expression of Glc1.8 changes the cell shape and the distribution of filamentous actin (Beck and Strand, 2005), suggesting that this protein may affect actin polymerization. By comparing the F/G-actin ratio in the pIZT/Glc1.8 stably transfected High Five cell line with the spreading wild type High Five cells, I found that in the stable line which are not able to spread, the F/G-actin ratio is higher than the spreading cells. By immunostaining, we knew that in the Glc1.8 expressing cells the F-actin is accumulated at the periphery of the cell (Beck & Strand, 2005). Although actin polymerization is required for protrusion formation, a filamentous actin cortex prevents the cells from adhering on foreign surfaces, which is the initial step for spreading (Cuvelier et al., 2007). This filamentous actin cortex needs to be depolymerized to promote cell adhesion. My data suggest that Glc1.8 expression enhances the amount of filamentous actin which may forms a cortex surrounding the cells and prevents the adhesion.

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Figure 4.1. Relative abundance of integrin subunit transcripts from wild type or MdBV-infected *P. includens* hemocytes (PiH) at 0, 8, 16, 24, 48 hours after seeded in culture wells. (A) Transcription level of Piα1; (B) transcription level of Piα2; (C) transcription level of Piα3; and (D) transcription level of Piβ. The transcriptional abundance of integrins in wild type *P. includens* hemocytes at the 0 time point was standardized as 1. The black bars represent the relative amount of each integrin transcript in wild type hemocytes while the gray bars represent transcript abundance of integrins in MdBV-infected hemocytes.

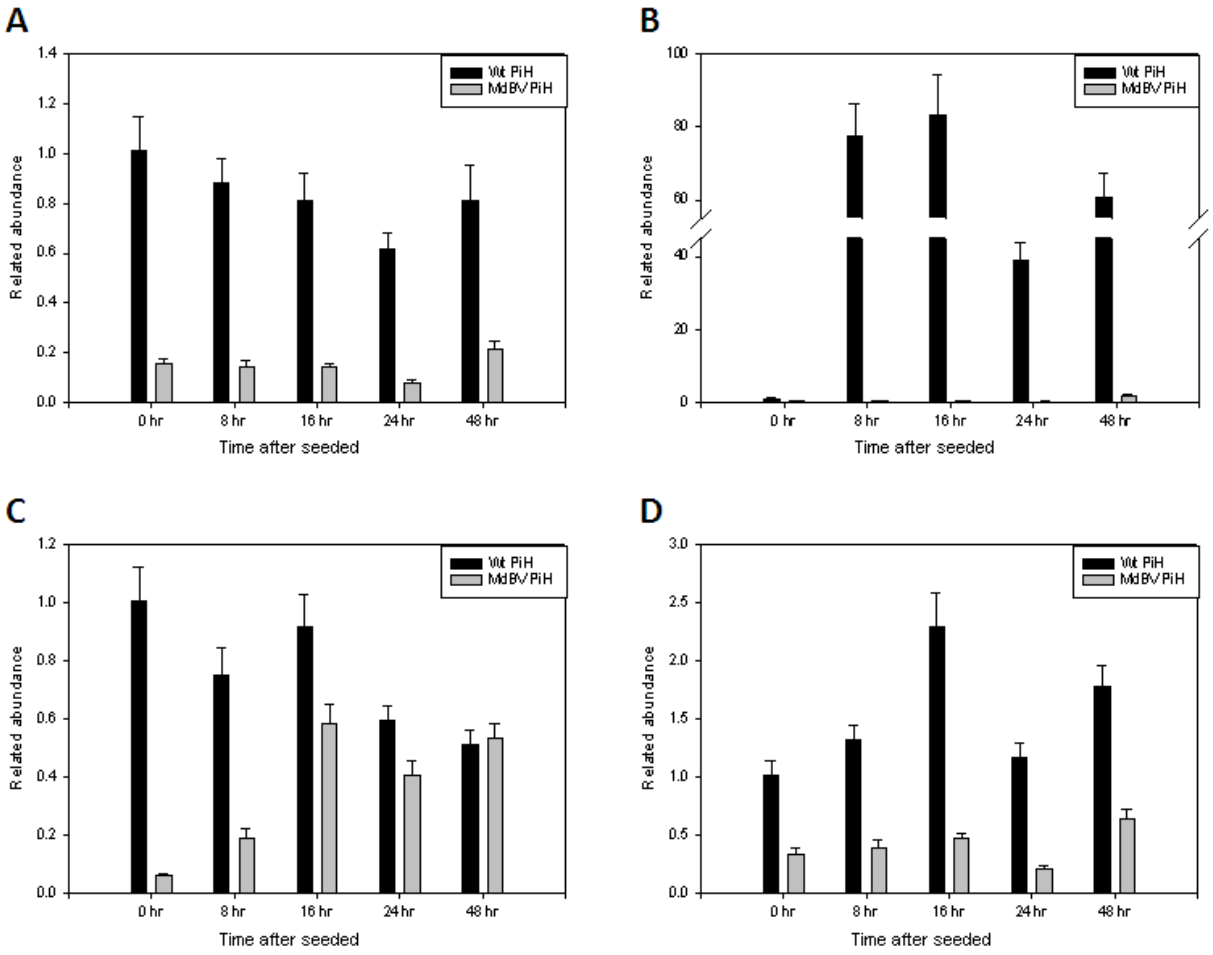

A
B
C
D
Wt PiH
MdBV/PiH
Related abundance
Time after seeded
0 hr
8 hr
16 hr
24 hr
48 hr

Figure 4.2. Activity of the ERK and JNK in High Five cells after the MdBV infection. The protein samples from High Five cells were extracted at 0, 0.5, 1, 3, 6, 12, 24 and 48 hours after MdBV infection. (A) The total ERK and phosphorylated-ERK in the un-infected High Five cells were detected by anti-ERK antibody and anti-phosphorylated-ERK antibody; (B) the total JNK and phosphorylated-JNK in the un-infected High Five cells were detected by anti-JNK antibody and anti-phosphorylated-JNK antibody; (C) the total ERK and phosphorylated-ERK in the MdBV-infected High Five cells were detected by anti-ERK antibody and anti-phospho-ERK antibody; (D) the total JNK and phosphorylated-JNK in the MdBV-infected High Five cells were detected by anti-JNK antibody and anti-phosphorylated-JNK antibody; (E) the MdBV protein Glc1.8 was detected with monoclonal antibody 55F2G7.

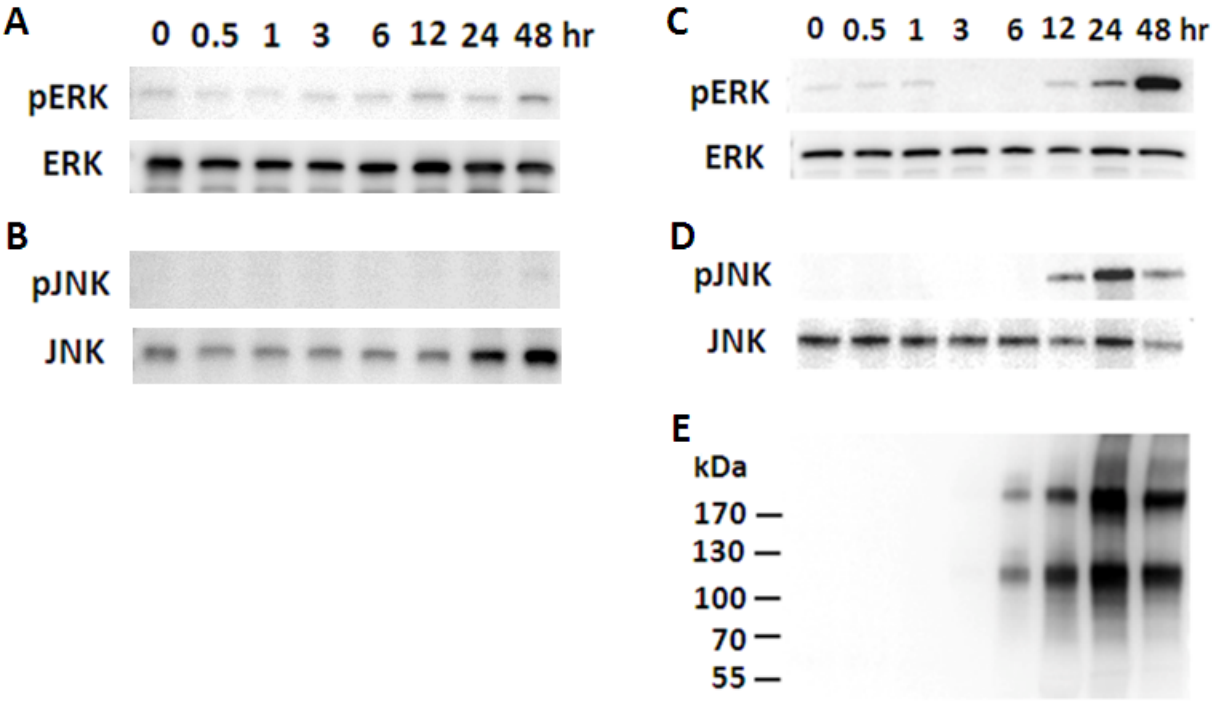

A
0 0.5 1 3 6 12 24 48 hr
pERK
ERK
B
pJNK
JNK
C
0 0.5 1 3 6 12 24 48 hr
pERK
ERK
D
pJNK
JNK
E
kDa
170
130
100
70
55

Figure 4.3. Activity of JNK in the wild type High Five cells, pIZT/Glc1.8 transfected High Five cells and *P. includens* hemocytes without or with MdBV infection after H_2O_2 challenge. The total JNK and phosphor-JNK were detected by anti-JNK and anti-phospho-JNK antibody, respectively. Lane 1 and lane 2 were the wild type High Five cells without or with H_2O_2 challenge; lane 3 and lane 4 were the pIZT/Glc1.8 stably transfected High Five cells without or with H_2O_2 challenge; lane 5 and lane 6 were the High Five cells transfected by empty pIZT vector without or with H_2O_2 challenge; lane 7 and lane 8 were High Five cells transiently transfected by pIZT/Glc1.8 without or with H_2O_2 challenge; lane 9 and lane 10 were *P. includens* hemocytes without or with H_2O_2 challenge; lane 11 and lane 12 were MdBV infected *P. includens* hemocytes without or with H_2O_2 challenge.

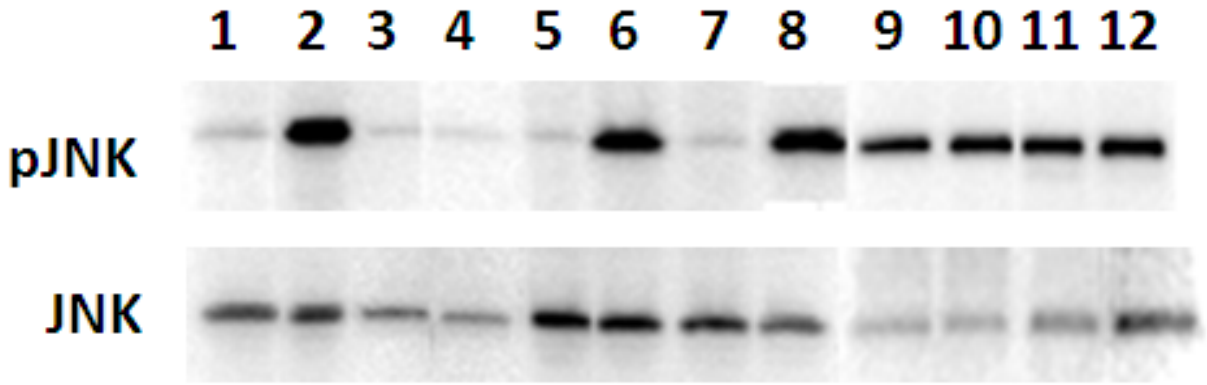
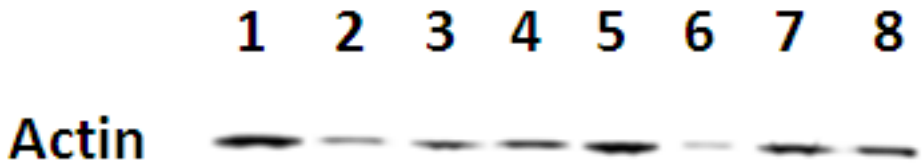

1 2 3 4 5 6 7 8 9 10 11 12
pJNK
JNK

Figure 4.4. F/G-actin ratio analysis in spreading wild type High Five cells and unadhesive pIZT/Glc1.8 stably transfected High Five cell line. The actin was detected by anti-actin antibody (Cytoskeleton). Lane 1, 3, 5 and 7 were G-actin in the samples while lane 2, 4, 6 and 8 were F-actin in the samples. Lane 1 and 2 are samples from spreading wild type High Five cells; lane 3 and 4 were positive control with high amount of F-actin; lane 5 and lane 6 were negative control with high amount of G-actin; lane 7 and lane 8 were samples from unadhesive pIZT/Glc1.8 stably transfected High Five cell line.


1 2 3 4 5 6 7 8
Actin

Chapter 5. Conclusions

Polydnavirus is an immunosuppression factor used by parasitoid wasps in Ichneunomonoidea. *Microplitis demolitor* bracovirus (MdBV) is symbiotically associated with the wasp *M. demolitor*. The whole genome of MdBV was sequenced and annotated (Webb et al., 2006). The MdBV genome has fifteen circular double stranded DNA segments (named A-O) and five gene families have been identified, including *ptp*, *ankyrin*, *egf*, *tRNA* and *glc*, which target different immune responses in the infected hosts (Beck and Strand, 2003; Beck and Strand, 2005; Beck and Strand, 2007; Pruijssers and Strand, 2007; Thoetkiattikul et al., 2005; Trudeau et al., 2000).

Among these gene families, *Glc*1.8 encodes for a transmembrane protein with five identical tandem repeats in its extracellular domain (Trudeau et al., 2000). Genomic sequencing showed that *Glc* genes were located only on segment O. Our previous annotation indicates that this segment encodes two copies of this gene. Although we observed two *glc* transcripts in MdBV infected host cells, the attempt to clone the other *glc* member was not successful. Given the tandem repeat structure of the *glc*1.8 genes and the fact that our genomic sequencing did not prove the presence of another *glc* gene, we predicted that the other *glc* gene should have a similar gene structure as *glc*1.8 but a different number of tandem repeats. To confirm the presence of a new *glc* gene, I used southern blotting against the digested MdBV genome with a *glc* repeat-specific probe. This probe recognized two genomic DNA bands which represent two different *glc* genes. Based on the sequence of *glc*1.8, the band of 3.6 kb size is *glc*1.8, while the 8.2 kb band represents the other *glc* gene in MdBV genome. I also analyzed the protein sample from

MdBV-infected cells and found that the anti-Glc1.8 antibody recognizes two protein bands at 115 kDa and 250 kDa respectively. Comparing my southern blotting and western blotting results with the previous northern blotting results, I found the new *glc* gene may contain twelve tandem repeats instead of five in *glc*1.8.

Although the existence of the other *glc* gene member is confirmed, the function of this new *glc* gene remains unclear. In the MdBV genome, other gene families contain multiple members. For example, there are twelve viral *ankyrins*, thirteen *ptps* and three *egf*s identified from the MdBV genome with different member having different tissue-specificity or activity (Webb et al., 2006). Previous experiments and the functional characterization in chapter 3 indicated that the extracellular repeats are important for the function. If the new *glc* gene contains more repeats as we predict, its anti-immunity activity should be stronger than that of *glc*1.8. Evidence supporting our hypothesis is that the adhesion and phagocytosis activity of the MdBV-infected cells are always lower than any Glc1.8 transfected cells (data not shown). Another evidence is that in the pIZT/Glc1.8 stably transfected High Five cells, the adhesion and phagocytosis activity is reduced more strongly than that in transiently transfected cells. By southern blotting, I found that there is a larger *glc* gene in the stable line which may have arisen as a result of recombination events. This new *glc* gene may be responsible for the stronger inhibition of the adhesion and phagocytosis.

Previous studies indicated that the Glc1.8 is a mucin-like protein with the tandem repeats predicted to be highly N-glycosylated. Unlike *ankyrins* and *ptps* which are common in BVs, among the identified PDV-encoded proteins, only HdGorfP30 from HdIV shares similar characterization with Glc1.8 (Galibert et al., 2003). However, the HdGorfP30 and Glc1.8 have no sequence similarity and the function of HdGorfP30 is still unclear. Ebola virus encodes

another membrane-associated glycoprotein (GP) which also contains a mucin-like domain. This viral GP is important for the fusion of the virus with the host cells (Simmons et al., 2002). Expressing of GP induces the host cells lose the adhesive activity, which is similar to Glc1.8. The mucin-like domain in the GP was proven to be essential for its anti-immunity activity. Similarly, the mucin-like repeats in Glc1.8 are also important for its function. To further characterize the tandem repeat domain of Glc1.8, I first purified Glc1.8 from the stably transfected High Five cell line. Both N-glycosidase digestion and N-glycosylation inhibition results confirms the present of the N-glycosylation on Glc1.8. Based on the MALDI/TOF-MS result, this protein is dominated by high mannose and fucosylated paucimannose oligosaccharides with Man3GlcNAc2 and Man3GlcNAc2Fuc accounting for more than 70 % of the glycan profile.

Similar to Ebola virus GP, the tandem repeats of Glc1.8 are important for its immunosuppressive activity. When I deleted the whole repeat domain, the protein had no function. Given that this repeat domain is highly N-glycosylated, I studied the functional importance of this N-glycosylation by creating a deglycosylated mutant, Glc1.8ΔNglc. This mutant has all predicted N-glycosylation asparagines changed to glutamines. My data show that the deglycosylated Glc1.8 has no anti-immunity function, which indicates that the glycosylation of the repeat domain is essential for the function. The possible mechanism can be that this large amount of glycans on the extracellular region forms a physical barrier which blocks the interaction between ligands and receptors so that the adhesion and phagocytosis activity of cells are blocked. Furthermore, I also created a cytoplasmic tail deletion mutant and found this mutant had reduced anti-immunity function. This result proves that besides the glycosylated repeat domain, the cytoplasmic tail also contributes to the function. This fact suggests that

MdBV-infection blocks cell adhesion not only by forming a glycan layer outside the cell surface but also by some regulation inside the cells.

In all these structural and functional experiments, since we cannot express recombinant proteins in *Pseudoplusia includens* hemocytes, I used High Five cells to express Glc proteins. Some characteristics of High Five cells make them suitable for our studies. First, High Five cells, which were generated from *Trichoplusia ni*, were hemocyte-like cells (Beck and Strand, 2003). Secondly, High Five cells are able to be infected by MdBV and show similar reactions as *P. includens* hemocytes after the viral infection. Finally, High Five cells are used broardly to express recombinant eukaryotic proteins given their ability for post-translational modification. Thus, it is very likely that my observation in High Five cells is similar as that in hemocytes.

As the MdBV infection inhibits cell adhesion, I investigated this inhibition in more detail in Chapter 4. Cell adhesion is regulated by some membrane factors, such as integrins (Hynes, 2002). Integrins are regulated by changing conformation or localization or by modulating their transcript level (González-Amaro and Sánchez-Madrid, 1999; Kim and Yamada, 1997). Experiments also indicated that Ebola virus GP reduced the amount of integrin subunits which are important for cell adhesion (Simmons et al., 2002). In this chapter, I first measured the relative transcript level of host integrin subunits after MdBV infection. For all four integrin subunits we identified from *P. incluens* hemocytes, MdBV-infection induces a significant decrease to their transcripts. Since integrins are also important for some viral infection, this downregulation could possibly contribute to blocking re-infection after MdBV-infection. However, since expressing single viral gene in host hemocytes is not possible up to now, we cannot determine the specific viral genes which are responsible for this integrin downregulation. Once activated, integrins initiate a wide range of signaling transduction pathways in the cells,

including the MAPK pathway. The MAPK pathway is activated by many mitogens and it regulates many physiological processes (Wu et al., 2008). Many viruses activate the MAPK pathway after infection to facilitate their own replication (Pleschka, 2008). Although the MdBV does not replicate in their lepidopteran host, my results indicate that the MdBV-infection activates the MAPK pathway in the High Five cells. However, in Glc1.8 expressing High Five cells, the MAPK pathway is not activated which indicated that Glc1.8 alone is not responsible for the MAPK activation after MdBV-infection.

As cells adhere or migrate on a surface, the cytoskeleton plays an important role for cells to change their shape (Small and Resch, 2005). It has been shown that during protrusion formation, globular actin is polymerized to filamentous actin. Given that Glc1.8 expression induces cells to become round and prevents them from forming any protrusions, I determined whether the actin polymerization is modified in the pIZT/Glc1.8 stably transfected High Five cell line. Compared to the well spreading wild type High Five cells, the F/G-actin ratio in Glc1.8 cell line is significantly higher. Previous experiments showed that in this Glc1.8 cell line, the filamentous actin accumulates in the periphery of the cell (Beck & Strand, 2005). Other researches shows that although actin polymerization is required for cell spreading, a high amount of filament actin forms a cortex around the cells which prevents the cells from adhering (Cuvelier et al., 2007). My data suggest that the adhesive inhibition by Glc1.8 is probably by this mechanism. Based on all the results in this dissertation, the possible mechanism for the anti-immunity activity of Glc1.8 might be that its extracellular glycans form a physical barrier, which interferes the interaction between ligands and cell membrane receptors, and its cytoplasmic tail interacts with some factors and finally modulate actin polymerization. Since several Glc1.8

mutants have been constructed, it is possible to determine the influence of the glycosylation and cytoplasmic tail of this protein on regulating actin polymerization.

Overall, my dissertation characterizes the glycan profile of a polydnavirus glycoprotein for the first time and proves the importance of this glycosylation. My data also provide evidence for possible mechanism used by MdBV to regulate host cell adhesion. This will contribute to the understanding of how MdBV and other polydnaviruses suppress host cellular immunity, which is essential for the survival of parasitoids.

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Appendix

List of Abbreviation

BV Bracovirus

CcBV Cotesia congregate bracovirus

CiBV Chelonus inanitus bracovirus

CpBV Cotesia plutellae bracovirus

CrBV Cotesia rubecula bracovirus

CRP Cysteine-rich protein

CsIV Campoletis sonorensis ichnovirus

EP Early-expressed protein

ERK Extracellular signal regulated kinase

FAK Focal adhesion kinase

GfBV Glyptapanteles flavicoxis bracovirus

GiBV Glyptapanteles indiensis bracovirus

HdIV Hyposoter didymator ichnovirus

HfIV Hyposoter fugitivus ichnovirus

IV Ichnovirus

JH Juvenile hormone

JNK c-Jun NH_3-terminal kinase

MAPK Mitogen-activated protein kinase

MdBV Microplitis demolitor bracovirus

MKK MAPK kinase

MKKK MAPK kinase kinase

PAP ProPO-activating proteinase

PDV Polydnavirus

PO Phenoloxidase

proPO Prophenoloxidase

PRRP Polar-residue-rich protein

PTP Protein tyrosine phosphatase

PTG Prothoracic gland

PTTH Prothoracicotropic hormone

SPH Serine proteinase homolog

TrIV Tranosema rostrale ichnovirus

TSP Teratocyte-secreted protein

VLP virus-like particle