Interaction of the baculovirus occlusion-derived virus envelope proteins ODV-E56 and ODV-E66 with the midgut brush border microvilli of the tobacco budworm, Heliothis virescens (Fabricius)

Wendy Olissa Sparks

Iowa State University

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Interaction of the baculovirus occlusion-derived virus envelope proteins ODV-E56 and ODV-E66 with the midgut brush border microvilli of the tobacco budworm, *Heliothis virescens* (Fabricius)

by

Wendy Olissa Sparks

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study committee: Bryony C. Bonning, Major Professor Lyric Bartholomay Larry Halverson Russell Jurenka F. Chris Minion

Iowa State University Ames, IA 2010

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In loving memory of my father, W. Spencer Wood—

Your love of life and laughter are sorely missed, but not forgotten
Table of Contents

ABSTRACT

Chapter 1: Introduction

On the nature of viruses 1
Virus physiology and nomenclature 2
Baculoviridae 4
The host and viral immunity 14
Baculovirus occlusion derived virus (ODV) 23
Receptor binding and fusion 38
Thesis organization 38
References 39

Chapter 2: ODV-E56 is PIF-8 and is non-essential to binding and fusion

ABSTRACT 55
BACKGROUND 56
MATERIALS AND METHODS 58
RESULTS 64
DISCUSSION 67
AUTHOR CONTRIBUTIONS 73
ACKNOWLEDGEMENTS 73
REFERENCES 77
TABLES AND FIGURES

Chapter 3: A peptide that binds the gut epithelium of *Heliothis virescens* has similarity to the baculovirus envelope protein ODV-E66 and impedes infection with wild type baculovirus

SUMMARY 85
INTRODUCTION 86
RESULTS 88
DISCUSSION 94
MATERIALS AND METHODS 101
ACKNOWLEDGEMENTS 105
REFERENCES 106
TABLES AND FIGURES 111

Chapter 4: Discussion and Conclusions

REFERENCES 120

ACKNOWLEDGEMENTS 128
Alphabaculoviruses of the family Baculoviridae infect the larvae of the Lepidoptera (moths and butterflies) when the occlusion-derived virus (ODV) released from the occlusion body (OB) binds and fuses to the midgut epithelium. Most alphabaculoviruses readily infect only a few species of caterpillars. The ODV contain more than 30 proteins, twelve of which are conserved across the alphabaculoviruses, including the envelope proteins ODV-E56 and ODV-E66. The mechanism of viral fusion and entry, as well as, the ODV envelope proteins implicated in this process, are unknown. A family of ODV envelope per os infectivity factors (PIFs) has been identified, which includes seven proteins that have significant effects on oral infectivity. Here, we assess the potential roles of ODV-E56 and ODV-E66 in oral infectivity. Bioassays showed that ODV-E56-negative virus was significantly less infectious per os, in both ODV and OB. However, the ODV-E56 negative purified ODV exhibited a two-log reduction in oral infectivity compared to ODV-E56 positive virus, whereas the ODV-E56 negative OBs exhibited a five-log reduction in infectivity. This suggests ODV-E56 may function in early interactions within the gut. The ODV-E56 negative viruses exhibited wild-type levels of binding and fusion, but viral DNA was not transcribed. ODV-E56 bound to 97 kDA protein from H. virescens midguts. Thus, ODV-E56 is not essential for cell fusion, but may function in cell signaling and post-fusion events. These results indicate that ODV-E56 is PIF-5.

Fifteen H. virescens gut-binding peptides were isolated using a phage display library, and two peptides showed similarity to ODV-E66. One phage peptide, HV1, exhibited strong binding to cryosections of fourth instar H. virescens midguts, and in competition assays with baculovirus resulted in decreased mortality and increased survival time. The homolog AcE66A to the second phage peptide (HV2), exhibited strong binding, but had no effect on mortality in competition assays. ODV-E56 and ODV-E66 are conserved proteins that localize to a very complex virion envelope, and further studies are needed to detail their functions. Increased knowledge of the determinants of virus infection may facilitate further development of these viruses for use as environmentally benign insecticides, as well as, greater understanding of viral mechanisms.
Chapter 1: Introduction

The introductory chapter is organized to first provide general information about viruses and virus genetics, and then to introduce the specific virus, a baculovirus, that is the focus of this dissertation. This introduction also provides a review of insect host interactions with viruses and the insect defenses in the midgut which may provide insight into viral protein functions that have evolved to counter the host immune system. Thus, this introduction is composed of three parts that provide background to: viruses and baculoviruses, insect-virus interaction, and the specific proteins of interest in this dissertation.

On the nature of viruses

Currently viruses represent both an immense challenge and a great potential. Viruses are obligate intracellular pathogens; they are unable to replicate without a host. While the exact nature of viruses as a life form is a topic of debate, the success of such a life cycle cannot be denied; viruses have been found in all types of life described to date. From the ancient archaea, to deep-sea bacteria, from the common dog to the elusive vole, from the tiny honeybee to the giant elephant, viruses pervade. We share viruses with our closest friends and unknown strangers, our dearest pets, our nameless livestock, and our fellow earth inhabitants, the wild animals upon whose habitats we encroach. The remnants of ancient retroviruses are passed down from our parents via our DNA. From our first moments in this world to our last breath, every organism is under attack from viruses. Even the viruses themselves have their own parasites- incomplete viruses and the viroids.

The challenge is obvious. Viruses challenge the life of their host(s). At best, they utilize valuable resources to support their own replication; at worst they can decimate entire civilizations and populations through plague or famine. The potential of viruses lies in harnessing their unique characteristics for use in (i) the control of pests, (ii) production of vaccines to save lives and prevent suffering, (iii) the study of genes unique to viruses to
elucidate how viral strategies might be employed to cure other diseases, stop other problems, and further our knowledge of our own biology.

**Virus physiology and nomenclature**

The term virus is used to refer to the physical properties of the pathogen, its life cycle and the disease it causes in the host, whereas the term virion explicitly refers to the physical structure responsible for infecting a host cell. A virion is composed of a set of instructions (RNA or DNA) wrapped in a protective shell (proteins and sometimes sugars, lipids and other molecules) that may then be further enveloped with cell membranes during production of the virion. Modern technological advances such as high throughput sequencing of viruses, RNA interference to knockout individual gene products, quantitative PCR, and improved microscopy techniques, have provided much needed information about virus biology. However, much is still unknown.

Despite their reliance on a cellular host, a few viral species are very stable in the environment, existing for years in the soil or permafrost. Such stability at low temperatures allows for long term storage within laboratory freezers (Smith *et al.*, 2004; Lapied *et al.*, 2009). By their very nature as obligate intracellular pathogens, viruses offer a window into host pathogen interactions, with different strains having almost symbiotic or platonic relationships with one host, while causing fatal infections in another. Viruses are the tiniest organisms described that contain their own nucleic acid in a protective protein coat. Some of the simplest viruses reflect just this, encoding only a few kilobases of nucleic acid in a simple protein shell, and depend on the host cellular machinery for replication. At the other end of the spectrum, the most complex viruses have large dsDNA genomes nearing 300 kB, exhibit phasic expression of genes, encode their own polymerases which recognize virus specific promoters, and produce complex enveloped virions containing both viral and host proteins. The largest virus, *Mimivirus*, has a genome of 1.2 megabases (Lapied *et al.*, 2009), significantly larger than those of the smallest archaea, *Nanoarchaeum equitans*, (491 kB) and the smallest bacterium described, *Mycobacterium genitalium* (580 kB) (Lapied *et al.*, 2009).
The viruses are classified using Linnaean taxonomy (rank based hierarchy) that was initially based on morphological characteristics, and then expanded to take into account genomic phylogeny. Viruses do not encode their own protein synthesis machinery; therefore, all viruses must produce mRNA in their life cycle for translation by host machinery in order to replicate. How the viruses do this is the basis for another useful categorization of viruses, the Baltimore classification. This classification separates viruses into seven classes based upon the type of nucleic acid present in the capsid that functions as the viral genome and how mRNA is produced for protein production in the cell (Baltimore, 1971). This classification provides insight into class-specific strategies, as well as overall mechanisms shared by all viruses. The Baltimore classes are as follows: I. dsDNA viruses (herpesviruses, baculoviruses, poxviruses), II. ssDNA viruses (+) sense DNA (paroviruses), III. dsRNA viruses (reoviruses), IV. (+)ssRNA viruses (picornaviruses, togaviruses), V. (-)ssRNA viruses (orthomyxoviruses, filoviruses), VI. ssRNA-RT viruses (positive sense RNA with a DNA intermediate such as retroviruses), and VII. dsDNA-RT (DNA viruses that utilize reverse transcription such as hepadnaviruses). Classes I and II most closely resemble the nucleic acids of their hosts, and in theory do not require any transformation of the viral genome for transcription and translation to occur, although many utilize virus specific genes. (-)ssRNA and dsRNA viruses contain extra steps in their life cycle to produce mRNA from their genomes. The retroviruses have diploid ssRNA(+) genomes that undergo reverse transcription and then fully integrate dsDNA provirus into the host genome, and hepadnaviruses undergo reverse transcription only in the packaged virion. Reverse transcription is unique to viruses, but the discovery of RNA interference has shown a role for the multiple forms of RNA in uninfected cells. In general, the RNA viruses, especially the reverse transcription viruses, have greater mutation rates due to lack of proofreading by the RNA polymerases. Viruses such as human immunodeficiency virus (HIV) use this to their advantage to evade the host immune response. The dsDNA viruses such as poxviruses have lower mutation rates, but an order of magnitude more genes, and recombination events can lead to great selective advantages leading to fixation of a given trait in a population (or disadvantages that are selected against).
The evolution of viruses is driven by three key elements: ability to infect a host, ability to evade the host immune system, and ability to maintain stability of the virion in the environment in between host organisms. As a result, viruses have co-evolved with their hosts, and for the majority of viruses have become less virulent to their host over time (Koonin et al., 2006). Viruses also must trade off between having a broad host range (generalist) versus a narrow host range (specialist) (Woolhouse et al., 2001). A broad host range increases the likelihood of encountering a host and continued survival of the virus, whereas a narrow host range allows for very specific adaptation to the host resulting in more efficient virus infection, albeit at the cost of potential extinction. Many viruses evade this potentially detrimental outcome by developing a latent phenotype either in the original host, an alternate species reservoir, or in an environmentally stable form. Viruses also exist as populations in nature, not as single genotypes. Small viruses such as HIV exhibit large numbers of polymorphisms existing as a cloud of viral related sequences termed a quasispecies, as single dominant genotypes are at a selective disadvantage (Domingo et al., 1998; Wilke, 2003). Likewise, the large dsDNA viruses, such as the poxviruses, herpesviruses, and baculoviruses, exhibit large-scale recombination, insertions and deletions, and transposon insertions, in addition to single nucleotide polymorphisms (Cory et al., 2005; Herniou et al., 2003). Overall, these attributes lead to a highly versatile, highly successful life form with a long history and no end in the immediate future.

**Baculoviridae**

This dissertation describes the interaction of two envelope proteins from the baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), and a highly susceptible lepidopteran host, the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). The following provides the necessary background information to understand the life cycle of the virus, the manner in which viral genes are expressed, elements of insect physiology and the immune system, and the critical reasoning behind why we chose to study the two gene products described in this dissertation.
**Historical perspective.** Fossil evidence of arthropods filled with occlusion bodies (OBs; proteinaceous crystals embedded with virions) dates back to 100 million years ago (Poinar & Poinar, 2005). Today, three different occluded viruses of insects exist: the baculoviruses, the entomopoxviruses, and the cypoviruses, and are thought to have a common ancestor (Anduleit *et al.*, 2005; Coulibaly *et al.*, 2007; Ji *et al.*, 2010). The epizootics caused by natural populations of baculovirus have been documented for hundreds of years, concurrent with the domestication of the silk moth, *Bombyx mori* that began as early as 3000 B.C. in China (Miller, 1997). The silk trade was both a major industry and a cultural icon for centuries, and along with the spice trade led to travel between Europe and Eastern Asia. Silk worm colonies were imported to Europe, and along with them, their pestilences including baculoviruses. Miller provides a translated passage from an Italian bishop with his description of the “wilting disease” as it became known in the early part of the 20th century:

“All at once in the weak ones, the skin appears yellow. Then they swell up and a foul inactivity comes in the bodies of those who have fallen down. Finally, they break open and everything is infected with repulsive putrid gore, diseased blood, from all sides flows from the bodies.”

Light microscopy allowed visualization of the sickness as the OB of the virus are very dense and reflect the light. It was at this point that the term polyhedroses became associated with the disease, as the occlusion bodies of the silk worm appeared polyhedral in shape (Rohrmann, 2008). With the invention of electron microscopy in the mid 20th century, virions were finally observed for the first time. The virions of the baculoviruses were found to be rod shaped (Bergold, 1947). It is this feature that led to the naming of the family; *baculum* is Latin for cane, walking stick, or staff (Rohrmann, 2008).

**Nomenclature.** Originally placed into multiple families, the baculoviruses were consolidated into a single family, the Baculoviridae in 1973 based on virion morphology and genome type (Rohrmann, 2008). The Baculovirus family infects only insect hosts, primarily lepidopteran larvae (moths and butterflies) (Jehle *et al.*, 2006). Previous classification separated the family into two genera, the nucleopolyhedroviruses (the subject of this dissertation) and the granuloviruses, based on virion morphology and life cycle
characteristics (Murphy, 1995). Having fully sequenced 44 of the baculoviruses identified from over 500 insect species, phylogenetic studies have revealed that the viruses diverge according to the order of their insect hosts, not just virion morphology (Herniou & Jehle, 2007; Herniou et al., 2003; Herniou et al., 2004; Rohrmann, 2008). Therefore, a new classification has been instituted resulting in four genera: Alphabaculovirus, Betabaculovirus, Gammabaculovirus, and Deltabaculovirus for lepidopteran nucleopolyhedroviruses, lepidopteran granuloviruses, hymenopteran nucleopolyhedroviruses and dipteran nucleopolyhedrovirus species, respectively (Jehle et al., 2006). This new nomenclature will be easy to amend to incorporate baculoviruses from other insect orders such as Coleoptera, Neuroptera, Thysanura, and Trichoptera. As the new classification has not become fully adopted, both classifications will be referred to in this dissertation.

Under the current nomenclature, the virus species name remains unchanged; it reflects the insect species from which the virus was first isolated. Most baculoviruses are highly host specific, with only one or two fully permissive hosts; however, this naming scheme results in confusion if multiple species are isolated from the same insect host or if the same species is isolated from two distinct insect species, both of which have occurred (Harrison & Bonning, 1999; Li et al., 2005). Therefore, it is likely that many of the more than 500 described baculoviruses represent strains of the same species. Much of the literature has focused on the type species for the Alphabaculovirus genus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac* MNPV). *Ac* MNPV is one of the few baculoviruses with a broad host range; the host range of *Ac* MNPV encompasses 39 species across 13 families of lepidoptera (Bonning & Hammock, 1992; Entwistle & Evans, 1985; Granados & Williams, 1986). The *Bombyx mori* NPV (*Bm* NPV), although sharing over 90% identity at the genome level, has a much more specific host range of the domestic silkworm and the wild silkworm *B. mori* mandarina (Gomi et al., 1999; Arunkumar et al., 2006).

Alphabaculoviruses are further characterized at the species level by the number of nucleocapsids (NC) packaged into the occlusion derived virion. Single nucleopolyhedrovirus (SNPV) contain a single nucleocapsid, whereas multiple
nucleopolyhedrovirus (MNPV) may package between 1 and 10 nucleocapsids per virion. *AcMNPV*, for example, packages several nucleocapsids per virion as shown by electron microscopy and sucrose gradient density segregation of virions. Further study of the ancestry of the baculoviruses and their insect hosts has shown this multiple phenotype to be a relatively new phenotype and that the ancestral virus would have been a virus with single nucleocapsid virions (Washburn *et al.*, 1999). Only viruses within the genus Alphabaculoviruses show the M phenotype.

Phylogenetically, the Alphabaculoviruses exhibit another evolutionary split into two groups. Group I contains at least 11 genes that Group II lacks (Ohkawa *et al.*, 2005; Okano *et al.*, 2006), and contains an additional orf encoding the budded virus (BV) fusion protein, GP64, utilized by the BV to fuse to host cell membranes (see Life cycle and virion morphology, below). Group I NPVs utilize GP64 as the fusion protein of BV, whereas group II NPV (as well as the remaining genera of Baculoviridae) utilize the F-protein. Interestingly, the *gp64* protein is homologous to the *gp64* fusion protein of the Thogotoviruses that belong to the Orthomyxovirus family. The Group I NPVs do encode and express the orf for the F-protein, but it no longer functions as a fusion protein in the group I NPVs (Pearson & Rohrmann, 2002). The F-protein is homologous to the F-protein the Errantiviruses, the insect retroviruses (Pearson & Rohrmann, 2004; Rohrmann & Karplus, 2001).

**Life cycle and virion morphology.** The epizootics caused by natural populations of baculovirus have been documented for hundreds of years, concurrent with the domestication of the silk moth, *Bombyx mori*. The polyhedra are extremely stable in the environment, resistant to heat and desiccation; however, they are very susceptible to UV radiation and alkaline pH (Carruthers *et al.*, 1988; Thompson *et al.*, 1981). When the lepidopteran host ingests a polyhedron (OB) from its environment, the alkaline juices of the midgut solubilize the polyhedron, releasing the ODV into the gut lumen. The ODV then cross the peritrophic matrix (PM) that lines the gut and make contact with the brush border microvilli of the columnar cells of the midgut epithelium. Cell entry occurs through binding to an unidentified cellular receptor(s) thought to have a protein component, and the ODV envelope fuses with the cell membrane (Horton & Burand, 1993). Fluorescence
microscopy studies showed fusion occurs at the apical tips of the microvilli, and the nucleocapsids are transported to the nucleus via the actin filaments in a manner that is not understood (Charlton & Volkman, 1993; Dreschers et al., 2001). Replication and construction of nucleocapsids and ODV occurs in the nucleus of the cell. Early in infection, the virions are packaged as budded virus, which acquire an envelope as they bud from the infected cell and spread systemically through the insect through clathrin mediated endocytosis. In non-permissive hosts, and for some species of nucleopolyhedroviruses, replication is limited to the midgut epithelium and does not spread throughout the host (Afonso et al., 2001; Lauzon et al., 2006). During the late stages of infection the virus shifts to producing occlusion-derived virus (ODV). The nucleocapsids are enveloped within the nucleus and then packaged within the polyhedrin matrix and further enveloped. Ultimately, the polyhedra filled cells lyse resulting in liquefaction of the entire host, distributing polyhedra into the environment once again.

Multiple mechanisms have been proposed for the spread of NPV from the gut to other tissues. Simultaneous infection of the gut cells and hemocytes of Trichoplusia ni was noted at 12 hpi (Barrett et al., 1998). This time point is too early for new BV to have been produced, and ODV are very poorly infectious to other tissues. The M phenotype with simultaneous release of multiple NC into a cell, may allow some nucleocapsids to travel to the nucleus and commence viral transcription and translation, and other NC to move directly to the basal membrane of the columnar cells and bud out, acquiring gp64 and/or the F-protein in the process. These BV then escape the gut system through hypothetical weak points in the basement membrane to infect hemocytes or the tracheoblasts that permeate the basement membrane of the gut (Engelhard et al., 1994; Federici, 1997). The hemocytes that come into contact with the tracheoblasts could then be infected. Once the hemocytes are infected, they circulate through the insect, infecting all other tracheoblasts, fat body, reproductive tissues, and finally the epithelial cells of the cuticle. One of the few tissues shown to be resistant to baculovirus infection is the Malpighian tubules which regulate water balance (Knebel-Mörsdorf et al., 1996). Likewise, after the initial infection, the gut clears the infected cells either through cell sloughing (an immune defense) or through the natural sloughing of the epithelial cells
which occurs during the molt. The gut then remains uninfected, allowing the infected insect to continue to feed throughout the infection (Engelhard & Volkman, 1995; Washburn et al., 1998).

**Genome and gene expression.** Baculoviruses have large, circular, supercoiled, double-stranded DNA genomes ranging from 80 to 180 kB. The viral genomes encode 120-160 open reading frames (orfs), of which many are of unknown function. Over half have been characterized in some manner, but mostly on an independent basis; the interaction of viral proteins is largely unstudied and unknown (Rohrmann, 2008). The type species, *AcMNPV*, contains a genome of ~150 kB, with ~151 orfs greater than 50 amino acids in length, of which ~93 have an associated function (Rohrmann, 2008).

Gene expression of baculoviruses has three distinct phases: early (before DNA replication), late (beginning after the initiation of DNA replication), and very late. Within an infected cell, gene expression is determined by the promoters associated with the viral genes. Early genes contain promoters recognized by the host RNA polymerase II, resulting in peak gene transcription six to twelve hours after infection (Fuchs et al., 1983; Hoopes & Rohrmann, 1991; Huh & Weaver, 1990). Early gene transcripts can be detected within 15 minutes of infection, and include ie-0 and ie-1, transcription factors which greatly enhance the expression of the other early genes by interaction with an activation region upstream of the promoter (Friesen, 1997). Early genes include that for the BV envelope protein *gp64*, host immune suppressors such as *p35*, and the viral proteins needed for DNA replication. Some early genes contain both an early and a late promoter, resulting in continual low level transcription throughout infection. Once DNA replication begins, the viruses begin to express the late genes; simultaneously, host cell nuclear transcription is mostly shut down (Friesen & Miller, 1986; Ooi & Miller, 1988). The late genes are transcribed by a virally encoded four subunit RNA polymerase that recognizes the baculovirus late promoter motif A(T/G)TAAG (Beniya et al., 1996; Guarino et al., 1998; Rohrmann, 1986). Late gene transcription occurs 6-24 hpi, and maximum production is observed at 24 hpi (Blissard et al., 1989). Nineteen late expression factors (lefs) have been described in *AcMNPV* that are required for optimal expression of late and very late genes (Bonning, 2005). Most are expressed in the early stage, with the exception of the very late
expression factor-1 (vlf-1) that regulates the very late genes (McLachlin & Miller, 1994; Todd et al., 1996). The two very late genes, polyhedrin/granulin and p10, are transcribed from 18-72 hpi from the very late promoter motif, (T/A)ATAAGNA(T/A/C)T(T/A)T, and the additional downstream burst element (Ooi et al., 1989; Rohrmann, 1986). The very late genes continue to be transcribed well after the late gene expression has been turned off (Thiem & Miller, 1990).

The majority of viral genome replication occurs between 6-18 hpi (Kool & Vlak, 1993). DNA replication is dependent on the production of early viral proteins, and various non-coding regions in the viral genome, including the homologous regions (hrs), which are dispersed throughout the genome (Kool & Vlak, 1993; Leisy et al., 1995; Pearson et al., 1993). Five genes are considered essential to DNA replication, and five are stimulatory (Bonning, 2005). The virus encodes its own DNA polymerase, but conflicting results place it in the stimulatory category (Ahrens & Rohrmann, 1995; Kool et al., 1994; Lu & Miller, 1995; Pearson et al., 1993). Host DNA polymerase combined with the essential viral proteins can replicate the DNA albeit at low levels not sufficient for viral survival. One of the essential genes, p143, encodes a helicase that contributes to host susceptibility. Inserting a portion of the BmNPV p143 into AcMNPV increased the host range of AcMNPV to include B. mori, and this host range expansion was subsequently mapped to two critical amino acids (Argaud et al., 1998; Maeda et al., 1993). The mechanism for DNA replication, whether a rolling circle mode, a theta mechanism, or a combination, is unknown.

**Genomics.** The baculoviruses exhibit strong co-evolution with their invertebrate hosts (Herniou et al., 2004; Okano et al., 2006). Computational analyses have identified 30 core genes conserved across all four genera, and there may be more homologs that have diverged to such an extent that they are not immediately recognized, as in the case of ODV-E18 (Herniou et al., 2003; McCarthy & Theilmann, 2008). Another limiting factor for computational analyses is the fact that only one member of the deltabaculoviruses, and two members of the gammabaculoviruses have been identified and sequenced. Beyond these conserved genes, the genomes differ dramatically and exhibit gene duplications, deletions, insertions, and inversions (Herniou et al., 2003). The baculoviruses have been
shown to contain retrotransposons, and an entire family of insect retroviruses, the errantiviruses, is thought to have resulted from the integration of a transposon which acquired an envelope gene from the baculoviruses (Jehle et al., 1996; Malik et al., 2000; Rohrmann & Karplus, 2001). However, gene order (of the 30 core genes) is conserved to a great extent in the Group I alphabaculoviruses (though not Group II) and the granuloviruses, although the order between these two groups differed dramatically (Herniou et al., 2003). Only one cluster of four genes is conserved across all baculoviruses, which includes helicase, pif-4, 38K, and lef-5. Helicase and LEF-5 are essential for replication and transcription, PIF-4 essential for oral infectivity of polyhedra, and 38K is found in the nucleocapsid (Fang et al., 2009a; Herniou et al., 2003; Wu et al., 2008). The early, late and very late genes are dispersed throughout the genome, on both strands of DNA, dependent on the promoter and cis elements for correct transcription. The baculovirus genomes exhibit a high degree of fluidity in the terms of horizontal gene movement from other viruses, bacteria, and hosts; recombination events during DNA replication, along with the retrotransposon activity are the most likely mechanisms for horizontal gene transfer. Three genes specific to the baculoviruses that infect the lymantriids, hrf1, ctl1, and ctl2, have also been identified, and may contribute to host specificity (Herniou et al., 2003). The lepidopteran species also have an additional 32 conserved genes, and an additional 13 are present in most of the alphabaculoviruses (Herniou et al., 2003). Twelve of these 13 have been shown to be auxiliary genes, non-essential to virus replication. Interestingly, several genes have been shown to be acquired separately, multiple times, again pointing to the fluid nature of baculovirus evolution. Examination of five group I alphabaculovirus genomes for genes undergoing positive selection identified nine genes of interest, ac38, ac66, arif-1, lef-7, lef-10, lef-12, odv-e18, and odv-e56 (Harrison & Bonning, 2004).

**Population dynamics.** Our understanding of baculovirus population dynamics is limited. Most work at the molecular level is done with a single strain of a virus species, one that has often been passaged through tissue culture multiple times, or through lab reared insects that may be of a more homogenous gene pool than found in the field. Under field conditions, baculoviruses exist as a population, and are commonly found with several
different viral species. Insects can be co-infected with both different baculovirus strains and different species, and the genetic mixing of the baculoviruses reflects this. In a single *Panolis flammea* larva, 24 unique *PaflNPV* genotypes were determined by restriction fragment length polymorphism (RFLP), and three hypervariable regions identified (Cory et al., 2005). The phenotypes of these cloned variants differed significantly in virulence, virus yield, and survival time (Hodgson et al., 2001). Populations of *SfMNPV* isolated from Nicaragua were found to have nine RFLP genotypes, eight of which were defective on their own (Lopez-Ferber et al., 2003). The most abundant defective genotype contained a 16.4 kb deletion, which included both PIF-1 and PIF-2. Maintenance of the defective genotypes would require simultaneous infection of the cell by both genotypes or that both genotypes be present in the same ODV. Most interesting from these studies, a mixture of the complete genome and the deletion mutants, at a ratio of 3:1 was more potent than the complete virus alone (Clavijo et al., 2009). Further, this difference was mapped to the deletion of both *pif-1* and *pif-2*. When insects were orally fed different ratios of the complete and deletion virus, the genotypic ratios stabilized to 85% virus with the complete genome to 15% deletion mutant virus after four per oral passages, irrespective of the starting doses (Clavijo et al., 2009). The advantage to the virus of not encoding these PIFs in the genome is unclear. Baculoviruses are so large that studies of these populations at the nucleotide level are not yet practical. Nevertheless, a comparison of two *MacoNPV-A* strains at the nucleotide level found 521 point mutations, and several insertions and deletions (Li et al., 2005). These two virus genotypes came from a single population of wild larvae during a natural outbreak of the virus. Interestingly, the strain that exhibited lower virulence was estimated to be present at 25%, similar to the *SfMNPV* studies (Li et al., 2005).

**Current topics in baculovirology.** Baculoviruses continue to be a plague upon lepidopteran colonies, both silkworm colonies and laboratory colonies of lepidopteran species. Natural epizootics occur regularly in some forest lepidopterans and have led to the successful modeling of baculovirus host interactions (Cory et al., 1997; Evans, 1986; Rothman & Myers, 1996).
Movement toward the creation of environmentally friendly insecticides led to great interest in the development of baculovirus treatments. This has led to the development of three baculovirus insecticides in three different systems. The most successful has been the treatment of the velvet bean caterpillar on soybean in Brazil. The success of treatment by spraying $1.5 \times 10^{11}$ polyhedra per hectare resulted in the development of facilities dedicated to production of AgNPV, and more than 30,000 hectares are treated annually (Szewczyk et al., 2006; Moscardi, 1999). The second example is the use of the betabaculovirus, *Cydia pomonella* GV (CpGV) against the codling moth, *Cydia pomonella*, which infest pear and apple crops. This has been used in several countries, both in Europe and North America (Oliveira et al., 2006). Virus resistance has been documented as a result of this treatment (Asser-Kaiser et al., 2007). The cotton bollworm, *Helicoverpa armigera*, is a major cotton pest that has developed resistance to several chemical insecticides. In China, *Hear* NPV has been applied to cotton fields, as well as, field trials of a recombinant virus expressing the scorpion toxin AaIT (Sun et al., 2002). The recombinant baculovirus kills the insects more quickly, leading to increased cotton yields (Sun et al., 2002).

Due in part to the availability of susceptible cell lines, BV have been well studied. This attribute has led to the investigation of using baculovirus BV for human gene therapy, for the study of basic viral mechanisms (the gp64 molecular structure was recently resolved and placed into a new class of fusion proteins), and the development of recombinant protein expression systems. Commercially, the baculoviruses have been exploited for many other applications including: 1. Expression of thousands of recombinant proteins for study of a variety of protein systems including cytochrome p450 and G-protein coupled receptors, 2. Use as display systems using fusions to the GP64 protein as well as the capsid protein VP39, 3. Production of viral like particles utilized in vaccine development for human papilloma virus, hepatitis C virus, and severe acute respiratory syndrome virus, and 4. Mammalian cell gene delivery vectors as baculoviruses are capable of entering a broad array of mammalian cell types, but the baculoviruses do not replicate in the mammalian host, although genes recognized by the mammalian host cell will be transcribed from the viral genomic DNA (Kost et al., 2005).
**The host and viral immunity**

Most viruses have coevolved with their host for millennia, to which baculoviruses are no exception. Fossil evidence for baculovirus infection of an insect host has been documented back to 100 million years ago (Poinar & Poinar, 2005). Such an ancient relationship reveals both a highly conserved innate immune response, as well as, highly specific insect-virus interactions. Much of what is known of the insect immune response to insect specific viruses comes from the study of baculoviruses with the exception of work with *Drosophila* viruses (Sparks *et al.*, 2007). No baculoviruses of *Drosophila* have been described to date. Insects exhibit a plethora of uniquely adaptive physiological traits, and this review will focus on what has been documented in the Lepidoptera and conserved systems from the model insect *Drosophila*.

**Immune defenses in the lepidopteran gut.** The insect exoskeleton is impenetrable, waterproof and cell-less at the outermost layer, all of which provide an unlikely environment for an obligate intracellular parasite such as a virus. This leaves two entrances into the insect body for horizontal virus transmission: via the tracheoles (the insect respiratory system which has several openings) and via the mouth. Of the over 250 insect viruses described by the International Congress of Virology (ICTV) only one, an iridovirus has clearly been documented to infect insects via the tracheoles, though that is not the primary mode of infection (Hunter *et al.*, 2003; Williams, 1998); oral infection is the primary mode of horizontal infection for all described insect viruses. Vertical transmission has been documented for several viruses, as well as, sexual transmission and via oviposition by parasitoids.

Throughout most of the larval stage caterpillars are eating machines; the majority of their body cavity is comprised of the gut tube. The gut has three distinct regions: the foregut, the midgut, and the hindgut. Only the midgut contains epithelial cells, but the peritrophic matrix (see below) and the cuticular sections of the foregut and hindgut are shed with each molt. The main target of viral infection is the columnar cells of the midgut epithelium as this is the only insect cell layer without a protective layer of cuticle (Tellam *et al.*, 1999). The columnar cells are the most predominant cell type in the epithelia, and
are most often noted for their brush border of microvilli. The microvilli serve to dramatically increase the surface area of the gut, improving the ability of these cells to absorb nutrients and produce digestive enzymes for secretion into the gut. As the most susceptible cells in the insect, the insect has developed multiple physical and physiological barriers to protect these vulnerable cells from virus infection.

Peritrophic matrix. The gut epithelium is protected from the gut contents by a physical barrier, the peritrophic matrix (PM), in most insects. The PM is best described as a porous extracellular matrix made up of chitin, sugars, and proteins. Two types of PM production have been described: Type I, produced by all of the cells of the midgut, and Type II, produced by the anterior cells of the midgut and being moved along the gut with the food. Interestingly, H. virescens is one of the few lepidopteran larvae to exhibit a Type II PM. In mosquitoes and hematophagous diptera, however, a type II PM is more resistant to pathogens (Lehane, 1997). The PM protects the midgut cells from abrasion by food while also acting as a sieve which potential pathogens must navigate (Lehane, 1997). Insects feeding on sterile diets of blood, nectar, phloem, or hemolymph usually do not have a PM, whereas insects feeding on contaminated diets of urine puddles, fecally contaminated water sources, muddy/brackish pools, and rotting flesh do contain PM (Lehane, 1997). Insects may also produce PM in one life stage (larva) but not as an adult (nectar feeding butterfly); or may produce PM only in response to specific diet exposure.

The PM is porous in nature, but the pore size ranges from 3-4 nm in Calliphora erythrocephala (Diptera), 21-29 nm for Lepidoptera, and 24-36 nm for Orthoptera (Peng et al., 1999). The pores allow digestive enzymes secreted by the columnar cells to travel to the gut lumen, and for the resulting digested nutrients to cross back to the microvilli for absorption. These pore sizes will inhibit many pathogens such as bacteria and large viruses. The size of an average AcMNPV ODV is 190 nm X 360 nm. PM of Trichoplusia ni larvae were almost impermeable to the virions, whereas Pseudaletia unipuncta PM allowed more ODV through indicating greater pore size (Peng et al., 1999). The entomopoxviruses (EPV) are at an even greater disadvantage in terms of PM penetration, ranging from 300-470 in diameter and 200-300nm in length (Goodwin et al., 1991). The insect dicistroviruses, small non-enveloped RNA viruses, such as cricket paralysis virus
(CrPV) and drosophila C virus (DCV) are spherical ranging from 27-30 nm (Moore & Eley, 1991; Bonning & Miller, 2010). Scanning electron micrographs show that some microvilli, including those of T. ni, can protrude through the pores of the PM providing direct access into the lumen of the gut (Adang & Spence, 1981). It is unclear if this is the case for all stages of the insect.

Proteins specific to the PM show strong non-covalent bonds with the PM, and high resistance to proteases. The resistance to the gut proteases is thought to be due to two types of modular cysteine rich domains, the peritrophin A domain and peritrophin B domain, that form disulfide bridges (Tellam, 1996). Peritrophin-like proteins can contain multiple copies of a peritrophin domain, although to date peritrophin A domains have only been identified in proteins specific to PM, a few nematode and arthropod chitinases, and baculoviruses (Tellam, 1996). Specific PM proteins have been characterized from the insect species Lucilia cuprina, Anopheles gambiae, Aedes aegypti, Chrysoma bezziana, Drosophila melanogaster, and T. ni (Devenport et al., 2005; Elvin et al., 1996; Guo et al., 2005; Shao et al., 2005; Shen & Jacobs-Lorena, 1998; Tellam, 1996; Wang & Granados, 1997a; b; Wang et al., 2004). The peritrophin proteins have been shown to interact with chitin which spans the matrix (Elvin et al., 1996; Shen & Jacobs-Lorena, 1998). The peritrophins are also rich in the amino acids proline, serine, and threonine that favor potential O-linked glycosylation, which may result in mimicry of cellular receptors bound by pathogens (Lehane, 1997).

The baculoviruses have evolved to contain several genes that provide a means to bypass these host defenses. Enhancin, named because it enhances viral infection, specifically degrades the peritrophin protein, insect intestinal mucin, allowing more virus to rapidly come into contact with the microvilli (Derksen & Granados, 1988; Lepore et al., 1996; Peng et al., 1999; Slavicek & Popham, 2005). Baculoviruses encode several chitinases that most closely resemble bacterial chitinases, but appear to be involved in host liquefaction, rather than virus entry (Hawtin et al., 1997; Rao et al., 2004; Wang et al., 2004). Several chitin binding proteins have been identified from EPV, as well as, the baculovirus homolog, gp37 (Dall et al., 1993; Hayakawa et al., 1996; Li et al., 2003). The baculovirus protein Ac150, and its homolog in H. armigera EPV, contain an integrin
domain and enhance infectivity as well (Dall et al., 2001; Lapointe et al., 2004; Zhang et al., 2005).

**pH.** The complex contents of the gut are the first line of defense. Many lepidopteran larvae have a basic pH. At the extreme end, a pH greater than 12 has been recorded in *Acherontia atropos* (Dow, 1992). This high pH has been shown to protect larvae from phytochemicals (Berenbaum, 1980). The baculoviruses and entomopoxviruses have adapted to the lepidopteran midgut, and require basic pH for activation of their virions (Bonning, 2005).

**Enzymes.** The columnar cells constantly secrete proteins into the midgut, the majority of which are proteases (Tellam et al., 1999). The proteases recognize a wide variety of cleavage motifs, and their primary role is to break down ingested food. The proteases are also active against any pathogen proteins, including proteins expressed on the surface of virions. These exposed proteins are needed for the initial binding of the virions to the target cells. The *Bombyx mori* NPV has been shown to be susceptible to a serine protease secreted *in vivo* in *B. mori* (the silkworm) (Nakazawa et al., 2004). Diet additives, such as fluorescent whiteners, result in increased infection by a recombinant *Bm* NPV (Wang et al., 2006). These insects exhibited significantly lower gut protease activity, and gut pH was decreased 0.5 –1.3, implicating both pH and protease activity as key components of innate immunity in the insect. Another digestive enzyme, lipase, also has antiviral activity. Lipase was expressed only by the midgut epithelia during active feeding stages of the insect (not during wandering or molting), and decreased the activity of *Bm* NPV (Ponnuvel et al., 2003). Baculoviruses are enveloped and would contain host lipids on the membrane. The lipase was expressed at constant levels irrespective of the presence or absence of virus.

**Diet.** The innate immunity of herbivores is affected to a surprising degree by the phytochemicals and nutrients in the diet (Duffey et al., 1995). *Helicoverpa spp.* have increased production of gut proteases that are unresponsive to the plant protease inhibitors of the plants upon which they feed (Volpicella et al., 2003). Larvae that fed on Scots pine needles had distinct *Panolis flammea* NPV genotypes from larvae that fed on lodge pole pine needles (Hodgson et al., 2004). *Heliothis virescens* larvae that fed on cotton were less
susceptible to baculovirus, than those feeding on lettuce; increased cell sloughing may occur in response to the physical damage caused by the cotton plant in the gut (Hoover et al., 2000). Increasing levels of the core element selenium in larval diet resulted in increased plasma levels of selenium and a decrease in AcMNPV susceptibility in *H. virescens* (Shelby & Popham, 2007). A specific class of enzymes, the selenoenzymes, require selenium for activity, and are involved in stress response and maintaining antioxidant levels (Beck et al., 2004).

**Cell sloughing and apoptosis.** Along with these various innate defenses at work in the insect gut, the insects utilize cell sloughing to clear infected cells from the gut. This has been well documented in *T. ni* infected with baculovirus. Immediately after ecdysis, these larvae lack PM and are more susceptible to viral infection and the PM is fully formed after three hours; however, resistance increases throughout an instar (Engelhard & Volkman, 1995). Infected larvae cleared all infected epithelial cells when molting to fifth instar, resulting in virus free insects if secondary infection of other tissues by BV had not yet occurred. This clearance of infected gut cells was also observed in *H. virescens* (Washburn et al., 1995). Both species showed no increase in resistance when BV was injected into the hemocoel at various time points (Washburn et al., 1995). Cotton feeding *T. ni* larvae were more resistant to baculovirus; cotton increases reactive oxygen species which may facilitate cell sloughing and rapid cell turnover (Hoover et al., 2000).

Apoptosis associated with infection by *Helicoverpa armigera* stunt virus (Tetraviridae), also resulted in increased cell sloughing (Brooks et al., 2002).

**Immune defenses against systemic infection.** Lepidopteran larvae exhibit physical, cellular, and humoral immunity that protect them from systemic viral infection. Although the invertebrates do not have the highly adaptive immune response observed in higher order vertebrates nor the degree of immune memory, the insects can and do mount different immune responses for different pathogens. The primary cell involved in immunity is the hemocyte. The hemocytes circulate through the hemolymph and can be recruited to areas of infection. The hemocytes express and secrete antimicrobial peptides, initiate encapsulation and melanization responses, as well as, the phenoloxidase cascade.
**Basement membrane.** The basement membrane (BM) is a physical barrier whose primary function is tissue support and separation of the gut and hemolymph. The BM protects the hemolymph from pathogens that have invaded the gut. The BM is another extracellular matrix, but is much more complex than the PM. It is composed of sheets of proteins that surround all organs of the insect including the gut, fat body, and tracheoles, and is used for structural support, filtration, and cell attachment, migration, and differentiation (Rohrbach & (Eds), 1993). The BM prevents the BV from crossing freely into the hemolymph to cause systemic infection; the virions are too large to freely diffuse (Reddy & Locke, 1990). Electron microscopy shows BV accumulate in the extracellular space between the midgut epithelium and the BM (Hess & Falcon, 1987; Tang et al., 2007). Some baculoviruses do not result in systemic infection, but are limited to the gut, and ancestral baculoviruses are thought to have been gut-limited (Reddy & Locke, 1990). Lepidopteran NPV appear to breach this defense by directly penetrating the membrane in places of structural weakness or using enzymes, or by infecting the trachea which serve the midgut tissues (Engelhard et al., 1994; Federici, 1997; Flipsen et al., 1995; Granados & Lawler, 1981; Ko et al., 2000). Currently, it is unclear if one mode of action is preferred, or if the mechanism varies by species. Enzymes that degrade the BM have also been identified in entomopoxvirus (Afonso et al., 1999). The baculovirus fibroblast growth factor (FGF) homolog may also attract hemocytes via chemotaxis; *fgf* has been found only in baculoviruses that exhibit systemic infection (Detvisitsakun et al., 2007).

**Phenoloxidase, melanization, and encapsulation.** Hemocytes in the hemolymph are capable of recognizing non-self and initiating an appropriate response. Hemocytes recognize extracellular pathogens, such as bacteria or parasites, via distinct pattern recognition motifs that are not found within the insect. Hemocytes recognize virus infected cells as well, but the recognition process is unclear. One possibility is that baculovirus infection and enlargement of the infected cells results in distortion of the basement membrane, which is then recognized and melanized by hemocytes. The cells underlying the distorted basement membrane would then be destroyed. Insects have a unique immune response involving melanization and encapsulation of foreign bodies (Shelby & Popham, 2006). Pro-phenoloxidase is secreted into the hemolymph. This form
is activated by serine protease cleavage, and in turn the phenoloxidase cascade oxidizes tyrosine residues to become quinines, which can then be polymerized to form melanin. The insect sequesters pathogens in capsules of cross linked hemocytes and melanin. This encapsulation is most often observed for extracellular pathogens. Interestingly, encapsulation of baculovirus infected cells has been observed in *H. zea* and *M. sexta* (Washburn *et al.*, 2000; Washburn *et al.*, 1996). Tracheal cells infected with AcMNPV became surrounded by hemocytes, and encapsulated tracheal cells were identical in morphology to encapsulated bacteria and parasites. Hemocytes, which are critical to baculovirus secondary infection of the host, are not encapsulated and baculovirus infected hemocytes have lost the ability to encapsulate; thus, infection of the hemocytes results in the insect succumbing to the viral disease (Trudeau *et al.*, 2001). The inability of AcMNPV to replicate in the hemocytes of *H. zea* correlates with the increased resistance of *H. zea* to AcMNPV. The hemocytes in this system remove virus from the hemolymph, but are not infected, and can still encapsulate infected trachea (Trudeau *et al.*, 2001). Phenoloxidase also has antiviral activity independent of the melanization/encapsulation response (Shelby & Popham, 2007).

**Apoptosis.** Beyond recognition of infected cells by hemocytes, infected cells also mount an immune response. Apoptosis, or programmed cell death, is a common response to viral infections in both invertebrates and vertebrates (Cashio *et al.*, 2005; Siegel, 2006). Our current understanding of apoptosis largely results from study of a baculovirus protein that inhibits apoptosis and has no host homolog. Apoptosis is initiated by the activation of caspases, which normally remain inactivated by the inhibitor of apoptosis proteins (IAPs) that bind to them (Salvesen & Duckett, 2002). Insect viruses lacking inhibitor of apoptosis proteins are significantly less virulent (Clem *et al.*, 1991).

The baculovirus AcMNPV encodes *p35*, a universal caspase inhibitor with no homologs in any organisms except other baculoviruses (Clem, 2005). Viruses lacking this gene induced apoptosis leading to reduced BV and ODV production in the insect (Clem *et al.*, 1991). Upon infection by the virus, cellular caspases cleave P35 which results in a conformational change and covalent bond formation with the caspase, inactivating the caspase, and thus preventing apoptosis (Clem, 2005). P35 and its homologs exhibit
slightly different affinities to the different types of caspases found in the cell (Clem, 2005; LaCount et al., 2000). In addition to the inactivation of caspases, p35 has also been implicated in the quenching of free radicals, which blocks caspase activation (Sahdev et al., 2003). In addition to the p35 protein, the baculoviruses contain another family of anti-apoptosis proteins, the IAPs proteins. The mode of action of IAPs is distinct from p35; IAP specifically blocks the activation of pro-Sf-caspase-1 in Sf cells (Seshagiri & Miller, 1997). Deletion of *iap-1* or *iap-2* in AcMNPV had little effect on apoptosis, compared to p35 deletion (Griffiths et al., 1999). AcMNPV IAP-1 shares only 28% identity with the *Op* MNPV IAP-1, while *Op* MNPV and *Cp* GV share 58% identity, suggesting a different origin and/or function for these proteins in these systems. Cellular homologs of IAP-1 have been identified in *S. frugiperda* and *T. ni* (as well as *Drosophila melanogaster* for which no baculovirus has been described), suggesting that IAP is of host origin, even if p35 is not (Clem, 2005). Further, the *Cp* GV IAP clustered with the lepidopteran IAP homolog, rather than the NPV homologs, and the entomopoxvirus IAP homologs formed a third distinct group in the phylogeny (Hughes, 2002). The endogenous IAPs all have specific caspase inhibition, rather than universal activity like that of P35 (Deveraux et al., 1997; Roy et al., 1997). A third IAP, *O. pseudotsugata* IAP-3, which clusters with the other lepidopteran homologs, exhibits E3 ubiquitin ligase activity (Blitvich et al., 2002; Green et al., 2004). This protein ubiquinates the pro-apoptotic protein *Drosophila* HID to block the apoptotic cascade (Vucic et al., 1997).

That virus infected cells can recognize infection and initiate apoptosis has been clearly demonstrated; however, the basis for this recognition is unknown. Potential cell recognition factors include synthesis of viral DNA, the late viral gene expression, and the obstruction of host transcription and translation (Clem, 2001). The addition of transcription inhibitors to Sf-21 cells results in rapid induction of apoptosis (Clem & Miller, 1994). Cells transiently transfected with the genes necessary for AcMNPV DNA synthesis yielded little DNA, unless co-transfected with *p35* or an active *iap* (Lu & Miller, 1995). The immediate early proteins, immediate early-1 (IE-1), and pe38 both induce apoptosis (Prikhod'ko & Miller, 1996; Prikhod'ko & Miller, 1999).
Cell signaling cascades/antimicrobial peptides. Apoptosis is not the only defensive response available to a cell. Several cell-signaling pathways result in differential gene expression associated with immune defense against viruses: the Toll, Imd (immune deficiency), and the Jak-STAT pathways. The transmembrane receptor Toll was first discovered in Drosophila, and the mammalian homologs, the Toll-like receptors (TLR) subsequently described. Although both mammals and insects contain approximately ten copies of Toll receptors, only Toll exhibits immune function in insects with the other nine related to development. In contrast, all TLR in mammals have immune-related functions (Imler et al., 2004). The Toll receptor is activated by an intermediate ligand, Spaetzle, a cysteine knot growth factor, unlike the TLR which interact directly with the pathogen (Lemaitre et al., 1996). Pattern recognition initiates a signaling cascade that activates Spaetzle, allowing it to bind Toll. Drosophila contains six spaetzle like genes, and these other copies may interact with the other insect TLR (Parker et al., 2001). Both Toll and Imd regulate the production of anti-microbial peptides in Drosophila, as well as, activating two members of the NF-KappaB transcription factors, Dif and Relish (Brennan & Anderson, 2004). Imd is a death domain adaptor protein that activates Relish, while Toll activates Dif as observed in Drosophila in response to Drosophila X virus (DXV) (Brennan & Anderson, 2004; Zambon et al., 2005). In flies lacking Dif, viruses replicated to higher titers and the insects died sooner; however, Relish mutants showed wild type levels, indicating that although DXV activates the Imd pathway, the antimicrobial peptides produced were ineffective against this virus (Zambon et al., 2005). Polydnaviruses also produce homologs to interferon kappa B proteins that inhibit NF-KappaB in mammals (Thoethiattikul et al., 2005).

Genetic and developmental immunity. In addition to these specific pathways and anti-viral peptides, several cases of genetic and developmental resistance to viruses have been described. Genetic resistance to baculovirus has been observed in both field and laboratory populations; a non-additive polygenic trait with autosomal inheritance was suggested by C. pomonella resistance to baculovirus (Briese, 1986; Eberle & Jehle, 2006). Developmentally, lepidopteran larvae become increasingly resistant to baculovirus throughout an instar; this is thought to be due to increased levels of cell sloughing, and
then shedding the entire midgut in ecdysis (Engelhard & Volkman, 1995; Kirkpatrick et al., 1998; Teakle et al., 1986). Some Lepidoptera, such as the gypsy moth larvae (L. dispar) show systemic resistance to baculovirus infection through unknown mechanisms; and others exhibit resistance in the penultimate instar, which suggests the resistance may be hormonally mediated (Engelhard & Volkman, 1995; Hoover et al., 2002; Kirkpatrick et al., 1998; Teakle et al., 1986). An anti-viral response that results in viral clearance in the hemolymph in L. dispar is also a possibility (Hoover et al., 2002). Another interesting potential genetic component is the insect dscam, an immunoglobulin like gene, shown to encode 38,000 and 31,000 alternatively spliced transcripts in D. melanogaster and Anopheles gambiae, respectively (Dong et al., 2006; Watson et al., 2005). This gene showed differential expression in response to different pathogens, in which the peptides were both secreted and membrane bound. This may provide a means for insects to react in a more precise manner to invading pathogens.

**Baculovirus occlusion derived virus (ODV)**

**Composition.** The ODV are composed of nucleocapsids that are identical to those in the BV, tegument proteins that create a matrix between the nucleocapsid and the envelope, and the envelope, a bilipid membrane studded with proteins. A large number of ODV specific tegument and envelope proteins have been described. In addition, the ODV package several proteins that are also present in BV, although the amount of protein present in their respective envelopes differs dramatically (Braunagel et al., 2003; Braunagel et al., 2004; Deng et al., 2007; Perera et al., 2007). Several viral proteins related to DNA replication have also been isolated from the ODV, but the significance of their presence is unclear (Braunagel et al., 2003; Deng et al., 2007; Perera et al., 2007). Specific proteins that interact with the ODV envelope and the polyhedrin protein have not been identified. The predominant lipids of the ODV envelope are phosphatidylcholine and phosphatidylethanolamine, compared to phosphatidylserine on the BV, and is likely
reflective of the differences between the host plasma membrane and inner nuclear membrane (INM) (Braunagel & Summers, 1994).

**Synthesis.** Originally, the ODV envelope was thought to be synthesized *de novo*, since the ODV never left the nucleus. Further examination, however, revealed that nucleocapsids acquire their envelope from invaginations of the INM (Hong *et al.*, 1997). The INM is part of a continuous membrane composed of the INM, the outer nuclear membrane (ONM) and the endoplasmic reticulum (Braunagel *et al.*, 2004; Rosas-Acosta *et al.*; Saksena *et al.*, 2004). Several of the envelope proteins have a conserved N-terminal sorting motif (SM) composed of a strong hydrophobic region followed by several charged amino acids that target the proteins to the INM. Pores exist between the INM and ONM capable of shuttling proteins up to ~76 kDa (Hinshaw *et al.*, 1992). Baculoviruses replicate and assemble in the nucleus, and therefore, all the structural proteins needed to form nucleocapsids and ODV must be transported to the nucleus. During baculovirus infection, the nucleus increases in size by a factor of 5 to 10 fold as it swells with occlusion bodies (Federici, 1997). Proteins targeted to the ODV accumulate in the nucleus in a region termed the ring zone. The mechanism by which the nucleocapsids are enveloped and then packaged into the polyhedra are not understood.

**ODV envelope genes and proteins.** Multiple methods (both biological and computational) have been used to identify ODV envelope proteins. Biological methods include antibody recognition, mass spectrophotometry, and deletion mutant analysis. Computational analyses have identified orfs, their promoters, conserved genes, and genes undergoing positive selection. Many proteins have been clearly identified by western blot, but have not been detected in proteomic analyses using mass spectrophotometry; this emphasizes the necessity of a multifactorial approach to understanding the complexity of the ODV envelope.

**Biological studies.** Braunagel et al. (2003) published the first comprehensive biological work identifying the ODV envelope proteins of *AcMNPV*. Using multiple techniques, the ODV were estimated to contain between 31 and 44 proteins, some of which are exclusively only found in the ODV. In addition to the proteins identified in this study, additional proteins shown to be important to oral infection of the baculovirus have been
identified. It has been postulated that the ODV purification process results in loss of some of these proteins that may only be weakly associated with the ODV envelope (Braunagel et al., 2003). More recent comprehensive studies on HearNPV and the dipteran CuniNPV ODV gave similar results, with both expected ODV specific proteins and unexpected DNA replication proteins identified (Deng et al., 2007; Perera et al., 2007). Two virus specific HearNPV proteins, Ha44 and Ha100, have been shown to be associated with the nucleocapsid and nucleocapsid and envelope, respectively (Deng et al., 2007). Most of the published work on ODV envelope proteins has been focused on only one or two proteins at a time, although FP25K mutants have been screened for effects on the production of both BV and ODV proteins (Braunagel et al., 2003). Table 1 describes the known ODV envelope proteins and their proposed functions. The proteins listed are identified by their AcMNPV orf and common name, with the exception of Ha44 and Ha100, which have no known homologs in AcMNPV (Deng et al., 2007).

**Computational studies.** Computational analyses showed that the genes p74, pif-1, pif-2, pif-3, odv-e18, odv-e56, odv-ec43 are conserved across all Baculoviridae, ac145 conserved in all but the dipteran baculoviruses, and pif-4, odv-e66 and odv-e25 are conserved across the lepidopteran baculoviruses (Herniou et al., 2003; McCarthy & Theilmann, 2008). In contrast, ac150 is found only in Group I alphabaculoviruses, while enhancer like genes are scattered throughout Group II alphabaculoviruses and some of the betabaculoviruses (Herniou et al., 2003; Rohrmann, 2008; Slavicek & Popham, 2005). The two virus specific HearNPV proteins, Ha44 and Ha100, show yet a different pattern, illustrating the complex evolution of baculoviruses (Deng et al., 2007). Ha44 is conserved in Group II alphabaculoviruses and the betabaculoviruses, whereas Ha100 is found only in Group II alphabaculoviruses (Deng et al., 2007).
Table 1. Characterization of proteins identified within the ODV envelope and conservation within the Baculoviridae. * potential PIF

<table>
<thead>
<tr>
<th>Protein</th>
<th>PIF</th>
<th>ORF</th>
<th>Conserved</th>
<th>Signal</th>
<th>Known function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P74/PIF-0</td>
<td>PIF-0</td>
<td>ac138</td>
<td>All</td>
<td>C terminal</td>
<td>Binding to midgut/ oral infection</td>
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<td>PIF-2</td>
<td>ac22</td>
<td>All</td>
<td>N-terminal SM</td>
<td>Binding to midgut/ oral infection</td>
</tr>
<tr>
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<td>PIF-3</td>
<td>ac115</td>
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<td>N-terminal SM</td>
<td>Oral infection of ODV</td>
</tr>
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<td>ac144</td>
<td>Alpha, Beta</td>
<td>Unknown</td>
<td>Multifunctional viral cyclin found only in ODV nucleocapsids</td>
</tr>
<tr>
<td>ODV-E35</td>
<td></td>
<td>ac143/144</td>
<td>?</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>ODV-E56</td>
<td>PIF-5</td>
<td>ac148</td>
<td>Alpha, Beta</td>
<td>C terminal</td>
<td>See Chapter 2</td>
</tr>
<tr>
<td>ODV-E66</td>
<td>PIF-6*</td>
<td>ac46</td>
<td>All</td>
<td>N-terminal SM</td>
<td>Similarity to hyaluronidase, increased speed of kill See Chapter 3</td>
</tr>
</tbody>
</table>

**Non-envelope structural proteins specific to ODV.** This group of proteins has been shown to be associated with the nucleocapsid, and many of the proteins are highly conserved. Mutation of these genes results in either lack of virion formation, or non-infectious virions. The gp41 is the main tegument protein between the nucleocapsid and the envelope, but how gp41 interacts with the envelope is unclear. Some of these proteins have potential transmembrane signals.

**BV/ODV-E26 (ac16).** This protein has been identified in both the BV and ODV envelope of AcMNPV (Beniya et al., 1998). It contains an N-terminal sorting motif (SM)
that targets the protein to the INM (Braunagel et al., 2009). Homologs are present in all Group I alphabaculoviruses (Herniou et al., 2003), and ODV-E26 regulates transcription of ie-1 and ie-0 (Nie et al., 2009).

**ODV-EC43 (ac109).** ODV-EC43 is encoded by one of the core baculovirus genes (Herniou et al., 2003). Deletion mutants could not be isolated from BmNPV (Gomi et al., 1999). Analysis of the AcMNPV and HearNPV ODV both showed localization of ODV-EC43 to the ODV (Braunagel et al., 2003; Deng et al., 2007; Fang et al., 2003). Interestingly, some homologs contain transmembrane domains, while others do not (Slack & Arif, 2007).

**GP41 (ac80).** GP41 is the tegument protein of the ODV and is found between the nucleocapsid and the ODV envelope. This protein exhibits O-linked glycosylation, and is implicated in helping nucleocapsids exit the nucleus to form BV (Olszewski & Miller, 1997; Whitford & Faulkner, 1992). GP41 is conserved across all baculoviruses (Herniou et al., 2003). The hymenopteran species *Neodiprion sertifer* NPV and *Nediprion lecontei* NPV are limited to infection of the midgut cells, and no BV have been observed (Garcia-Maruniak et al., 2004; Lauzon et al., 2004).

**VP91.** VP91 is a capsid-associated protein found in both BV and ODV (Russell et al., 1991). VP91 is also conserved across all baculoviruses (Herniou et al., 2003). All homologs of VP91 exhibit a strong N-terminal hydrophobic domain similar to ODV envelope proteins, and localize to the same region in the nucleus, the ring zone (Russell & Rohrmann, 1997; Slack & Arif, 2007).

**ODV envelope structural proteins.** Currently four proteins are referred to as structural proteins of the ODV envelope. The term “structural protein” indicates that the protein is found in the structure of interest, in this case the ODV. However, this should not be extrapolated to infer that it is essential to maintaining the structure of the virion. Currently, ODV-E18, ODV-E25, ODV-E56, and ODV-E66 are classified simply as structural proteins, and by default the remaining proteins localized on the ODV envelope, P74, Pif-1, Pif-2, Pif-3, Pif-4, Ac150, HA-100, HA-44, and enhancin are also structural proteins (Rohrmann, 2008; Slack & Arif, 2007).
The functions of the following ODV envelope proteins are unclear:

**ODV-E18 (ac143).** ODV-E18 is thought to be a true structural protein, that is, essential for the proper assembly of ODV. Although the predicted molecular weight of the *orf* *ac143* is 9.5 kDa, polyclonal antibody identified an 18 kDa protein in ODV envelope preparations separated by SDS-PAGE (Braunagel *et al*., 1996b), hence the name ODV-E18. The protein does not appear to be post-translationally modified, but exists as a dimer found on the ODV envelope and virion associated INM. ODV-E18 contains an N-terminal sorting motif for trafficking to the INM (Hong *et al*., 1997). Attempts to make polyhedra-positive deletion mutants of ODV-E18 have been unsuccessful in both *BmNPV* and *AcMNPV* (Gomi *et al*., 1999; Harrison, personal communication). Interestingly, deletion of *odv-e18* prevented BV production (McCarthy & Theilmann, 2008). Although originally not identified as a core baculovirus gene across all taxa of baculovirus, a recent re-examination of the genomes found that *odv-e18* is a core gene (McCarthy & Theilmann, 2008). The single dipteran baculovirus contains a highly divergent homolog of *odv-e18*. Within the five alphabaculovirus Group I genomes assessed for orfs undergoing diversifying selection, *odv-e18* showed significant rates of non-synonymous change compared to the rate of synonymous change (Harrison & Bonning, 2004). This was true even between the highly similar RoMNPV and AcMNPV genomes. Since deletion of this *orf* is lethal for the formation of BV, ODV, and polyhedra, novel methods will be needed to study the exact nature of this protein, as well as, compensating for its role in ODV-E35 (see below).

**ODV-E25 (ac94).** The ORF *odv-e25* is a core lepidopteran gene, and deletion of this gene appears lethal (Gomi *et al*., 1999; Herniou *et al*., 2003). The protein has been studied in three different species of baculovirus (*Orygia pseudotsugata* MNPV, *AcMNPV*, and *Helicoverpa armigera* NPV), and referred to as 25K or P25 before the standard nomenclature and name that is known today, ODV-E25 (Braunagel *et al*., 2003; Deng *et al*., 2007; Russell & Rohrmann, 1993). ODV-E25 contains the conserved sorting motif composed of a highly hydrophobic region followed by a few charged amino acids at its N-terminus that is sufficient to target the protein to the INM (Hong *et al*., 1997; Saksena *et
The ODV-E25 protein is uncleaved in the ODV envelope. ODV-E25 is also expressed on BV envelopes, but at much lower levels (Russell et al., 1991).

**ODV-E35.** ODV-E35 is a protein of 35 kDa detected immunologically in AcMNPV infected cells. This protein is thought to be a heterodimer of ODV-E18 and ODV-EC27, as it cross reacted with antibodies specific to both ODV-E18 and ODV-EC27 (Braunagel et al., 1996b). Examination of the AcMNPV genome showed that ODV-E18 and ODV-EC27 are bicistronic, that is, translated from a single mRNA. These two orfs are in close proximity in the alphabaculoviruses and the gammabaculoviruses but not in the betabaculoviruses (Slack & Arif, 2007). The ODV-EC27 of the betabaculoviruses has an N-terminal signal, which is not the case for homologs in other baculoviruses. The ODV-E35 heterodimer could be formed through alternative splicing or frameshifting, both of which have been observed in baculoviruses (Braunagel et al., 1996b). The authors suggest ODV-E35 may stabilize the ODV envelope interaction with the nucleocapsids.

**The per os infectivity factors (PIFs).** Deletion of several ODV proteins results in a profound effect on the oral infectivity of the virus. These ODV proteins are called per os infectivity factors (PIFs) (Kikhno et al., 2002) (Table 1). As indicated by the name, these proteins are essential for oral infectivity, but have no effect on the infectivity of the BV. Single deletion mutants of the genes encoding these proteins result in loss of oral infectivity of the OB. The term PIF has been clearly attached to 5 proteins; the proteins PIF-1 through PIF-4 have been named solely for this characteristic, while the P74 protein has retroactively been designated as PIF-0 or just PIF (Fang et al., 2009a; Kikhno et al., 2002; Ohkawa et al., 2005; Pijlman et al., 2003; Slack & Arif, 2007; Slack et al., 2001; Yao et al., 2004). Unfortunately, the definition of a “PIF” is ambiguous, which has resulted in two proteins being referred to as PIF-4, and several others listed as potential PIFs (Lapointe et al., 2004; Slack & Arif, 2007; Zhang et al., 2005). In addition to the loss of OB infectivity, PIFs P74, PIF-1, and PIF-2 all show loss of binding and fusion to the midgut in addition to loss of oral infectivity in deletion mutants (Haas-Staplethon et al., 2004; Ohkawa et al., 2005). PIF-3 showed loss of oral activity, but no effect on binding (Ohkawa et al., 2005). Currently, both Ac150 and Ac96 have been referred to as PIF-4 in
the literature, leading to confusion (Fang et al., 2009b; Slack & Arif, 2007; Zhang et al., 2005). Furthermore, both of these proteins have properties unlike the previously described PIFs; Ac150 shows a loss of oral infectivity only when fed as OB, but not as ODV, and both Ac150 and Ac96 are found in both ODV and BV (Fang et al., 2009b; Lapointe et al., 2004; Zhang et al., 2005). Mutants lacking functional Ac150 showed a significant decrease in oral infectivity of OB when fed to *H. virescens*, *T. ni*, and *S. exigua*, and in the discussion, this protein is mentioned as being potential PIF-4, but was not named so in the paper. The loss in oral infectivity of the OB was on the order of a 5-fold decrease, compared to decreases of $10^5$ for the other defined PIFs. For purposes of understanding wild type virus transmission, Ac150 is better classified as an enhancer of oral infection, not an essential PIF. Likewise, deletion mutants of the 11K protein, Ac145, also exhibited significant less *per os* infectivity (Lapointe et al., 2004). The effect was even stronger in double deletions of Ac145 and Ac150. As with Ac150, Ac145 is observed in both the BV and the ODV; however, *ac145* is conserved across the alpha and betabaculoviruses (Herniou et al., 2003; Lapointe et al., 2004). We propose that both Ac145 and Ac150 be grouped with Enhancin, and not be referred to as a PIF.

**P74-PIF-0 (ac138) – the first protein to be described as a PIF.** Of all the ODV envelope proteins, the greatest amount of work has been done with P74 in various baculovirus species including *AcMNPV*, *BmMNPV*, and *Chositoneura fumiferana MNPV*, *HearNPV*, *Culex nigripalpus iNPV* (Deng et al., 2007; Faulkner et al., 1997; Haas-Stapleton et al., 2004; Kuzio et al., 1989; Perera et al., 2007; Rashidan et al., 2005; Yao et al., 2004; Zhou et al., 2005). It was the first baculovirus protein shown to be a virulence factor and localized to the occlusion bodies as shown through production of non-infectious polyhedra when P74 was deleted (Kuzio et al., 1989). Polyhedra and ODV formation appeared indistinguishable by electron microscopy, but the polyhedra were not infectious to their hosts. P74 was shown to be a part of the ODV in 1997 (Faulkner et al., 1997). This localization was confirmed with the *AcMNPV* ODV composition study in 2003 (Braunagel et al., 2003). More recent analyses of the ODV composition of *HearNPV* and *CuniNPV* confirmed the presence of these homologs in the ODV envelope (Deng et al., 2007; Perera et al., 2007). Computational analyses including genomes from all four
genera showed that *p74* is a core baculovirus gene (Herniou *et al.*, 2003). Among five Group I alphabaculoviruses with very different host ranges, *p74* is under strong purifying selection, as indicated by the ratio of the rate of non-synonymous to synonymous change (Harrison & Bonning, 2004). A recent study found that successful oral infection required the presence of all the conserved PIFs of the ODV - P74, PIF-1, PIF-2, and PIF-3 (Song *et al.*, 2008).

There is evidence for proteolytic cleavage of ODV envelope protein P74 when exposed to brush border membrane vesicles (BBMV) derived from midgut epithelium (Slack & Lawrence, 2005; Slack *et al.*, 2008). The authors speculate that the alkaline environment of the lepidopteran midgut primes P74 for this event. Tryptic activation of the viral envelope fusion protein is a strategy utilized by a wide array of viruses including Vaccina virus, Sendai virus, and influenza (Ichihashi & Oie, 1992; Itoh *et al.*, 1987; Steinhauer, 1999). P74 has 2 transmembrane domains at the C terminus, while the N terminus is extracellular. Cleavage of the transmembrane domains results in a soluble protein, whereas the observed cleavage occurs on the N-terminus, and can be inhibited (Slack *et al.*, 2008). Thus, it is believed that P74 directly inserts itself into the envelope membrane via the two C-terminal transmembrane domain motifs. Mixtures of *p74* deletion mutants expressing eGFP and wild type polyhedra resulted in infected cells expressing eGFP indicating that P74 does not need to be physically attached to the virion to enable infection (Haas-Stapleton *et al.*, 2004). This result was further supported by demonstration that co-feeding purified P74 and a P74 null mutant to larvae will rescue the loss of infectivity of the knock-out virus (Zhou *et al.*, 2005). ELISA assays showed that P74 binds to host BBMV in a saturable and specific manner, and pull down assays using purified polyhistidine tagged P74 were used to isolate a 35 kDa protein found in the susceptible host *Spodoptera exigua*, but not from the resistant host *Helicoverpa armigera*. The 35 kDa protein was not sequenced, and hence its identity is unknown.

**PIF-1 (ac119).** The properties of PIF-1 were discovered through routine *per os* screening of deletion mutants (Kikhno *et al.*, 2002). The loss of oral infectivity prompted the creation of the term PIF. After the discovery of another PIF, this gene was designated PIF-1. This protein is ~60 kDa in *SpliNPV*, and contains the N-terminal SM (Slack & Arif,
The deletion mutants show no differences in polyhedra formation or ODV, but do result in lack of binding to the midgut epithelia (Kikhno et al., 2002; Ohkawa et al., 2005). PIF-1 has clearly been shown to be present on the ODV envelope, and is conserved across all baculoviruses (Herniou et al., 2003; Song et al., 2008).

**PIF-2 (ac22).** PIF-2 was discovered in the same manner as PIF-1 (Pijlman et al., 2002). The protein is ~44 kDa in SpexMNPV, and also contains an N-terminal SM (Slack & Arif, 2007). Deletion mutants lacked binding activity (Ohkawa et al., 2005). PIF-2 was detected in proteomic analyses of the ODV envelope, as well as by immunoblot (Braunagel et al., 2003; Song et al., 2008). As with PIF-1, PIF-2 is conserved across all baculoviruses (Herniou et al., 2003). Interestingly, in competition studies with wild type virus, PIF-1 deletion mutants, and PIF-2 deletion mutants, a mixture of wild type and deletion mutants resulted in increased potency (Clavijo et al., 2009). Proposed hypotheses to explain this observation include that higher levels of PIFs may cause fusion of ODVs leading to the multiple nucleocapsids per virion and few foci in infected insects, or that higher levels of PIFs may saturate the receptors impeding the efficiency of infection. Only the alphabaculoviruses exhibit the M phenotype, however.

**PIF-3 (ac115).** PIF-3 was also identified through a screening of deletion mutants for oral infectivity (Ohkawa et al., 2005). This protein is ~23 kDa in AcMNPV and contains an N-terminal transmembrane domain (Ohkawa et al., 2005; Slack & Arif, 2007). The deletion mutants exhibit decreased oral infectivity, but show no decrease in binding (Ohkawa et al., 2005). PIF-3 is conserved across the Baculoviridae, confirmed to be in the ODV envelope by immunoblotting, but was not isolated in proteomic analyses (Braunagel et al., 2003; Herniou et al., 2003; Song et al., 2008). The role of PIF-3 in oral infection appears to be downstream of the initial binding and fusion of the membranes.

**PIF-4 (ac96).** Computational analysis of the conserved baculovirus genes identified ac96 as a late gene conserved across all baculoviruses (Herniou et al., 2003). Deletion of this gene revealed that this was another PIF that results in lack of oral infectivity of the polyhedra, with no impact on BV infectivity (Fang et al., 2009a). PIF-4 contains an N-terminal sorting motif, and was observed at the predicted molecular weight of 19 kDa (Fang et al., 2009a). This protein was detected in the proteomic analysis of
CuniNPV but not AcMNPV, but localization to the ODV envelope was confirmed by immunoblot (Braunagel et al., 2003; Fang et al., 2009a; Perera et al., 2007). Intriguingly, PIF-4 localizes to both ODV and BV envelope fractions. The *pif-4* deletion mutant contained a small portion of the N-terminus of the orf, and this may be sufficient to maintain infectivity of the BV.

**Auxiliary proteins.**

*Enhancin.* Enhancins are metalloproteases found in various alpha and betabaculoviruses (Lepore et al., 1996). These enzymes degrade the proteins of the peritrophic membrane, enhancing the virion’s ability to infect the midgut. The enhancin of *LdMNPV* was found to associate with the ODV envelope, and a study of *TnGV* found enhancin at levels up to five percent of the mass of occlusion bodies (Hashimoto et al., 1991; Slavicek & Popham, 2005). Like the 11 K proteins, *enhancin* may be present in multiple copies (Hayakawa et al., 1999; Popham et al., 2001). *LdMNPV* contains two copies, both of which have an effect on infectivity when deleted; single deletions showed two to three fold decreases, and the double deletion decreased infectivity by a factor of 12 (Popham et al., 2001).

*Ac145 (11K protein).* Ac145 and Ac150 were discovered based upon a computational analysis that identified homologous proteins present across invertebrate viruses (Dall et al., 2001; Lapointe et al., 2004). Members of one of the groups identified were named the “11K genes”, as their predicted molecular weight was 11 kDa. Homologs were present in baculoviruses and entomopoxviruses. They too contain an N-terminal hydrophobic domain, and a core C6 motif (peritrophin A domain), which frequently interacts with chitin, and is found in ecdysis related proteins (Aguinaldo et al., 1997). The presence of Ac145 is conserved only in the alpha and betabaculoviruses, and the number of copies of the gene varies dramatically; the betabaculoviruses contain many more copies (Lapointe et al., 2004). Ac145 was detected in both BV and ODV, and polyhedra and ODV production were not affected by *ac145* deletion. Neonate *H. virescens* showed an increased LC$_{50}$ for double deletion mutants of Ac145 and Ac150, but not for single deletion mutants. Deletion mutants of *AcMNPV*
ac145 resulted in a small, but significant, increase in lethal dose for neonate T. ni, but a double deletion of Ac145 and Ac150 was not significantly different from wild type (Lapointe et al., 2004). The variation in type and number of the 11K proteins suggests that the 11K proteins may enhance infection of different hosts.

**Ac150 (11K protein).** Ac150 was first described as an 11 K protein, which contained an N-terminal SM in addition to a peritrophin C domain, present in both BV and ODV (Lapointe et al., 2004). Ac150 acted synergistically with Ac145 to enhance infection, but deletion of Ac150 alone had no effect (Lapointe et al., 2004). An Ac150 deletion mutant showed lack of oral infectivity for the polyhedra, but ODV were as infectious as wild type virus (Zhang et al., 2005). Ac150 was not detected in the ODV of AcMNPV (Braunagel et al., 2003). This orf is not conserved, even among the alphabaculoviruses (Herniou et al., 2003). As a result, Ac150 was not explicitly named PIF-4, although it was still described as a PIF in the discussion. It is this orf that is referred to as PIF-4 in a recent baculovirus ODV review (Slack & Arif, 2007). Ac150 did not interact with chitin or the PM, and its enhancing effects on the polyhedra are unknown (Lapointe et al., 2004; Zhang et al., 2005). In a natural infection the insects would ingest polyhedra, not liberated ODV, and would be exposed to the enhancing effects observed with ingestion of the polyhedra. A recent study of BmNPV field isolates found multiple subtypes of the Ac150 homolog, all of which had the N-terminal and C-terminal domains, but varied with respect to the presence of an interior RGD motif (Hao et al., 2009). Deletion mutants of two of the Ac150 subtypes exhibited an increase in survival time, but not a decrease in oral infectivity in agreement with the first study (Hao et al., 2009). Subtypes with the RGD motif produced higher numbers of polyhedra both in vitro and in vivo.

**Ha44, Ha100 and other virus specific genes encoding ODV envelope proteins.** Proteomic analysis of the HearNPV ODV envelope identified two novel proteins, HA44 and HA100 (Deng et al., 2007). No bioassay data have been presented to date for viruses lacking the genes encoding these proteins. The proteomic analysis of CuniNPV ODV identified 23 novel proteins specific to that species, although they may also be encoded by other deltaviruses. The number of species-specific genes and/or copy number of these
genes is likely to increase as more genomes are sequenced and proteins analyzed via proteomic analysis. The effects of these individual proteins may be minor, but in concert may contribute to the different host ranges of these viruses.

**ODV-E56 and ODV-E66, the focus of this dissertation.**

**ODV-E56 (ac148).** The *odv-e56* orf is a core lepidopteran gene (Herniou *et al.*, 2003). It was first identified as a partial orf next to the orf encoding IE-1 in OpMNPV, with a homolog found in the completely sequenced AcMNPV genome (Guarino & Summers, 1987; Theilmann & Stewart, 1991). The entire gene was sequenced from OpMNPV, and named *odvp-6e*, as it was the sixth ODV protein to be described that was present in the ODV envelope (Theilmann *et al.*, 1996). The protein was concurrently identified as ODV-E56 in AcMNPV, based on molecular weight and the transition to a more informative protein name (Braunagel *et al.*, 1996a). ODV-E56 transcription initiates from a late gene promoter motif TTAAG at 18 hours post infection (hpi) coincident with viral DNA replication (Theilmann *et al.*, 1996). Expression of ODV-E56 increased to steady state levels by 48 hpi, and then decreased, but was still detectable at 120 hpi. Two mRNA transcripts were observed by northern blot: a 1.2 kB and a 5.0 kB transcript; the 1.2 kB transcript was associated with the TTAAG consensus site.

ODV-E56 has a predicted size of 40.24 kDa in OpMNPV, and size of 40.8 kDa in AcMNPV (Braunagel *et al.*, 1996a; Theilmann *et al.*, 1996). In all baculoviruses in which ODV-E56 has been examined, however, it has a larger size on SDS-PAGE, indicative of extensive post-translational modifications. In OpMNPV, a 40 kDa protein was observed at 48 hpi in infected Sf9 cells, but a 54.3 kDa was the strongest band detected by western blot of purified ODV (Theilmann *et al.*, 1996). The antibody to OpMNPV ODV-E56 (produced from Ld652Y cells) was found to cross react to AcMNPV ODV-E56 in infected Sf9 cells; OpMNPV and AcMNPV share 69.8% identity and 82.5% similarity. In AcMNPV, 67 kDa and 56kDa bands were the predominant forms (Braunagel *et al.*, 1996a). The AcMNPV ODV-E56 was unresponsive to tunicamycin and N-glycosidase, suggesting the protein was not N-glycosylated at the four predicted sites. ODV-E56 is distinct from most of the other ODV envelope proteins in that it does not contain an N-terminal sorting
motif for trafficking to the INM. Instead, it has a C-terminal hydrophobic domain that functions in this capacity (Braunagel et al., 1996a). It is very similar to P74 in this respect, as well as, the presence of potential membrane insertion signal peptide motif in the interior region of the protein (Slack & Arif, 2007). The multiple forms observed may be due to trypsin cleavage sites, as well as, post-translational modification (Theilmann et al., 1996).

Computational analysis indicated that odv-e56 is undergoing positive selection across five species of Group I alphabaculoviruses (Harrison & Bonning, 2004), while the other PIFs are undergoing strong purifying selection. As these viruses exhibit very different host ranges, and this gene is conserved across all baculoviruses, odv-e56 was a good candidate for study of genes involved in baculovirus oral infectivity and host specificity. We recently showed that deletion mutants were not infectious per os to neonate larvae, and that swapping AcMNPV and RoMNPV ODV-E56 did not expand host range in several lepidopteran species that show significant differences in susceptibility to these two viruses (Harrison et al., 2010). ODV-E56 is the subject of Chapter 2 of this dissertation.

**ODV-E66 (ac46).** ODV-E66 was first described as a ~66 kDa protein from AcMNPV named PDV-E66 (Hong et al., 1994). ODV-E66 is conserved only among the lepidopteran baculoviruses (Herniou et al., 2003). It was shown to be an exclusive ODV envelope protein, transcribed from two late motif TAAG initiation sites, with transcripts observed at 12-72 hpi, and protein at 24-72 (Hong et al., 1994). A hydrophobic domain is present at the N terminus, as found in ODV-E25, which was sufficient to target reporter proteins to the INM (Hong et al., 1997; Hong et al., 2004). ODV-E66 was found to be uncleaved in the ODV envelope, not N-glycosylated, and capable of self insertion into microsomal membranes (Hong et al., 1997). Several transmembrane domains are predicted in the central domains of ODV-E66, but are not conserved (Slack & Arif, 2007). The orientation of ODV-E66 is unclear, one study suggests that the C-terminus is exposed on the ODV surface, but others show that ODV-E66 interacts with the major viral capsid protein, VP39, and ODV-E25, which remains associated with the nucleocapsid after treatment with detergent (Braunagel et al., 1999; Braunagel et al., 2004). There is also evidence for trypsin cleavage, in the form of a 60 kDa protein, which could result in a
conformational change of ODV-E66 (Hong et al., 1994). A recent study of the enzyme hyaluronanidase encoded by the Jaagsiekte sheep retrovirus (JSRV) using a baculovirus expression system, showed that ODV-E66 has hyaluronidase activity (Vigdorovich et al., 2007). This enzyme digests hyaluronan, an extracellular polysaccharide that provides structure for the cells, and has been identified in bee venom and human sperm (Arming et al., 1997; Markovic-Housley et al., 2000). The hyaluronidase in bee venom is a major allergen (Markovic-Housley et al., 2000). JSRV targets the lung epithelia of sheep, and utilizes glycosylphosphatidylinositol-anchored hyaluronanidase on the cell surface as the virus receptor. This lung hyaluronidase shows little enzymatic activity, and the viral envelope proteins bind to a region distinct from the site of enzymatic activity (Vigdorovich et al., 2007). Hyaluronan is a major component of the PM, and hyaluronidase activity of ODV-E66 may actively degrade the PM, enhancing oral infection (Lehane, 1997). Since the ODV-E66 protein exhibits hyaluronidase activity, it is unlikely that the virus utilizes hyaluronan on the cell surface as a virus receptor.

Unidentified proteins of the ODV. A thorough investigation of the ODV envelope composition of the two lepidopteran baculoviruses and the dipteran baculovirus has been completed (Braunagel et al., 2003; Deng et al., 2007; Perera et al., 2007). Each study identified the majority of the proteins described above, several DNA binding proteins thought to be packaged due to close proximity, but having no real function in ODV infectivity, and 14, 2, and 23 virus specific proteins in AcMNPV, HearNPV, and CuniNPV, respectively. In each of these studies, the ODV were derived from infected insects. Multiple orfs of unknown function exist in all three viruses. Three proteins were not identifiable by their sequence in the HearNPV study. Polyclonal antibody developed against ODV did not show cross-reactivity with lysates from host cells suggesting there is minimal host protein present in the ODV (Braunagel et al., 2003). Both AcMNPV and HearNPV ODV exhibit two protein bands around 20 and 25 kDa which did not have any BLAST matches for sequence, and could represent host proteins. Vaccinia virus, of the Poxviridae, contains 23 host proteins in addition to 75 viral proteins (Chung et al., 2006).
Receptor, binding, and fusion

Very little is known about the baculovirus ODV receptor(s). ODV are poorly infectious to existing insect cell lines derived from non gut tissues (Volkman, 1976). The midgut lines that have been developed, have been difficult to maintain, with the exception of a cell line from *T. ni* midgut tissue (Granados, 1986). Specific binding of ODV to a protein receptor in the midgut through binding and fusion was shown in 1993 (Horton & Burand, 1993). The reaction was saturable and competitive, suggesting that a specific receptor(s) was involved. Further, protease treatment of the midgut resulted in a significant decrease in binding, as did treatment by alpha-deglycosidase. The treatments of midguts with neuraminidase, phospholipase, and beta-deglycosidase had no effect on ODV binding. Although binding was higher at an acidic pH, fusion was not, indicating that the mechanism of ODV entry into the cell was not adsorptive endocytosis. P74 has been shown to interact with a 35 kDa gut protein, but the identity of that protein is unknown (Zhou *et al.*, 2005). Potential ODV receptors may include the alkaline phosphatases, aminopeptidases, and cadherins, which are present at high levels in the midgut, and are bound by the *Bacillus thuriengiensis* toxins (Jurat-Fuentes *et al.*, 2003; Zhang *et al.*, 2008). Evidence suggests that different viruses use different receptors in different host insects; in competition assays, in *S. frugiperda*, *SfMNPV* bound a different receptor than the *AcMNPV* ODV (Haas-Stapleton *et al.*, 2005).

Thesis organization:

This thesis is written in manuscript format. The second chapter is focused on the role of the ODV-E56 protein in initial infection of the lepidopteran host, the tobacco budworm, *Heliothis virescens* (Fabricius). The third chapter addresses the binding of peptides identified through a phage bio-panning technique; these peptides exhibit similarity to ODV-E66 and had significant effects on baculovirus infection. Finally, the last chapter of this thesis contains the overall conclusions from this work and discusses areas for potential future investigation.
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Chapter 2:

**ODV-E56 is per os infectivity factor-5, but is not essential for baculovirus binding and fusion**

W. O. Sparks¹*, R.J. Harrison²#, and B.C. Bonning¹

¹ Genetics Program, Department of Entomology, Iowa State University, Ames, IA 50011, USA
² Invasive Insect Behavior and Biocontrol Laboratory, Agriculture Research Service, United States Department of Agriculture, Beltsville, MD 20705

**ABSTRACT**

**Background:** Baculovirus occlusion-derived virus (ODV) infects the insect host via the midgut epithelium. Although the mechanism of viral fusion is unknown, a family of *per os* infectivity factors (PIFs) has been shown to function in initial infection. We previously reported that the ODV envelope protein ODV-E56 was essential for oral infection of neonate *Heliothis virescens* (Harrison et al, 2010, J. Gen. Virol., in press).

**Results:** Here, we present a more comprehensive study of ODV-E56 in fourth instar *H. virescens*. Bioassays were conducted with the recombinant clone AcIE1GFP-e56LacZ(+), with *odv-e56* disrupted through insertion of a *lacZ* cassette. AcIE1GFP-e56LacZ(+) was five logs less infectious than the control *odv-e56*-positive virus following ingestion of polyhedra, and 200-fold less infectious following infection with ODV. In contrast, budded virus, which serves to disseminate infection within the host insect, was equally infectious for ODV-E56 negative and positive viruses following injection of larvae. Co-feeding larvae with purified ODV-E56 and AcIE1GFP-e56lacZ(+) did not restore oral infectivity. Binding and fusion assays showed that AcIE1GFP-e56LacZ(+) bound and fused at levels similar to wild type virus at supersaturating doses of ODV, compared to virus lacking P74-PIF-0 (ac138) which exhibited minimal levels of binding and fusion. Only insects fed polyhedra of virus constructs containing ODV-E56 and dissected 24 hours post inoculation exhibited strong

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* Primary author of the work presented in this chapter
# Generated the deletion mutants used in this study and described in Harrison et al., J. Gen. Virol., 2010, in press
eGFP foci. No fluorescence was seen in larvae infected with virus constructs lacking ODV-E56. This result suggests that although viruses that do not express ODV-E56 fuse and enter the cell, the viral DNA does not undergo transcription and translation. Purified ODV-E56 bound to several proteins in midgut-derived brush border membrane vesicles from fourth instar *H. virescens*. Although the function of ODV-E56 remains to be clearly defined, these results indicate that ODV-E56 is the sixth *per os* infectivity factor (PIF) protein to be described, PIF-5.

**BACKGROUND**

Baculoviruses are large dsDNA viruses, which have been isolated from the insect orders Lepidoptera, Diptera, and Hymenoptera (Jehle, 2006). Baculoviruses have proven extremely useful as protein expression vectors in tissue culture, potential vectors for gene therapy, and have been used as natural insecticides to successfully stem pest outbreaks. The virus infects insects through a bi-phasic life cycle that produces two distinct forms of virions for within- and between-host viral transmission. Infection begins when the insect ingests occlusion-derived virus (ODV) packaged in an environmentally stable crystal (polyhedron or granule) composed of the viral protein polyhedrin or granulin, known as occlusion bodies (OBs). The OBs dissolve in the basic pH of the insect gut, releasing the ODV, which then bind and fuse to the brush border microvilli of the midgut epithelium columnar cells (Horton and Burand, 1993). The genus *Alphabaculovirus* includes the lepidopteran-specific nucleopolyhedroviruses (NPV), which package multiple nucleocapsids (NC) into a single ODV, a unique characteristic of baculoviruses. The nucleocapsids are transmitted to the nucleus where both viral replication and assembly occur. The cells first produce budded virus (BV), which buds out from the cell cytoplasm, and infects neighboring cells via endocytosis. ODV are produced in the nucleus late in baculovirus infection and are occluded within the polyhedrin matrix, which is further enveloped. Eventually the host insect dies, infected cells lyse, and polyhedra are released back into the environment.

Although the BV phenotype has been well characterized, little is known about the ODV beyond characterization of individual ODV proteins. Over 44 proteins have been identified in ODV, several of which are unique to the ODV envelope, using membrane
fractionation and library screening of mutants (Braunagel et al., 1996; Deng et al., 2007; Fang et al., 2009; Perera et al., 2007; Slack and Arif, 2007). ODV enter the cell via binding and fusion (Horton and Burand, 1993). Most described baculoviruses infect only a few host species, and the ability of the ODV to bind and fuse to the gut epithelia is a large component of virus specificity and host susceptibility. A core set of 30 genes found in all completely sequenced baculovirus genomes to date has been characterized (Jehle et al., 2006; McCarthy and Theilmann, 2008). An additional 22 genes are conserved among the lepidopteran baculoviruses of *Alphabaculovirus* and *Betabaculovirus*; 19 of which have known functions (Fang et al., 2009; van Oers and Vlak, 2007). Among all 52 lepidopteran core genes, 14 are ODV envelope proteins or found within the ODV (excluding polyhedrin), while 9 of the 30 core baculovirus genes encode proteins that are present in the ODV. Notably, a class of proteins has been described, the *per os* infectivity factors (PIFs), which include five ODV envelope proteins that play a role in oral infectivity: P74-PIF-0 (*ac138*), PIF-1 (*ac119*), PIF-2 (*ac22*), PIF-3 (*ac115*), and PIF-4 (*ac96*) (Fang et al., 2009; Haas-Stapleton et al., 2004; Hao et al., 2009; Kikhno et al., 2002; Kuzio et al., 1989; Ohkawa et al., 2005; Pijlman et al, 2003; Yao et al., 2004). The first four PIFs are conserved across the Baculoviridae, and PIF-4 is conserved across the lepidopteran baculoviruses. Deletion of these proteins results in a profound decrease in the oral infectivity of the OB. The impact of deletion of each of these five PIF proteins varies and potential interactions between the proteins have not been determined. P74-PIF-0 is the best characterized of the PIF proteins (Faulkner et al., 1997; Haas-Stapleton et al., 2004; Slack and Arif, 2007; Yao et al., 2004; Zhou et al., 2005).

We previously conducted a computational analysis of five Group I alphabaculoviruses for genes that were undergoing positive selection (Harrison and Bonning, 2004). Two ODV envelope proteins, ODV-E56 and ODV-E18, were identified as undergoing positive selection, whereas, the PIFs P74-PIF-0, PIF-1, PIF-2, PIF-3, and PIF-4 exhibited purifying selection. Genes exhibiting positive, or diversifying, selection have greater amino acid sequence variation, which may lead to different functions beneficial to the virus, ultimately leading to fixation of these changes in the population. No bioassay data for ODV-E56 deletion mutants were available, thus we were interested in determining the role of ODV-E56 in oral infection. We constructed four deletion mutant viruses in which *odv-e56*
was disrupted via insertion of a lacZ cassette, and the deletions did not impact polyhedra formation (Harrison et al., 2010). Initial bioassays of oral infectivity in neonates of the highly susceptible lepidopteran *Heliothis virescens* revealed a profound loss of oral infectivity for AcIEGFP-e56lacZ(+). Here we present an in depth examination of the oral infectivity of *odv-e56* deletion virus through bioassays of polyhedra, ODV, and BV in fourth instar *H. virescens*. Binding and fusion of virions lacking ODV-E56 was examined using oral inoculation of R-18 labeled ODV. Orally infected larvae were dissected 24 hpi to determine whether translation of viral genes occurred in infected cells as indicated by GFP expression in the midgut. On the basis that P74-PIF-0 rescued oral infectivity when co-fed with *p74-pif-0* deletion mutant virus (Yao et al., 2004), we produced recombinant, N-terminal polyhistidine-tagged ODV-E56. This protein was assessed for its ability to rescue the infectivity of AcIEGFP-e56lacZ(+), and for binding to purified BBMV from fourth instar *H. virescens*. Our results indicate that ODV-E56 is PIF-5.

**MATERIALS AND METHODS**

*Virus construction, amplification, and purification.* The construction of the ODV-E56 deletion mutants has been described in Harrison et al., 2010 (Fig. 1). Briefly, transfer vectors in which *odv-e56* was disrupted with a LacZ cassette in each orientation were created in a plasmid containing the 7.1kB *Clai*-F fragment of AcMNPV-C6. The (+) designated virus contains the cassette in the reverse orientation relative to the orf, while the (−) construct is in the same orientation as the orf. This plasmid was then recombined with either the recombinant virus AcIE1TV3.EGFP which contains eGFP under control of the immediate early −1 (ie-1) gene promoter, or AcMLF9.EGFP, which contains eGFP under control of the late p6.9 gene promoter, via co-transfection of Sf-9 cells with viral DNA and the transfer vectors using Cellfectin (Invitrogen). The *ie-1* promoter is turned on immediately in the infected cell, albeit at low levels, and continues to be expressed throughout infection (Huijskens et al., 2004). The *p6.9* promoter, on the other hand, is a strong viral late promoter that is active beginning at 18 hours post infection (hpi), and peaking at 24 hpi (Todd et al., 1996). Several rounds of plaque purification were carried out to isolate LacZ-positive and polyhedra-positive plaques. To ensure that any phenotypic differences were due only to the
inactivation of odv-e56, revertant viruses were created using transfer vectors with odv-e56 in its native location, and selecting for LacZ-negative/polyhedra-positive plaques. In theory, a short peptide of the first part of the odv-e56 orf could be transcribed and translated in the disruption mutants, but this potential protein was not detected by western blots of infected Sf9 cells using anti-E56 antibody that recognizes the N-terminus (Harrison et al., 2010). The virus AcIEGFP-p74-lacZ(+) was created in the same manner using an initial plasmid containing the HindIII-Q fragment of AcMNPV-C6. The hsp70-lacZ expression cassette was inserted into the Sac II site of the p74 ORF, and the resulting transfer vector was co-transfected with AcIE1TV3.EGFP to make a virus with an inactivated p74 gene. The structure of all viruses was confirmed by restriction endonuclease digests, and virus constructs are shown in Figure 1.

**BV preparation.** BV were amplified using Sf21 cell culture. Cells (1 X 10^6) were incubated with virus constructs, and cells harvested at 48 hpi. Cells were pelleted at 2000 rpm for 10 minutes and the supernatant containing the BV transferred to a new tube. BV was quantified by plaque assay using standard methods (King and Possee, 1992).

**Polyhedra preparation.** Each of the recombinant viruses was amplified in H. virescens to produce polyhedral stocks. BV was injected into fifth instars via the proleg, and polyhedra isolated from the cadavers using standard methods and stored at 4°C (O’Reilly et al., 1992). Polyhedra were subsequently purified by centrifugation through a 25% w/w sucrose in PBS cushion, and then further purified in 45-65% w/w sucrose in PBS linear sucrose gradients created using Gradient Master (BioComp). The banded polyhedra were collected using a needle and syringe and washed in PBS. Purified polyhedra were resuspended in small volumes of sterile water with 0.1% sodium azide and quantified using a hemocytometer.

**ODV preparation.** ODV was purified using sucrose purified polyhedra solubilized with .1M NaCO₃ + .1M NaCl for 10 minutes on ice in amber microcentrifuge tubes to protect ODV from light. The alkaline solution was neutralized with 1 M Tris pH 7.3 then placed in an orbital shaker at room temperature at 150 rpm for one hour. Virus suspensions were briefly spun in a microcentrifuge at 2000 rpm for 5 minutes. The supernatants were immediately loaded onto 63%, 57%, 35%, 25% sucrose (w/v in TE buffer) step gradients in
Beckman Ultra Clear tubes. Samples were overlaid with mineral oil, balanced, and spun for 1 hour at 26100 rpm (90000g) in an SW28 rotor at 4°C. White ODV bands (observed at the interfaces between 57 and 63, and 35 and 57% interfaces) were harvested by puncturing the side of the tube with an 18-gauge needle with a glass syringe. Samples were diluted with water to twice the volume, and pelleted at 26100 rpm for 30 minutes. ODV pellets were resuspended in small volumes of PBS, stored in amber microfuge tubes at 4°C for no more than one week. ODV were quantified using the BioRad protein detection system.

**Insects and bioassays.** *Heliothis virescens* larvae (BioServe) were reared on artificial diet (Southland Products, Inc.) at 28°C with 12 hour day/night cycles. Fourth instar larvae were used for all experiments into the nature of ODV-E56. For amplification of OB using budded virus constructs, early fifth instar larvae were inoculated with BV via proleg injection. For OB bioassays and microscopy work, third instar larvae showing head capsule slippage were starved overnight, and then fed the respective dose of polyhedra on a small diet cube. After 24 hours, larvae that did not consume the entire cube were discarded, and diet was returned to those that remained. ODV bioassays were conducted by starving the larvae overnight and then droplet feeding virus to the larva in a total volume of one to two microliters. Only insects that consumed the entire dose of virus were returned to diet. For BV bioassays, fourth instars were injected via the proleg using a microapplicator (Burkhard) with a 32-gauge sharp needle affixed to a 1 mL syringe. Likewise, ODV were administered orally using a 32-gauge blunt needle inserted into the midgut for binding and fusion assays (Li et al., 2008; Sparks et al., 2008). Bioassays were performed in triplicate on sample sizes of 15-30 insects per dose, for at least five doses of virus. After infection, insects were inspected twice daily for death, and death by polyhedrosis confirmed by light microscopy. Data were analyzed using Polo Plus version 2.0 (LeOra software), and statistical significance determined by the LD ratio test (Robertson and Preisler, 1992).

**BBMV purification.** Midguts were collected from newly molted fourth instar *H. virescens* dissected in ice-cold MET buffer (300 mM mannitol, 2 mM EDTA, 17 mM Tris, pH 8.0). The midguts were rinsed free of the peritrophic membranes, placed in small aliquots of buffer, flash frozen using liquid nitrogen, and stored at -80°C. Brush border membrane vesicles were prepared in accordance to a modified method of Wolfsberger...
(1987). Briefly, midguts were quick thawed in a water bath, mixed with 9 times their wet weight of MET buffer, and homogenized using a Wheaton homogenizer for 9 strokes at speed 3. An equal volume of 24 mM MgCl₂ was added and mixed 3 times. A small sample of the crude homogenate was taken, and the remaining sample spun at 4500 rpm for 15 minutes in an SW28 Beckman rotor. The supernatant was transferred to a fresh tube and spun at 15000 rpm for 45-60 minutes. The supernatant was removed, and the pellet resuspended in equal volumes of MET and MgCl₂; and the high and low speed centrifugation repeated as above. The final supernatant was decanted, and the final BBMV pellet resuspended in 100-300 µL of 0.5X MET buffer in water, aliquoted, and flash frozen in liquid nitrogen. Samples of crude homogenate, cell pellet, and supernatant were assayed for protein concentration and leucine aminopeptidase activity. Protein concentrations were determined using BioRad Protein Detection system. For leucine aminopeptidase activity, 50 µL of 50 mM NaPO₄ buffer pH 7.2 and 2 µL sample were placed in a 96 well plate. At a ratio of 43.5 µL of 50 mM NaPO₄ buffer to 6.5 µL fresh 24 mM L-leucine p-nitroanilide (Sigma), 50 µL of this solution was added to the sample wells, and the plates immediately read at 412 nm for 5 minutes using a Vmax spectrophotometer (Molecular Devices). Readings were normalized to protein concentrations, and preparations exhibiting at least an eight-fold increase in aminopeptidase activity relative to the original crude homogenate were used for further assays.

**Octadecyl Rhodamine B-Chloride (R18) Labeling/Binding and Fusion studies.**

ODV were labeled with octadecyl rhodamine B chloride (R18) using a modified method of Ohkawa et al.(2005). ODV were prepared as described above, with the addition of 10 µL of (R18) being added to the ODV (from 1 X 10¹⁰ polyhedra) on sucrose gradients. After centrifugation, the labeled ODV appeared as a major magenta band in the gradient between the 35% and 57% steps and a minor band between 57% and 63%. The bands were collected and diluted 2X with water. The ODV were pelleted, and then resuspended in minimal amounts of PBS (100-200 µL). ODV were quantified using the BioRad protein assay, and the fluorescence units determined using a spectrophotometer with an excitation 556 nm and emission 583 nm. Only virus labeled in the range of 5 X 10⁵ to 2 X 10⁶ fluorescence units per microgram (FLU/µg) were used for further study (as determined by Ohkawa et al.,2005).
Newly molted fourth instars were orally inoculated using a 32-gauge blunt needle in doses ranging 2-6 μg of virus within 15 minutes of the molt. Insects were dissected 1 hpi in low light conditions in Separation Buffer (SB) (100 mM NaCO₃, 100 mM KCl, 100 mM EGTA, pH 9.5). Midguts were cut longitudinally and rinsed free of peritrophic matrix, then placed in 6-well dissection dishes containing 200 μL of SB on ice for 15 minutes. After this incubation the midgut epithelia was separated from the basement membrane using forceps and a dissecting probe. The gut and buffer were transferred to a microfuge tube containing 800 μL of SB, and flash frozen in liquid nitrogen. Tubes were stored at -80°C until fluorescence was determined. Samples were thawed and added to cuvettes containing 1 mL of SB. The midgut epithelial cells were dispersed by pipetting the solution up and down six times. Fluorescence was determined using a spectrophotometer, and normalized to background and volume. This measurement in relative fluorescent units (FLU) represented the total amount of viral fusion. After the first measurement was taken, Triton X-100 was added to 1% of the total volume, to disrupt any remaining dimers of R18, and the samples read again. This measurement, corrected for volume, represented the total amount of R18 bound to the gut. The data are presented as the percentage of virus bound and fused based on the fluorescence of the dose of virus given to the insect, normalized for any background fluorescence. The data presented represent the mean of six individual insects per treatment group and were analyzed by one-way ANOVA and student’s t-test.

**Microscopy.** Fluorescence of infected tissues was captured on a Zeiss Axioplan microscope. Newly molted fourth instar *H. virescens* were fed a solution of polyhedra on a small diet cube. Insect midguts were dissected out at 24 hpi in 1% formalin in Neutral Buffer, rinsed free of the peritrophic matrix, and fixed for 1-2 hrs in 500uL of formalin buffer. Midguts were washed in PBS for 5 minutes, excess PBS allowed to drip off, then embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (Ted-Pella, Inc.) in the chamber of a Universal cryostat. Guts were sectioned at 20 microns thick and mounted onto Probe-On Plus slides (Fisher Scientific). Sections were air-dried for 1 hour, and circled with a PAP-pen, to reduce the amount of reagent required for the following steps. Sections were washed in PBS to remove excess O.C.T., then incubated in 3% Triton X-100 in PBS for 30 minutes to solubilize membranes. Slides were washed in PBS, and then
incubated with 40-120 μL of DAPI (Invitrogen) and phalloidin (Invitrogen) in water (7 μL of DAPI to 1 mL total, 20 μL of phalloidin to 1 mL) for 30 minutes in a covered container. Slides were then washed two times in PBS, overlaid with Fluorogel (E.M.T.) coverslipped and sealed with finger nail polish. Slides were stored in the dark at 4°C until image capture. Images were captured on a Zeiss Axioplan microscope with a TRITC filter (red), FITC filter (green), and DAPI filter (blue). Samples were examined from six to ten cross-sections from three to four insects per treatment. Uninfected fourth instar *H. virescens* were used for scanning electron microscopy of the midgut epithelia. For transmission electron microscopy (TEM), insects were fed AcIE1TV3.EGFP at a dose exceeding the LD₉₀ (2 μg ODV), and dissected 1 hpi. Whole midguts, minus the peritrophic matrix, were fixed and electron microscopy (EM) performed according to standard protocols as described in Tang et al. (2007).

**Recombinant ODV-E56 protein purification.** The *odv-e56* gene was PCR amplified from AcMNPV C6 genomic DNA (Primers: F: GCAGGTAACGATGAGTTTTTTTCA, R:CGCAAGCTTTTATCGAGGGGGCCG), cloned into pBADhisC (Invitrogen), and transformed into Top-10 *E. coli* (Invitrogen). 500 μl of overnight cultures were re-inoculated into 50 mL Low-salt Luria Broth (LB) medium + ampicillin (50 μg/mL) and incubated at 37 °C according to the manufacturer’s protocol. When the cultures reached an OD₅₉₅ of 0.4, they were induced with 0.004% arabinose for 12-24 hours at 30°C in an orbital shaker at 150 rpm. The polyhistidine-tagged protein was batch-purified using Ni-NTA agarose under both native and denaturing conditions following the manufacturer’s instructions (Qiagen), and then desalted and concentrated with a Microcon YM-30 centrifugal filter. The resulting protein was analyzed by SDS-PAGE and western blot to confirm expression of polyhistidine-tagged ODV-E56 using anti-polyhistidine (Sigma) and anti-ODV-E56 sera (kindly provided by Sharon Braunagel, Texas A&M University).

**SDS-PAGE, western and far-western blots.** Bacterial lysates and Ni-NTA purified polyhistidine-tagged ODV-E56 protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean 3 cell (Bio-Rad) following the manufacturer’s instructions. Samples were loaded into 12% bis-acrylamide gels with 4% stacking gels, along with unstained broad range molecular weight markers.
(Bio-Rad) and prestained All Blue Broad Range markers (Bio-Rad). The proteins were visualized using Coomassie blue stain. Gels used for western blots were transferred to PVDF membrane (Hyclone) using a semi-wet transfer XCell II Mini-blot module (Invitrogen) for 2 hours at 25V. Membranes were blocked overnight in PBS, 4% milk, 0.1% Tween, washed in PBS 0.1% Tween three times for 5 minutes with agitation, then incubated with mouse anti-His antibody (Sigma) or rabbit anti-ODV-E56 (Summers lab). Blots were washed as before, then probed with secondary antibody of goat anti-mouse-HRP (Sigma) or goat anti-rabbit-HRP (Sigma). The ECL ladder (GE Healthcare) was incubated with S protein-HRP for the final 30 minutes of the secondary antibody incubation. Bound antibody and weight markers were visualized using the Amersham ECL Plus Western Blotting Detection system (GE Healthcare). Fluorescent western blot signals were detected using a Typhoon 9410 Variable Mode Imager set at an excitation wavelength of 457 nm and emission wavelength of 532 nm. Far-westerns were performed as above with the addition of incubation of native or denatured ODV-E56 protein in sodium phosphate buffer pH 9.0 for one hour at room temperature or TBS pH8.0 at 4°C for one hour. The membranes were then washed three times with TBS-0.1% Tween. ODV-E56 was detected using anti-his or anti-ODV-E56 antibody as previously described.

RESULTS

**ODV-E56 is essential for the oral infectivity of both ODV and OB.** The odv-e56 orf was disrupted by insertion of a lacZ cassette under the control of the *Drosophila melanogaster hsp70* promoter as described in Harrison et al., 2010 (Figure 1). Bioassays with fourth instar *H. virescens* revealed that polyhedra of the construct AcIE1GFP-e56lacZ(+) have significantly lower per os infectivity (Table 1). The dose of OB necessary to observe any mortality was five logs higher than that of the other constructs and wild type virus (Figure 2: Table 1). The LD50 of the backbone recombinant AcIE1TV3.EGFP, the revertant AcIE1GFP-e56R, and wild type AcMNPV-C6 OB were not significantly different from one another as determined by probit analysis (p>0.05; LD ratio test)(Robertson and Preisler, 1992). We conducted bioassays with ODV isolated from polyhedra of AcIE1GFP-e56lacZ(+) and AcIE1TV3.EGFP, which also showed a significant difference in infectivity.
(p< 0.05, LD ratio test); the LD50 of the deletion mutant was 200 times greater than the *odv-e56* containing virus. The ODV LD50 of AcIEGFP-e56LacZ(+) was 2425 ng compared to 11.79 ng of ODV for AcIETV3.EGFP. In contrast, the BV of all constructs tested by proleg injection were not significantly different in infectivity (p>0.05: LD ratio test).

**Confirmation of loss of per os infectivity.** Deletion mutants were created in two recombinant viruses expressing eGFP under the immediate early promoter or the late p6.9 promoter. Table 2 shows the bioassay results for all four deletion mutants. AcIE1GFP-e56lacZ(-) exhibited a similar phenotype as the (+) construct, as did the Acp69GFP-e56lacZ(+) construct, requiring doses of greater than 1 X 10^6 OB to observe larval mortality. Acp69GFP-e56lacZ(-) had an LD50 of 1666 OBs, reflecting contamination with wild type virus as described previously (Harrison et al., 2010). The orientation of the LacZ cassette within the virus did not impact oral infectivity, nor did the location of the *egfp*. The bioassay results for viruses containing *odv-e56* were not significantly different from one another (p>0.05; LD ratio test).

**ODV-E56 deletion mutants bound to and fused with the columnar epithelia of *H. virescens.*** In order to assess the interaction of AcIE1GFP-e56LacZ(+) with the brush border microvilli *in vivo*, binding and fusion studies were conducted as previously described (Ohkawa et al., 2005). *H. virescens* larvae were orally inoculated with excess purified ODV labeled with the fluorescent probe Octadecyl Rhodamine B-chloride (R18). The knockout virus AcIEGFP-e56lacZ(+) was compared to the wild type virus AcMNPV-C6 (positive control) and the *p74-pif-0* deletion mutant, AcIEGFP-p74lacZ(+) virus (negative control; Figure 3). AcIE1GFP-e56LacZ(+) bound and fused to BBMV at levels similar to the wild type virus, AcMNPV-C6. The *p74-pif-0* deletion virus, AcIEGFP-p74lacZ(+), exhibited no binding, as described previously (Haas-Stapleton et al., 2004). One-way ANOVA for both binding and fusion indicated a significant difference among treatment groups (p < 0.05), with a least significant difference of the mean 0.84 and 0.52, respectively. The wild type and ODV-E56 negative virus were not significantly different from one another in binding or fusion, but the *P74-PIF-0* virus was significantly lower in binding and fusion.

**Electron microscopy of midgut.** The appearance of the surface of the brush border microvilli of fourth instar *H. virescens* is shown in Fig 4A. A TEM cross-section of the
microvilli with an adjacent ODV from the AcIE1TV3.EGFP construct is shown in Fig. 4B, which illustrates that AcIE1TV3.EGFP ODV are similar in diameter to the microvilli. One ODV appears to be shedding its envelope, although this may be an artifact of preparation (black arrow). In Fig. 4C, the nucleocapsids in the basal body of the cell appear to be drifting apart from one another (black arrow). This figure illustrates how the ODV fuse to a microvillus that is equivalent in diameter.

**Fluorescence microscopy of infected midguts at 24 hpi.** To address whether the *odv-e56* deletion virus was entering the cell, being transported to the nucleus, and initiating gene expression, we used fluorescence microscopy. Cryo-sectioning of midgut tissue into approximately 20-micron thick sections reduced the gut auto-fluorescence, and fixation of the tissues in formalin containing buffer had no effect on detection of eGFP expressing foci. The AcIE1TV3.EGFP virus had observable foci at doses of 1 X 10^7 (Fig. 4D). No GFP was detected in the negative control sections infected with wild type AcMNPV-C6 (Fig. 4E), or in the knockout AcIE1GFP-e56lacZ(+) virus (Fig 4F). Staining with DAPI and phalloidin allowed the nuclei and actin filaments to be observed. The muscles of the gut strongly stain with the actin stain, but actin within the microvilli is also observed. Differences between the actin filaments of uninfected and infected cells were not detected at this resolution. Figure 5 shows the bright field, FITC and merged FITC and DAPI images from midguts infected with the 6 constructs: AcIE1TV3.EGFP, AcIE1GFP-e56lacZ(+), AcIE1GFP-e56lacZ(-), AcMLF9.EGFP, Ac69GFP-e56lacZ(+), and Ac69GFP-e56lacZ(-) at a dose of 1.5 X 10^{10} polyhedra. Here, we clearly see the AcIE1TV3.EGFP virus infected cells exhibit lower GFP expression (Fig. 5A) and thus weaker foci than those of the AcMLF9.EGFP and the Ac69GFP-e56lacZ(-) virus (Fig. 5D,F). At these high doses for the ODV-E56-positive viruses, we see a plethora of foci in these representative sections, while no foci are observed in the AcIE1GFP-e56lacZ(+) infected gut tissues (Fig. 5B,C). The Ac69GFP-e56lacZ(-) virus exhibited an intermediate phenotype in the number of foci observed in the gut compared to the other two, reflecting contamination with wild type virus (Harrison et al., 2010) (Fig. 5F). The other two deletion mutants, AcIE1GFP-e56lacZ(-) and Ac69GFP-e56lacZ(+) showed a phenotype like AcIE1GFP-e56lacZ(+) with no foci detected at these levels (Fig. 5C,E). Here, ODV-E56 is required for baculovirus gene expression.
**Interaction of recombinant ODV-E56 with BBMV proteins.** Full length ODV-E56 with an N-terminal polyhistidine tag (His-ODV-E56) was expressed in *E. coli*, and purified under native or denaturing conditions (Fig. 6A). The native protein was ~ 66 kDa, and under denaturing conditions a dominant 45 kDa band was observed, along with minor 40 kDa and 66 kDa bands (Fig. 6A). All three bands were also detected by both the anti-E56 antibody (data not shown) and the anti-his antibody, suggesting the 40 kDa degradation product of His-ODV-E56 was not degraded at the N-terminus. The polyhistidine tag is estimated to add 6 kDa to the size of the protein. The predicted molecular weight of *AcMNPV* ODV-E56 is 40.8 kDa, but has previously been observed at ~56 kDa (Braunagel et al., 2003). Full-length ODV-E56, both native and denatured, bound to a high molecular weight protein band from BBMV preparations separated by SDS-PAGE and immobilized on PVDF membrane, when incubated under basic conditions (Fig. 6B). The identity of this protein is unknown. Co-feeding larvae with His-ODV-E56 and either polyhedra or ODV of AcIE1GFP-e56lacZ(+) did not restore oral infectivity of the virus (data not shown).

**DISCUSSION**

This study shows that the lack of ODV-E56 in the virion has a profound impact on the oral infectivity of the virus in both its polyhedral and ODV forms in fourth instar *H. virescens*. This result is in agreement with our earlier studies using neonate *H. virescens* (Harrison et al., 2010). The ODV-E56 deletion mutant AcIE1GFP-e56lacZ(+) required five logs more virus to achieve 50% mortality in fourth instar *H. virescens* than virus AcIE1TV3.eGFP. This degree of impairment is similar in scale to that observed for deletion of several PIFs tested in fourth instar *H. virescens* (Haas-Stapleton et al., 2004; Ohkawa et al., 2005; Zhang et al., 2005). The impairment in oral infectivity observed for ODV-E56 deletion mutants, concurrent with no observable impact on BV infectivity, places ODV-E56 within the growing family of *per os* infectivity factors.

Interestingly, the impact of *odv-e56* deletion on oral infectivity differed between the polyhedra and the ODV. Here, the *odv-e56* deletion mutant ODV were two logs less infectious than wild type virus ODV, in contrast to the polyhedra deletion mutants, which were five logs less infectious. It is unclear why the ODV and polyhedra *odv-e56* deletion
mutants differ in their infectivity relative to odv-e56 positive virus, yet both deletion mutants still cause a significant impairment in *per os* infectivity. Possible explanations include: (i) ODV-E56 functions in release of ODV from OB, or in penetration of the peritrophic matrix; (ii) OB lacking ODV-E56 contain fewer ODV than wild type virus; (iii) ODV become entrapped within the polyhedral envelope protein that surrounds the polyhedra and hence access to the host gut epithelium is restricted in infections with OB. The only other ODV envelope protein deletion mutant for which ODV LD50s have been calculated is the enhancin like factor Ac150(*ac150*), whose *per os* infectivity enhancing effects are observed only for polyhedra, but not the ODV (Zhang et al., 2005). The LD50 of ODV deletion mutants of Ac150 are not significantly different than wild type. *ac150* deletion mutants did not exhibit binding activity to the gut in binding and fusion studies, and in conjunction with the bioassays, this protein is thought to be involved in events upstream of virus binding, such as facilitating movement of the virions out of the dissolved OB and/or through the peritrophic matrix (PM). Here, with ODV-E56, the ODV of *odv-e56* deletion mutants are still significantly less infectious than the wildtype ODV, suggesting a role in events early after ingestion of OB. Cross sections of polyhedra examined by EM did not reveal any differences for the *odv-e56* deletion mutants when compared to wild type virus (Harrison et al., 2010); however, the number of NCs per ODV, and ODV per OB were not quantified. Alternatively, alkali liberated purified ODV may simply be more infectious than the polyhedra-derived ODV. The alkaline gut pH dissolves the polyhedrin protein, but the polyhedral envelope remains, entrapping the ODV within (Rohrmann, 2008). The gut proteases (and likely viral proteases packaged within the OB) as well as the physical disturbance by the movement of the gut ultimately disrupt this membrane. Fewer polyhedra-derived virions may actually be coming into contact with the gut, that is, some may remain entrapped (compared to alkaline treated OB *in vitro*). More research is needed to ascertain if the *in vivo* effects observed with lab purified ODV are truly representative of ODV solubilized from OB in a wild type infection.

Unlike the other PIFs that are strongly associated only with the ODV envelope and exhibit strong effects on oral infectivity of polyhedra (P74-PIF-0, PIF-1, and PIF-2), the ODV-E56 deletion mutant did not exhibit any impediment in binding and fusion to the gut *in
vivo. ODV-E56 has similarities to PIF-3 (ac115) in that profound effects on oral infectivity were observed, but no effect on binding and fusion was detected (Ohkawa et al., 2005). Western blots have clearly shown PIF-3 is associated with the ODV envelope (Song et al., 2008). The association of PIF-3 with the ODV may be weaker, however, as two of the three proteomic analyses have not identified this protein by mass spectrophotometry. PIF-3 is thought to have a role downstream of the binding and fusion event. Likewise, the role of ODV-E56 may promote necessary elements of viral infection of the cell after fusion of the viral and cellular membranes.

We then examined cryosections of midgut tissue for eGFP expressing foci of viral infection. Foci were not detected in cryosections of insects infected at an LD_{50} (~150) though doses of 1 X 10^7 OB did result in observable foci. Doses of 1.5 X 10^{10} OB were used to easily visualize eGFP expressing foci in the midgut by the _odv-e56_ positive virus constructs AcIE1TV3.EGFP and AcMLF9.EGFP at 24 hpi. As expected, the foci of constructs with _egfp_ under control of the _ie-1_ promoter were weaker than those under control of the very strong late promoter of _p6.9_. Examination of the midguts infected with the viral constructs AcIE1GFP-e56lacZ(+), AcIE1GFP-e56lacZ(-), and AcP69GFP-e56lacZ(+) revealed no foci of infection. This suggests two hypotheses: (1) At this dose of polyhedra, near the LD_{50} of the _odv-e56_ negative virus, virus infection of a cell is a very rare event, and thus not captured in our cryosections, as observed by the lack of foci detected using LD_{50} doses of AcIE1TV3.EGFP, and (2) Virus binds and fuses to the cells, but the viral DNA is not transcribed and does not produce eGFP. Hypothesis (1) suggests a role for ODV-E56 in events upstream of binding and fusion, such as release from the polyhedra and efficient movement through the PM. Hypothesis (2) however, suggests a potential role of ODV-E56 in cell signaling after fusion, possibly initiating the reorganization of the actin cytoskeleton to transmit the nucleocapsids from the apical microvilli to the basal part of the cell as observed in Fig. 4C.

The fluorescence microscopy of the deletion mutant AcP69GFP-e56lacZ(-) known to be contaminated with wild type _AcMNPV_ is also interesting. Detection of eGFP expressing foci indicates that the nucleocapsids of the deletion mutants are entering the cell and being transported to the nucleus for transcription. Cellular entry could be facilitated either through
infection by ODV that contain both wild type and deletion mutant nucleocapsids, multiplicity of infection (more than one virion infecting the cell), or both. Both scenarios have been documented in the literature (Cory and Myers, 2003), and are a key element in the virulence of baculovirus populations in the field. There is also evidence to suggest that mixtures of wildtype and pif knockout polyhedra produce a more virulent population than the wild type alone (Hao et al., 2009). One hypothesis presented by Hao et al., is that the deletion mutants lacking both PIF-1 and PIF-2 may fuse faster in conjunction with wild type virus facilitating the fusion step; that is, binding of multiple pif proteins on the wild type ODV may actually slow the binding and fusion process overall. Recent studies with four single deletion mutants of p74-pif-0, pif-1, pif-2, and pif-3 found that mixtures of these viruses were also non-infectious to the host per os (Song et al., 2008). This result suggests that virus infection requires all four of these PIFs present on the same ODV, and is not unexpected given their conservation across all baculoviruses.

As observed in previous studies, we detected multiple bands of ODV-E56 at sizes greater than the predicted molecular weight. ODV-E56 appears to have a high degree of post-translational modification, and although N-glycosylation sites have been described, treatment with N-glycosidases resulted in no change to protein size (Braunagel et al., 1996). In addition, the antibodies used to detect ODV-E56 both targeted the N-terminus (the polyhistidine tag and the N-terminal 135 aa of ODV-E56), so some forms may not be detected. ODV-E56 and ODV-E18 are the only described ODV envelope proteins observed at sizes highly discrepant from their predicted molecular weights. These modifications may serve to protect the ODV in the gut, or be needed for specific ligand interactions (either with the polyhedra, PM, or cellular receptor(s). P74-PIF-0, the only PIF shown to be exposed on the surface of the ODV envelope thus far, has been shown to undergo protein cleavage by insect midgut trypsins (Faulkner et al., 1997; Slack et al., 2008). Transmembrane prediction suggests ODV-E56 is very similar to P74-PIF-0, containing two C-terminal transmembrane domains; however, its placement on the interior or the exterior of the virus envelope is unknown (Theilmann et al., 1996; Slack and Arif, 2007).

ODV-E56 was found to bind to an unidentified high molecular weight band in preparations of BBMV from fourth instar *H. virescens*. In vitro binding of ODV to BBMV
may not be representative of \textit{in vivo} activity, or may support a role of ODV-E56 in cell signaling after viral fusion. ODV-E56 may interact with a surface protein after fusion and mixing of the cellular and viral membranes, resulting in necessary cellular reorganization needed to transport the nucleocapsids to the nucleus. The magnitude of actin microfilament reorganization required to transport the large nucleocapsids from the distal microvilli to the nuclei of the cell is substantial (Volkman et al., 1982). Our SEM studies show that the ODV are similar in diameter to those of the microvilli that they fuse with. Alternatively, the post-translational modifications of ODV-E56 may make it sticky in nature, allowing it to bind both PM and proteins on the BBMV; some proteins of the PM are secreted from the microvilli into the extracellular space (Lehane, 1997). PIF-3, which exhibits a similar phenotype to ODV-E56, was not tested for midgut binding partners, and thus far, only P74-PIF-0 has been shown to bind to an unidentified 35-kDa protein (Yao et al., 2004).

Despite the strong binding of both native and denatured ODV-E56 to gut BBMV, co-feeding with expressed ODV-E56 did not rescue the \textit{odv-e56} deletion viruses as was shown for P74-PIF-0 (Yao et al., 2004). In its individual form, ODV-E56 may not be stable in the gut long enough to achieve rescue, or, alternatively, may have the wrong conformation. It should also be noted that the P74-PIF-0 that successfully rescued deletion mutants was expressed in insect cells, whereas our ODV-E56 protein was expressed in \textit{E. coli}. Differences in post-translational modifications may occur and result in a non-active form of the protein. P74-PIF-0 requires trypsin activation, and ODV-E56 may require this as well. Further study in this area is needed.

The family of PIFs will most certainly continue to grow, both in number and in mode of action. Oral infection is a defining moment in the life cycle of a virus; without access to a host cell it cannot replicate. As mentioned earlier, 25-30\% of the core baculovirus genes are ODV proteins, and six of the core 30 genes are PIFs once ODV-E56 and PIF-4 (\textit{ac96}) are added (Fang et al., 2009; Herniou et al., 2003). Many genes conserved across the lepidopteran baculoviruses are still of unknown function. In addition, the three proteomic analyses have identified a plethora of virus specific proteins, which may be included in the OB or ODV and may be essential to oral infection of specific hosts (Braunagel et al., 2003; Deng et al., 2007; Perera et al., 2007). Current studies of the PIFs already indicate that they
have different functions in different host insects, or different homologs have different functions within the same host (Hao et al., 2009; Ohkawa et al., 2005; Zhang et al., 2005). The PIFs also appear to have different affinities to the ODV envelope, as several of the PIFs were not detected in the proteomic analyses, although western blotting has clearly localized them to the ODV envelope. The different methods used in these studies may make some PIFs susceptible to disassociation or degradation.

The next important avenue of study is in beginning to understand how ODV proteins work in concert on the surface of baculovirus ODV to mediate binding, fusion and oral infection. All studies to date have focused on single gene deletion virus constructs (with the exception of the double knockouts of the enhancin like factors Ac145 and Ac150; Lapointe et al., 2004), or the expression of a single ODV protein. The true nature of these protein interactions within the ODV envelope is unknown. Also unclear from the current studies of the PIFs, is whether the loss of oral infectivity (and ODV binding and fusion for P74-PIF-0, PIF-1, PIF-2) is due to the lack of the protein on the ODV envelope or by mis-folding/different presentation of other ODV envelope proteins which are then unable carry out their function in viral infection. Future work on the interaction of these PIFs is necessary.

The baculoviruses have progressed several areas of study due to their novel genes and strategies. The study of the BV fusion protein GP64 has recently helped create a new class of viral membrane fusion proteins with a unique mechanism of fusion and distinct protein conformation. Future study of the ODV may reflect another novel entry mechanism, and understanding the mechanism(s) of ODV entry may lead to breakthroughs in other areas as well.

In summary, ODV-E56 is a per os infectivity factor, non-essential to binding and fusion of the ODV. ODV-E56 enhances OB infectivity at levels larger than those observed for the ODV, suggesting a role for ODV-E56 in early release of the ODV from the polyhedra. In addition, ODV-E56 exhibits binding to midgut BBMV proteins, suggesting an additional function of ODV-E56 at the cell level. Further study will be needed to clarify the role of ODV-E56 in oral infectivity. We propose to call ODV-E56 PIF-5 after P74-PIF-0, PIF-1, PIF-2, PIF-3, and PIF-4 (Sparks, 2010).
AUTHORS CONTRIBUTIONS
WOS designed and performed the experiments described herein and wrote the manuscript.
RLS created the virus constructs used in these experiments and contributed to the editing of
the manuscript. BCB contributed to experimental design and editing of the manuscript.

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Microscopy and NanoImaging Facility for their assistance in cryosectioning and microscopy.
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9797-9802.
nucleopolyhedrovirus identified two new occlusion-derived virus-associated proteins,
californica multiple nucleopolyhedrovirus core gene ac96 encodes a per Os
envelope protein of Autographa californica nucleopolyhedrovirus required for
binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus


to midgut cells of *Heliothis virescens* larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. *J Virol* **79**(24), 15258-15264.


Table 1. ODV-E56 is required for optimal oral infectivity of OB and ODV. Fourth instar *H. virescens* ingested polyhedra (OB) or occlusion-derived virus (ODV), or were injected with budded virus (BV) via the proleg. Experiments were performed in triplicate, using 20-30 insects per dose of virus. The LD$_{50}$ for each virus treatment in each of the three virion forms was calculated using the software package POLO-Plus (LeOra Software). The lower and upper bounds of the 95% confidence interval (C.I.), sample size (N), heterogeneity (Het), slope, and LD Ratio are indicated. LD$_{50}$ with the same letter are not significantly different from one another. The LD Ratio test was performed to compare treatments to the *AcIE1TV3.EGFP* virus, and LD Ratios with confidence intervals that include 1.0 indicate the LD$_{50}$ of the viruses compared are not significantly different from one another (p > 0.05) (Robertson and Preisler, 1992). n/d indicates not determined.

<table>
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<th>Treatment</th>
<th>95% C. I.</th>
<th>95% C. I.</th>
</tr>
</thead>
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<td></td>
<td>LD$_{50}$ Lower</td>
<td>Upper N Het Slope</td>
</tr>
<tr>
<td>OB</td>
<td></td>
<td></td>
</tr>
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<td>AcMNPV-C6</td>
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<td>203.4 345 0.46 1.582</td>
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<td>196.6a 144.9</td>
<td>281.4 395 1.108 1.612</td>
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<td>7.1X 10$^8$ 311 0.55 1.157</td>
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<tr>
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<td>142.7 480 0.53 1.721</td>
</tr>
<tr>
<td>ODV</td>
<td></td>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>BV</td>
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<tr>
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<td>n/d n/d n/d n/d n/d n/d n/d n/d n/d</td>
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Table 2. Oral infectivity of OB of recombinant baculoviruses. Fourth instar *H. virescens* were fed OB on a small diet cube. Experiments were performed in duplicate or triplicate, using 15-30 insects per dose of virus; at least five doses of each virus were used. The LD$_{50}$ for each virus treatment was calculated using the software package POLO-Plus (LeOra Software). The lower and upper bounds of the 95% confidence interval (C.I.), sample size (N), heterogeneity (Het), slope, and LD Ratio are indicated. LD$_{50}$ with different letters are significantly different, p < 0.05. The LD Ratio test was performed to compare treatments to the AcIE1TV3.EGFP virus, and LD Ratios with confidence intervals that include 1.0 indicate the LD$_{50}$ of the viruses compared are not significantly different from one another (p < 0.05) (Robertson and Preisler, 1992). * contaminated with wild type virus (Harrison *et al.*, 2010).

<table>
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<tr>
<th>Treatment</th>
<th>LD$_{50}$</th>
<th>95% C.I. Lower</th>
<th>95% C.I. Upper</th>
<th>N</th>
<th>Het</th>
<th>Slope</th>
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<td>1.612</td>
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<td>4.9 x 10$^7$ b</td>
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<td>295</td>
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<td>0.0516</td>
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<td>1.602</td>
<td>0.87</td>
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Figure 1. Recombinant virus constructs. 

A. Wild type AcMNPV-C6 virus orf orientation at the polh locus in the left column, the odv-e56 locus in the right. 

B. Insertion of eGFP under the control of the ie-1 promoter, or C. p6.9 promoter, and disruption of the odv-e56 locus with lacZ reporter gene under control of the SV40 promoter in the positive and negative orientation designated (+) and (-), respectively (A and B from Harrison et al., 2010).
Figure 2. Deletion of *odv-e56* impairs infectivity of occlusion bodies (OB). Log transformed mortality dose response curves calculated by POLO-Plus (LeOra software). The viruses from left to right: *AcMNPV-C6*, *AcIE1GFP-e56R*, *AcIE1TV3.EGFP*, and *AcIE1GFP-e56lacz(+)*. 
Figure 3. Deletion of *odv-e56* doses not impact ODV binding and fusion to *H. virescens* midgut epithelial cells. Sucrose purified ODV were labeled with R-18 at levels not exceeding $1 \times 10^6$ FLU/µg ODV and orally inoculated into newly molted fourth instars (2.6 µg AcIE1GFP-e56LacZ(+), 1.8 µg AcMNPV, and 2.2 µg AcIE1GFP-p74LacZ(+)). Each column represents the mean of six larvae with standard error bars. Solid bars indicate the percent of the ODV that bound to the midgut, and striped bars indicate the amount that fused. One way ANOVA indicated the treatments were significantly different ($p < 0.05$) for both binding and fusion, with a 5% LSD of 0.84 and 0.54 %, respectively as indicated by a,b for binding and c,d for fusion.
Figure 4. Viral infection of midgut epithelial cells. A. SEM of fourth instar midgut microvilli. Bar indicates 5 µm. B-C. TEM of microvilli infected with AcIE1TV3.EGFP ODV. Bar indicates 2µm. B. The envelope (env) of an ODV with multiple nucleocapsids (NC) in contact with microvilli. C. The NC dispersing within the cytoplasm of the cell after fusion of the ODV with the microvilli. D-F. eGFP expression in midgut 24 hpi with 1 X 10^7 polyhedra for the following viruses. Bar indicates 100 µm. The presence or absence of odv-e56 and egfp in each virus in D-F is indicated.
Figure 5. ODV-E56 is required for expression of baculovirus genes. Fourth instar *H. virescens* were orally inoculated with 1.5 x 10^10 polyhedra; midguts were dissected at 24 hpi and cryosectioned for fluorescence microscopy. Nuclei are stained blue with DAPI, viral foci are green due to eGFP expression indicative of viral expression. This figure contains representative images from six to ten cryosections from three to six insects infected with: A. AcIE1TV3-EGFP, B. AcIE1GFP-e56lacZ(+), C. AcIE1GFP-e56lacZ(-), D. AcMLV9-EGFP, E. AcP69GFP-e56lacZ(+), and F. AcP69GFP-e56lacZ(-). The left hand column is the brightfield image, the middle column the FITC filter to detect the eGFP expressing viral foci, and the right hand column the overlay of DAPI and FITC images. The presence of absence of ODV-E56 in the viruses is indicated at left. Bar indicates 100 µM.
Figure 6. Purification of recombinant ODV-E56 and binding to *H. virescens* gut BBMV proteins. A. Nickel column purification of recombinant ODV-E56 under native and denaturing conditions. MW ladder, LacZ positive control, and eluted fractions of Native purified protein and denatured purified protein are shown. ODV-E56 was observed at ~66 kDa, when purified under native conditions and at ~45 kDa when purified under denaturing conditions. B. Far-western blot to show binding of ODV-E56 to *H. virescens* gut BBMV proteins. BBMV, ODV proteins (ODV), low speed cell pellet from BBMV preparation (cellular debris), and crude midgut homogenate from midguts for BBMV isolation are shown. Membranes were incubated with denatured His-ODV-E56 or native His-ODV-E56, as indicated, and detected using anti-His antibody. Binding to a ~97 kDa band was observed in BBMV samples (black arrows).
Chapter 3:

A peptide that binds the gut epithelium of Heliothis virescens has similarity to the baculovirus envelope protein ODV-E66 and impedes infection with wild type baculovirus

W. O. Sparks\textsuperscript{1*}, A. Rohlfing\textsuperscript{2#}, and B.C. Bonning\textsuperscript{1}

\textsuperscript{1}Interdepartmental Genetics Program and Department of Entomology, Iowa State University, Ames, IA, U.S.A
\textsuperscript{2}Program for Biological and Biomedical Sciences, Harvard University, Cambridge, MA, U.S.A.

SUMMARY:

Lepidopteran nucleopolyhedroviruses (NPV) of the family Baculoviridae initially infect their host species via the midgut epithelium through binding and fusion of the occlusion-derived virus (ODV). ODV contain upward of 30 proteins, and the mechanism of virus binding and fusion to the midgut epithelial cells is unknown. We screened a phage display library against brush border membrane vesicles (BBMV) derived from fourth instar Heliothis virescens midgut epithelia to identify gut binding peptides. Two phage clones expressing gut-binding peptides with similarity to the ODV-E66 protein from five species of alphabaculoviruses were isolated. ODV-E66 localizes to the ODV envelope and is conserved across lepidopteran baculoviruses. Chemically synthesized versions of these two peptides HV1 and HV2, and their homologs (AcE66-A and AcE66-B) from Autographa californica multiple nucleopolyhedrovirus (AcMNPV) bound to unfixed cryosections of whole midgut tissues. Interestingly, both one peptide from the phage bio-panning (HV1) and one peptide selected for its identity to AcMNPV (AcE66-A) exhibited the strongest binding. Two control peptides, an aphid gut binding peptide and a negative control peptide, bound at intermediate and low levels, respectively (AcE66-A, but not HV1, exhibited binding to H. virescens gut BBMV proteins separated by SDS-PAGE and detected by far-western blot). Competition assays with the strongest binding peptide, HV1, and purified AcMNPV ODV resulted in decreased mortality at an LD\textsubscript{50} dose, and a significant increase in survival time at

* Primary author of the work presented in this chapter
# Contributed to the phage bio-panning of H. virescens midguts
lethal concentrations of virus. These results suggest that HV1 competes with the virus for interaction with the viral receptor.

INTRODUCTION

Baculoviruses are large dsDNA viruses that infect arthropod hosts, primarily lepidopteran species. Of the four genera within this virus family, the most work has been performed with the genus Alphabaculovirus, which infects Lepidoptera (moths and butterflies). Baculoviruses are distinct in that they produce two different virion forms; budded virus (BV) which functions to disseminate the virus within an infected host, and the occlusion-derived virus (ODV), which serves to spread virus between hosts. The ODV of the alphabaculoviruses are further enclosed in a highly stable crystalline protein matrix to form polyhedra, also known as occlusion bodies (OB). The polyhedrin matrix protects the ODV from desiccation ensuring continued survival of the virus despite the brevity of the host’s life span. When ingested from the environment, the highly alkaline pH of the host midgut dissolves the OB, releasing the ODV into the gut lumen. There, the ODV cross the peritrophic matrix lining the gut and bind and fuse to the distal ends of the brush border microvilli of the columnar cells of the midgut epithelia (Horton & Burand, 1993).

The mechanism by which the ODV bind and fuse to their target cells, the columnar cells of the midgut epithelium, is unknown. Early studies indicated ODV bind and fuse to a cell surface protein receptor as opposed to entry via endocytosis (Horton & Burand, 1993). Over 30 proteins have been shown to localize to ODV or be involved in oral infectivity (Braunagel et al., 2003; Deng et al., 2007; Perera et al., 2007). Additional studies have led to the description of a family of per os infectivity factors (PIFs), containing six ODV envelope proteins that facilitate oral infectivity in some manner. These proteins include P74-PIF-0 (ac138), PIF-1 (ac119), PIF-2 (ac22), PIF-3 (ac115), PIF-4 (ac96), and PIF-5 (odv-e56/ac148) (Fang et al., 2009; Kuzio et al., 1989; Ohkawa et al., 2005; Pijlman et al., 2003; Sparks, 2010). Several other ODV envelope proteins have been described, though the role in initial infection is unclear, including ODV-E18, ODV-E25, ODV-E35, and ODV-E66 (Braunagel et al., 1996; Braunagel et al., 1999; Braunagel et al., 2004).

ODV-E66 localizes to the ODV envelope, and is a core gene in the lepidopteran baculoviruses (Braunagel et al., 2003; Herniou et al., 2003). A considerable amount of work
has been done on the nuclear trafficking of ODV-E66 and also ODV-E25. Both contain an N-terminal sorting motif (SM) comprised of a strongly hydrophobic region followed by charged amino acids which are sufficient to target the proteins to the inner nuclear membranes from which the ODV envelope is made (Hong et al., 1997). Preliminary knockout studies of ODV-E66 suggested a decreased virus fitness as indicated by an increased survival time of infected larvae (Gomi et al., 1999).

Although our knowledge of the complexities of the ODV envelope has increased, very little is known about the nature of the target receptor(s) for the ODV in their insect hosts. In contrast to the BV, which have a much simpler envelope composition and readily infect most insect cell lines, most insect cell lines are not easily infected by ODV, and stable cell lines of midgut have been difficult to create and maintain (Granados & Williams, 1986; Rohrmann, 2008; Volkman et al., 1976). A study with P74-PIF-0 co-precipitated a 35-kDa protein from the midgut brush border membrane vesicles (BBMV) of Spodoptera exigua, but the identity of this protein is unknown (Zhou et al., 2005). Genetic studies have documented one gene correlated with insect resistance to Bombyx mori NPV (Zhang et al., 2005), and Spodoptera frugiperda NPV may bind a different receptor than other NPVs (Haas-Stapleton et al., 2005) suggesting different baculovirus species utilize different receptors in different host species.

Phage bio-panning has been used in several insect pathogen systems, as well as, a diverse number of virus/toxin receptor studies (Fernandez et al., 2008; Ghosh et al., 2001; Larson et al., 2005; Ning et al., 2010). Our goal was to gain insight into the nature of the proteins that interact and bind to baculoviruses within the midgut of the model host species, Heliothis virescens. Here, we describe the successful bio-panning of a random phage library against H. virescens BBMV. The selected phage peptide sequences included two clones with similarity to the baculovirus occlusion derived envelope protein ODV-E66. Notably, each of the 17 selected peptides had similarity to known gut binding pathogens including a midgut specific insect pathogen, as well as human pathogens of worldwide medical importance. Here we demonstrate competition of one of these peptides with Autographa californica MNPV (AcMNPV) for binding to the H. virescens midgut.
RESULTS

**Phage Bio-panning.** The phage bio-panning was conducted on fourth instar brush border membrane vesicles (BBMV) from *H. virescens* midgut. A sodium carbonate buffer, pH 9.0 was used to mimic the pH of the midgut in which the microvilli extend. Neither whole guts nor BBMV preparations could withstand multiple rounds of washing to remove excess unbound phage. Therefore, we used a modified method of BRASIL (Giordano *et al.*, 2001). This method uses a single organic phase and centrifugation to separate unbound phage (which remained suspended at the top phase) from the phage bound to BBMV pellet. After three rounds of phage bio-panning, we selected twenty clones for sequencing. This generated 17 sequences without stop codons within the recombinant peptide region (which would truncate the phage surface protein). Of these 17, there were two sets of identical sequences, as well as, one pair with high similarity (Table 1). Across the 17 peptides, Proline was in highest abundance (26), followed by Phenylalanine (18), Leucine (17), Threonine (16), Alanine (13), Histidine (12) and Tryptophan (11). Glutamic Acid (9), Aspartic Acid (7), and Valine (7) were intermediate; Methionine (4), Arginine (4) and the remaining residues occurred only twice each (excluding the fixed Cysteine bridges). Phenylalanine, Histidine and Tryptophan have been shown to be over-represented in sugar binding sites of proteins (Boraston *et al.*, 2004; Cheng *et al.*, 2009).

**BLAST results.** A BLASTP search of the short peptides, both the complete 12 amino acid sequence including the conserved Cysteine at position 2 and 11, and the eight internal amino acids, revealed two very interesting results: multiple hits for matches to other known gut pathogens, and peptide homology to the ODV-E66 protein of baculoviruses (Table 1). The similarity of these BLAST hits ranged from 5/8 amino acids (aa) to 8/8 aa identical amino acids (both with and without gaps). As a result of the small length of these peptides, the scores of the hits were all less than 40, and E-values were all positive even for sequences with 100% identity; however, higher scores and lower E-values still indicated greater similarity. As expected, many hits returned were for unknown proteins deduced from computationally predicted open reading frames (orfs); thus, it is unknown whether these reflect similarity of function. Eleven of the 17 peptides returned bacteriophage proteins. Bacteriophage are thought to be the most abundant life form on the planet, exceeding
unicellular organisms by at least an order of magnitude (Koonin et al., 2006). Given the diversity of the phage and their bacterial hosts (including symbiotic gut bacteria), the large number of bacteriophage protein matches is not surprising. Another gene with multiple hits was the envelope protein from various lentiviruses of the family Retroviridae. This genus of retroviruses mutates rapidly due to lack of proofreading the RNA-dependent RNA-polymerase. Following these, we observed several pathogens; some with insect vectors or insect hosts. The recently discovered *Pseudomonas entomophila* is a pathogenic bacterium infecting *Drosophila* midguts (Vodovar et al., 2006). *Shrimp white spot syndrome virus* was originally classified as a baculovirus based on phenotype, until molecular studies necessitated the formation of a new family- the Nimaviridae (van Hulten et al., 2001; Vlak et al., 2005).

Giardia is an anaerobic protozoan parasite that binds to the brush border microvilli in the small intestine in mammals when ingested from contaminated water, soil, or food (Khanna et al., 1988). Likewise, Salmonella is an opportunistic pathogen of epithelial cells through adhesion and invasion, while *Vibrio cholerae* adheres and releases toxins (Burkholder & Bhunia, 2009; Faruque et al., 2003). Leptospira is another bacterium shown to adhere to epithelial cells (Levett, 2001). Most intriguing were the hits for *Leishmania, Trypanosoma,* and *Plasmodium,* which have complex life cycles involving sand flies, tsetse flies and mosquitoes, respectively, and cause leishmaniasis, sleeping sickness and Chagas disease, and malaria in humans (Morel & Lazdins, 2003; Welburn et al., 2009). Sindbis virus and Dengue virus also infect the midgut epithelia of *Aedes aegypti* (Lambrechts et al., 2009; Pierro et al., 2008). The proteins that showed similarity were primarily proteins of unknown function in these species.

Selected peptides also had similarity to baculovirus proteins from various species within Baculoviridae. Two of the peptides had similarity to the occlusion derived envelope protein ODV-E66, a lepidopteran specific envelope protein observed in both alpha-(nucleopolyhedro) and beta- (granulo) –baculoviruses (Herniou et al., 2003; van Oers and Vlak, 2007). Four peptides had similarity to five different baculovirus proteins derived from predicted orfs of unknown function, including the *AcMNPV orf-75* homolog that is a conserved lepidopteran baculovirus gene. There was also similarity to the VP1054 capsid protein (another core baculovirus gene) from two species of betabaculoviruses and to
AcMNPV orf-25 homologues (a core lepidopteran gene involved in binding to ssDNA). Also of note, only one peptide showed similarity to a species of baculovirus known to infect *H. virescens* (*AcMNPV orf*-75 homolog of *Rachiplusia ou* MNPV); however, the susceptibility of *H. virescens* to each of the baculovirus species with BLAST hits to the phage peptides has not been determined (Herniou *et al.*, 2004; Rohrmann, 2008).

**ODV-E66 alignment.** The two peptides with similarity to ODV-E66 were then aligned with the lepidopteran ODV-E66 baculovirus sequences identified from BLASTP using *AcMNPV* as the consensus sequence (Figure 1A). The phage peptides localized to two regions within ODV-E66, across six different species of baculovirus (Figure 1B). Region 1 encompassed amino acids 84-96 of ODV-E66, and region 2 included residues 179 to 192 of ODV-E66. The peptide SCWAVFSATLCT, designated HV1, had similarity to *Agrotis segetum* NPV (*AgseNPV*) ODV-E66 and both ODV-E66 homologs (*orf 77/78* and *orf 143/144*) in both species of *Mamestra configurata* NPV-A and –B (*MacoNPV*-A, *MacoNPV*-B) in region 2 of ODV-E66. The second peptide, HCSVWHVFAQCT, designated HV2, had similarity to several baculoviruses at two different locations: *Hyphantria cunea* NPV (*HycuNPV*) in region 1, and *AgseNPV* and *MacoNPV*- A and *MacoNPV*-B in region 2 (6/8 aa identity- one alanine serine mismatch which has a higher score than the 6/8 identity with *MacoNPV*- A which has a G –A match) and a 5 aa identity match of the VWHVF in *Spodoptera exigua* NPV (*SpexNPV*). As indicated previously, ODV-E66 is a core lepidopteran baculovirus protein. Although none of these matches included a species known to easily infect *H. virescens*, both peptides showed similarity to both Group I and Group II NPVs as illustrated in Figure 1B. Both species of *MacoNPVs* have two copies of ODV-E66 (*orf 77/78* and 143/144). Interestingly, from our alignment we see that the *orf 77/78* shares greater similarity with Group I *AcMNPV*, whereas *orf 143/144* has greater similarity to the Group II ODV-E66 proteins, as well as the HV1 peptide. Overall, region 1 of ODV-E66 showed greater sequence variation across all baculovirus species than region 2, which was more conserved at the amino acid level.

**ODV-E66 transmembrane prediction.** ODV-E66 contains a conserved transmembrane domain at its N-terminus, but the orientation of ODV-E66 in the ODV membrane is unclear. Using TMPred, TMHMM, and HMMTop software (Krogh *et al.*, 2004).
2001; Hofman et al., 1993; Tusnady and Simon, 2001), three other significant transmembrane domains were identified near amino acids 208, 294, and 347 in AcMNPV ODV-E66. The strongest possible transmembrane models are indicated in Figure 2 based on the output from these three membrane prediction software packages. TmHMM and HMMTOP favored the single transmembrane domain found in the N-terminus, while TMPred favored the three transmembrane domain model followed by the 2 transmembrane domain model. These additional transmembrane domains fall just under the score of 500 for significance in TMPred, while significant in HMMTop. Both the HV1 and HV2 peptides fall within loop regions of the protein (Figure 2). The conserved sorting motif (SM) on the N-terminus is indicated, as is a conserved domain found in the N-terminal alpha helical domain of Group 8 lyases (Vigdorovich et al., 2007).

**Peptide synthesis and binding.** The peptides of interest were synthesized containing a spacer and a 5-FAM (carboxy-fluoroscein) label for ease in detecting peptide binding. Since the alignment of the phage peptides HV1 and HV2 to AcMNPV ODV-E66 showed little similarity, we hypothesized that these regions of AcMNPV ODV-E66 were also involved in binding to the midgut. Thus two peptides were created containing 8 amino acids with identity to the AcMNPV ODV-E66 sequence found in region 1 and 2, flanked by Cysteines to force a loop structure, and terminal amino acid similar to the alignment. Two control peptides were also synthesized: the aphid gut binding peptide GBP3.1, and a negative control peptide, C6 (Liu et al., 2010). These short peptides were also analyzed for their hydrophilicity and antigenic index (Figure 3). Of the six peptides, only GBP3.1 showed a strong hydrophilic region and a strong antigenic index at a neutral pH. Differences between the phage peptides and the AcMNPV peptides were noted. Both HV1 and HV2 were strongly hydrophobic, whereas AcE66-A and AcE66-B had regions of slight hydrophilicity. Similarly, both AcE66-A and AcE66-B had a greater antigenic index compared to the phage peptides, and were classified as belonging to turns in secondary protein structure.

The high background fluorescence of the midgut made detection of peptides bound to whole midgut unsatisfactory. Thus, the midguts were dissected in 10% formalin Neutral buffer, and cryosectioned in approximately 20 uM thick sections. Initial binding assays using these fixed cryosections showed no binding by any of the FAM-labeled peptides.
Since formalin causes cross-linking between proteins, we attempted cryosectioning of insects dissected in PBS buffer. The gut sections were incubated with the peptides in a sodium carbonate buffer at pH 9.0 to mimic the gut pH. Under these conditions, binding was easily detected; representative images from six insects using the same exposure times are shown in Figure 4A. Qualitatively, the synthetic peptides showed distinct differences in binding as indicated by a range of fluorescent signal. The negative control, PBS, showed little fluorescence in the cryosections as compared to the whole gut mounts (data not shown). The HV1 peptide exhibited strong binding, whereas its regional homolog in AcMNPV ODV-E66, peptide AcE66B, showed very low binding. Inversely, the HV2 peptide showed an intermediate level of binding, while the peptide AcE66-A, exhibited extremely high levels of binding. The negative control peptide, C6, weakly bound to the midgut (as observed in aphid midguts as well (Liu et al., 2010). Interestingly, the other control in this experiment, the aphid gut binding protein, GBP3.1, showed moderate levels of binding to the caterpillar midgut.

Having observed that GBP3.1 bound to the H. virescens gut, we were interested in determining if any of these peptides would also bind to the aphid gut. HV1 was chosen due to its strong binding to H. virescens. Figure 4B shows the results of binding studies with the pea aphid, Acyrthosiphon pisum, and the green peach aphid, Myzus persicae. The HV1 peptide bound to the midguts of both species of aphids, at levels similar to GBP3.1.

**Peptide competition assays with baculovirus.** Having confirmed the binding of the peptides selected for H. virescens midgut binding at levels higher than the control peptide, we then wanted to see if the peptide(s) would compete for virus binding in vivo (HV1 was chosen as it exhibited the greatest level of binding in the midgut). We again used newly molted fourth instar H. virescens, and the recombinant virus AcIE1GFP.TV3 as described in Harrison et al. (2010). Initial experiments co-feeding HV1 peptide and AcIE1GFP.TV3 OB via droplet feeding showed no significant differences between virus and virus-peptide fed insects (data not shown). Likewise, no effect on insect growth and development was observed in the control group fed peptide only. Fearing the synthetic peptides were not surviving the harsh conditions of the midgut long enough to compete with ODV that were being liberated from the OB in vivo, we then co-fed peptide and sucrose purified ODV. In
this manner, the virus and the potential peptide competitor are hypothesized to compete for
the same target, at the same time. The results of competition assays using a lethal dose of
virus and a molar excess of peptide are shown in Table 2. Insects fed peptide alone
displayed no detectable differences in insect growth or vitality compared to negative
controls. All insects receiving virus only succumbed to polyhedrosis, as did all insects fed a
mixture of virus and peptide. Thus, none of the peptides were able to prevent infection of the
insect at this dose of virus and peptide. In replicates co-fed the HV1 peptide and virus
however, it was noted that for almost 75% of the insects the survival time had increased.
These insects died as late fifth instars whose development had been delayed by the
baculovirus (the buffer fed negative control insects pupated several days before these insects
died). Chi-square tests showed these numbers to be significant (p <0.05) for the HV1
peptide. Inversely and unexpectedly, the presence of the C6 peptide resulted in a
significantly shorter survival time for the two replicates performed (Table 2).

We then assessed the ability of the HV1 peptide to compete with various doses of
ODV (Figure 5). At dose of 0.05 and 0.5 μg ODV, a similar effect of decreased mortality at
fourth instar was observed (Figure 5B). At an extremely high dose of 1.5 μg ODV, this
effect was lost, and survival time of the peptide treated insects was not significantly different
from that of insects fed virus alone. At the other end of the spectrum, at an LD_{50} dose of 0.06
ng, a significant decrease in larval mortality compared to the virus only treatment was
observed (Figure 5A).

**Peptide binding to midgut BBMV.** The ability of the synthetic peptides HV1,
AcE66-A, and C6, to bind to midgut BBMV from fourth instar *H. virescens* was assessed
using far-western blotting (Figure 6). Each blot contained a sample of BBMV, as well as,
samples from the low speed spin that pellets larger cellular particles in the generation of
BBMV, and the crude homogenate of the midguts. No binding was observed for the HV1
peptide to the BBMV, the low speed cell pellet or the crude homogenate. The AcE66-A
peptide, however, exhibited many bands ranging the length of the blot, in all three samples:
the BBMV, low speed cell pellet, and crude homogenate. The negative control peptide, C6,
exhibited no binding to any samples.
DISCUSSION

Isolated peptides similar to ODV-E66. This study utilized phage bio-panning with a random phage library to detect fifteen unique H. virescens gut-binding peptides. The random peptides expressed by the phage are very short- only 12 amino acids in length. As a result, when probed against the known sequence repository, short sequences will yield matches with low scores, and with E-values much greater than 1, even when identity is high. Thus, no significance can be assigned to the sequence homology between these phage peptides and the proteins with similarity, but must be tested through other means. That being said, two of the phage peptides selected by the bio-panning process (HV1 and HV2) had similarity to ODV-E66. \textit{adv-e66} is a core lepidopteran gene, conserved across the alpha and betabaculoviruses (Herniou \textit{et al.}, 2003), whose function in the ODV envelope is unknown. ODV-E66 contains a 33 aa N-terminal domain, the sorting motif (SM) that is sufficient to traffic the protein to the inner nuclear membrane (INM), which is conserved in many ODV envelope proteins (Hong \textit{et al.}, 1994; Hong \textit{et al.}, 1997; Braunagel \textit{et al.}, 2004). Neither of the phage peptides localized within this domain. In addition to a predominant 66 kDa form, a 60 kDa form of ODV-E66 has also been observed, and is believed to be a result of trypsin cleavage (Hong \textit{et al.}, 1994).

Interestingly, the peptides aligned to two regions within ODV-E66 across five different species of alphabaculoviruses. The alphabaculoviruses exhibit an ancient phylogenetic split into two groups, Group I and Group II; Group I contains 11 additional genes that Group II lack, and Group I utilizes GP64 as the fusion protein for BV fusion, unlike the rest of the baculoviruses which utilize the F protein for this purpose (Okano \textit{et al.}, 2006). The phage peptides HV1 and HV2 showed similarity to both Group I and Group II alphabaculoviruses, and a few residues were conserved even among the betabaculoviruses (data not shown). Three species of baculoviruses - \textit{Mamestra configurata} NPV- A (MacoNPV-A), \textit{Mamestra configurata} NPV – B (MacoNPV-B), and \textit{Agrotis segetum} NPV (AgseNPV)- all contain two copies of ODV-E66, though the duplicate copies are not believed to be gene duplication events but rather acquired independently (Herniou \textit{et al.}, 2003; Ijkel \textit{et al.}, 1999; Li \textit{et al.}, 2002a; Li \textit{et al.}, 2002b). The greatest sequence divergence in ODV-E66 was observed in the species that contain two copies of ODV-E66, suggesting the second copy
may be evolving new functions as it is no longer constrained to perform the function of a single ODV-E66 gene. The gut systems of the Diptera and Hymenoptera differ greatly from the Lepidoptera, and the baculoviruses from these species may contain a highly divergent and therefore unrecognized ODV-E66 homolog, as was recently shown for ODV-E18 (McCarthy & Theilmann, 2008). Also probable, ODV-E66 may have an essential function only in the Lepidoptera, reflecting co-evolution with specific host species as is well documented for the Baculoviridae (Herniou et al., 2004). Intraspecies polymorphisms have been observed in the C-terminus of ODV-E66 in the field isolates of MacoNPV-A, as well (Li et al., 2005).

The study of the nuclear trafficking of ODV-E66 found the C-terminus of the protein is exposed in the cytoplasm, which would also suggest an external exposure after envelopment (Braunagel et al., 2004). However, a yeast two-hybrid study found ODV-E66 binds to the major capsid protein, VP39, suggesting an internal localization (Braunagel et al., 1999). The trypsin cleavage of ODV-E66 observed by Hong et al., 1997 would also suggest an external exposure for the protein. We utilized several different software programs to examine the predicted transmembrane domains. Four high scoring transmembrane models were predicted. Two programs predicted a single transmembrane domain, the SM, as described before (Braunagel et al., 2004); however, a third identified 3 other significant transmembrane domains. These three domains were just beneath the significance threshold of the other two programs. In each of the models, both HV1 and HV2 (and the homologs AcE66-A and AcE66-B) were located outside of the membrane, and externally in three of the four predictions. The potential trypsin cleavage site (Hong et al., 1994) provides evidence for extracellular presentation of the majority of the protein based on these models. Alternatively, these hydrophobic domains may be completely independent of the membrane, and contribute to homo- or heteromer formation within the virion, or interact with gut structures or the cell. Until tested in the biological system, the reliability of the transmembrane domain motif software in correctly choosing the correct orientation of the cytoplasmic and surface loops is weak; the strength of each software prediction lies within identifying membrane-spanning motifs. Further work is needed to clarify the surface presentation of this protein.
AcMNPV ODV-E66 has hyaluronidase activity in High5 cells infected with the virus (Vigdorovich et al., 2007). Computational analyses have also indicated high similarity of ODV-E66 to hyaluronidase, an enzyme that can degrade hyaluronan, a polysaccharide component of the extracellular matrix (Vigdorovich et al., 2007). The residues 137 to 298 of AcMNPV ODV-E66 have a significant hit with the N-terminal alpha helical domain of the polysaccharide lyase family 8, which includes the hyaluronidases. Structural studies of the enzymes in this family indicate conserved alpha and beta domains that fold to form an active cleft in between (Jedrzejas et al., 2002). Hyaluronan is the major glycoaminoglycan (GAG) found on the PM of Bombyx mori, and thought to be the predominant GAG of the tsetse fly (Glossina mortens mortens), as well (Lehane et al., 1996; Nisizawa et al., 1963). Thus, the hyaluronidase activity of ODV-E66 could degrade this component of the PM, increasing ODV access to the microvilli of the midgut epithelia resulting in greater oral infectivity. It is unknown if both ODV-E66 homologs found in the MacoNPV- A and –B viruses maintain hyaluronidase activity. Hyaluronan is secreted directly from the plasma membrane, and binding to the hyaluronan on the cell surface may also contribute to overall increased binding and fusion of the virus.

**Synthesized peptides bind to midgut tissues.** We synthesized the two phage peptides with similarity to ODV-E66, HV1 and HV2, along with the sequence alignment homologs in AcMNPV (AcE66-A and AcE66-B) in addition to a negative control peptide C6, and a positive aphid gut binding peptide GBP3.1 described previously (Liu et al., 2010). Each of the peptides was qualitatively assessed for its ability to bind to cryosections of fourth instar H. virescens midgut tissue. HV1 and AcE66-A exhibited strong binding to the midgut tissues, followed by intermediate levels of binding exhibited by AcE66-B, GBP3.1 and HV2. The negative control peptide exhibited low-level binding above the buffer alone treatment. AcE66-B exhibited a weaker signal than its homolog HV1, and AcE66-A exhibited a stronger signal than its homolog HV2, which had the lowest signal except for the negative control peptide C6. The sequence divergence between HV2 and AcE66-A was greater than that of HV1 and AcE66-B, and the distinct difference in binding may reflect this. The lower binding of HV2 was also surprising, since this peptide had similarity to species of baculovirus in both region 1 and region 2. Aromatic residues including tyrosine,
phenylalanine, tryptophan and histidine are over represented in active sites that bind sugars, DNA and RNA, as are asparagines, glutamine, lysine and arginine. The side chains of these amino acids make planar projections that serve as hydrophobic platforms for sugar binding sites, and the greater exposed surface area of these residues allows greater hydrophobic interaction as well (Boraston et al., 2004; Cheng et al., 2009). Both the HV1 and GBP3.1 peptides bound to both aphids and H. virescens midguts. The life cycles, diet, midgut structure and pH of these insects differ radically, but the binding of these peptides suggests the presence of some common moieties on the surface of the midgut cells, and sugars would be likely candidates.

Of the six peptides tested for binding in this study, only the negative control peptide C6 was able to bind formalin fixed midgut epithelia; and its signal was not increased in unfixed tissues. The other peptides exhibited no binding under these conditions. Formalin causes cross-linking between terminal amino groups of basic amino acids with the nitrogen in the peptide bond; this cross-linking occurs both inter-and intra-molecularly (Nirmalan et al., 2008). N-glycosylation profiles remain unchanged between frozen and fixed specimens though appear present at lower intensity in the fixed samples (Tian et al., 2009). Likewise, trypsin cleavage was unchanged as determined by peptides ending in arginine or lysine, and formalin is thought to preserve phosphorylation as well (Krutzik & Nolan, 2003; Tian et al., 2009). The effects of formalin on other post-translational modifications, such as ubiquitination or sulfonation are unknown, but thought to be unchanged unless the samples remain in the formalin for excess time periods (Kiernan, 2000). The matrix of cross-linked proteins may limit the surface presentation of these other molecules on the cell surface. Thus, the exclusive binding of these peptides to unfixed tissues suggests a protein-protein interaction between the peptides (and possibly ODV-E66) and the midgut.

**Peptide competes with baculovirus for binding.** Following the bio-panning and identification of similarity to ODV-E66, we set out to assess the ability of the phage to interact with the midgut of H. virescens in various ways. Insects were co-fed phage and wild type polyhedra, and no effect on mortality was observed (data not shown). These results were not surprising, as the stability of the phage in the midgut milieu of proteases is unknown. Co-feeding polyhedra and the synthesized peptides showed no detectable
differences. When the *H. virescens* larvae were co-fed ODV and peptides, a significant effect was observed for HV1. Insects fed high doses of ODV and molar excess of the HV1 peptide exhibited a longer survival time. When assessed for effects across a broad range of ODV doses, a significant decrease in mortality was observed at an LD$_{50}$. At higher doses, an increase in survival time was detected until a dose of 1.5 µg of ODV. These results suggest that at low levels of virus infection, the HV1 peptide is able to compete with the virus for initial binding and fusion. Hypothetically, at higher levels of virus infection, the HV1 peptide prevents some of the virus from gaining entry into the cells, effectively reducing the virus load on the insect and increasing survival time.

It is unclear from these results if the HV1 peptide ligand is a receptor for the baculovirus *AcMNPV*, or is in close proximity to the baculovirus receptor, and stearically inhibits the virus from binding and fusion. The longevity of these peptides *in vivo* is also unknown. At higher viral loads, the decrease in competition may indicate degradation of the peptide at higher levels than degradation of the virions; after the peptide is degraded, intact virions would then be able to initiate infection. The loss of any competition as observed by decreased mortality or increased survival time observed at the high dose of 1.5 µg ODV, similarly could be the result of greater stability of the virions in the gut. An alternative hypothesis for all the results above is that the HV1 peptide can also bind to the ODV, either with ODV-E66 or other envelope proteins. It is unknown if ODV-E66 forms homo- or heteromers with other proteins.

Partial deletion mutants of *BmNPV ODV-E66* also resulted in increased survival time (Gomi *et al.*, 1999). This suggests inhibition of initial virus binding to host receptor(s); however, binding inhibition can be the result of several upstream events from dissolution of the polyhedra to ODV traversing the PM. *In vitro*, the HV1 peptide bound directly to midgut cells without any PM present; however, it is unknown if the *in vivo* decrease in survival time was a result of competition for a viral receptor. ODV-E66 may be only one of many ODV proteins interacting with the target receptor(s) to which HV1 bound. The entry of ODV into the columnar cells has been shown to involve a protein receptor; though midguts treated with N-glycosidase were still susceptible to baculoviruses (Horton & Burand, 1993). It is unknown if multiple host proteins function as receptors, or if the virus utilizes lipids, sugars,
or other naturally occurring molecules on the cell surface as co-receptors. Alternatively, the HV1 peptide may bind the hyaluronan present in the peritrophic matrix (PM), impeding the ODV from crossing the PM and entering the microvilli of the target cells of the midgut epithelia.

**Peptides bind to midgut BBMV proteins.** ODV-E66 has hyaluronidase activity, indicating it binds and degrades hyaluronan, a component of the PM (Vigdorovich et al., 2007). The peptide binding studies were performed using *H. virescens* midguts that had been rinsed free of the PM, suggesting the peptides were binding to ligands on the cell membrane or ligands very closely associated with the membrane. Hyaluronan is secreted from synthetases on the plasma membrane and the peptides could be binding to this form of hyaluronan; however, none of the peptides align with conserved residues implicated in binding within the cleft of the enzyme (Jedrzejas et al., 2002; Zhang et al., 2009). Microvilli contain many lipid rafts composed of cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)-anchored proteins, and other surface proteins are heavily glycosylated to protect against invading pathogens (Zhang et al., 2008). We then used Far-Western blotting to determine if these peptides were binding to specific proteins in fourth instar midgut BBMV preparations. As expected, the negative control peptide, C6, exhibited no binding. Interestingly, the HV1 exhibited no binding. If the target ligand of HV1 is a protein, it may require secondary, tertiary, or quaternary structures that are disrupted by the SDS-PAGE. Alternatively, the target ligand for HV1 may not be a protein or a moiety associated with a protein, thus binding will not be observed using this method. Both hypotheses would account for the binding to the cryosections of midguts and competition with baculovirus; although if a protein is not involved the lack of binding to formalin fixed tissues is not explained. The receptor(s) for ODV are unknown, and warrant further study, for which this peptide could be a very useful tool. The peptide AcE66-A, in contrast, exhibited binding to multiple protein bands at a variety of sizes in both the BBMV samples and the low speed cell pellets. The identified bands appear to be the same across all three samples, suggesting proteins that are associated with both the distal microvilli and the basal cell. The multiple bands suggest this peptide may be binding to a common moiety on all these proteins; post-translational modifications such as sugars would be a potential target.
Thus, AcE66-A appears to be binding to several proteins, accounting for the binding in the cryosections, but this binding did not impede AcMNPV infection in *H. virescens*. The identity of these proteins is unknown, and warrants further study to determine what the common target is.

**Peptide similarity to peptides from pathogens that interact with gut tissues.** The clones isolated from our phage bio-panning showed similarity to proteins of several pathogens that reside in the insect gut including *Leishmania, Trypanosoma*, and *Plasmodium* species. The HV1 peptide, WAVFSATL, which exhibited such strong binding to *H. virescens* midgut and several species of aphid guts, also exhibited high similarity to these species. The proteins with similarity were hypothetical proteins for these species, so we are unable to infer possible biological importance. However, each of these pathogens traverses an insect gut, and it is possible that these proteins play a role in the initial infection of the insect host. Without testing these peptides in these other insects, we cannot conclude anything beyond noting these similarities of short sequences. The ability of the HV1 peptide to bind both *H. virescens* and two species of aphids indicates the need and the potential to identify common features shared across insect species; these targets could be very useful to the development of novel tools with both medical and agricultural applications.

Overall, these results again show the utility of phage libraries in expanding our knowledge and developing novel tools. This same library was used to identify the SM1 peptide in *Anopheles stephensi* that can block infection by *Plasmodium berghei* (Ghosh et al., 2001) and the aphid GBP3.1 peptide competes with *Pea enation mosaic virus* for aphid gut binding (Liu et al., 2010). Interestingly, the mosquito gut binding peptide bound to fixed tissues (though the bio-panning was done via injection of live *Anopheles gambiae*). Use of this library against the pea aphid gut via feeding showed very different results; 4 rounds of panning limited the library to one dominant peptide both times the experiment was conducted (Liu et al., 2010). Two peptides were identified that bind alkaline phosphatase (ALP) in fixed midguts of *H. virescens* and compete with the Bt toxin Cry11Aa for binding using a nonamer phage display library (Fernandez et al., 2008). Each of these bio-panning experiments identified strongly binding peptides with very different amino acid sequence and secondary profiles. This adds credence to the diversity of the phage libraries used and the
diversity of the gut. At this time, receptors are known for only two of these gut-binding peptides (ALP) as stated above. The peptides from these studies could be targeting similar ligands, as lack of amino acid similarity does not preclude similarity of function. The results of this bio-panning experiment strongly suggest a role for ODV-E66 in the initial stages of ODV infection of the lepidopteran baculoviruses. We have identified a gut binding peptide, HV1, capable of impeding AcMNPV infection in *H. virescens* that may provide insight into the gut receptor(s) for this virus in this species. Further, we found that this peptide also binds to midgut tissue of aphids. Future work will be needed to assess the potential use of these peptides in identifying receptors and determining modes of action for pathogens and toxins in the midgut.

**MATERIALS AND METHODS**

**Insects and brush border membrane vesicle (BBMV) preparation.** *Heliothis virescens* (BioServe) were reared in a growth chamber on a 12 hr day: night cycle at a constant 28°C on artificial diet (Southlands Inc.). For bioassays, insects at third instar head capsule slippage were starved overnight and then inoculated with virus on a small diet plug. The following day, insects that had not consumed the entire diet cube were discarded, and food provided for the remaining insects. Larvae were inspected twice daily for mortality. For any questionable deaths, the cadavers were examined under light microscopy to confirm polyhedrosis.

For BBMV preparations, we used a modified method of Wolfersberger (1987). Briefly, newly molted fourth instars were dissected in MET buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris, pH 7.5) on ice. Midguts were removed, cut lengthwise, and rinsed free of the peritrophic matrix. Guts were rinsed one more time in fresh buffer, placed in a tube with MET buffer, then flash frozen in liquid nitrogen. Guts were stored at -80°C for up to six months. Guts were quickly thawed in a 37°C water bath, and then homogenized with 9 mL MET buffer, for 9 strokes at 3000 rpm using a Wheaton homogenizer. Then 9 mL of 24 mM MgCl$_2$ was added and homogenized for 2 strokes. A sample aliquot of the crude homogenate was taken, and then the remaining homogenate centrifuged for 2500 g for 30 minutes. The supernatant was decanted and spun at 30000 g for 1 hr. The resulting high
centrifugation pellet was resuspended in equal volumes of MET buffer and 24 mM MgCl₂ and the high- and low-speed spins repeated. The final high-speed pellet was resuspended in a small volume of 0.5X MET in water (100-200 μL) and 10-20 μL aliquots flash frozen in liquid nitrogen. These preparations were stored at -80°C for up to three months. The BBMV protein concentration was quantified by the Bradford method using a BioRad Protein Assay (Bio-Rad). The quality of the BBMV was determined using an assay of aminopeptidase (APN) activity. Samples were thawed, and cell pellet and crude homogenate diluted 1:10 to 1:50. Fresh 50 mM NaPO₄ buffer, pH 7.2, was made from 100 mM stocks of Na₂HPO₄ and NaH₂PO₄ solutions; 50 μL of buffer was placed in wells, with 2 μL of sample. Then 43.5 μL of NaPO₄ with 6.5 μL of 24 mM Leucine p-nitroanilide (in methanol) was added to each well. The samples were mixed, and read using a Vmax spectrophotometer (Molecular Devices) kinetic read at 420 nm for 5 minutes. The slopes were normalized for protein concentration, and BBMV whose APN activity was at least 8 fold greater than the crude homogenate were used for further study.

**Phage bio-panning.** The f88.4-LX8 phage display library (Bonnycastle et al., 1996) was used for bio-panning against the *H. virescens* gut. This library contains the random peptide XCXXXXXXXXCX fused to the N-terminus of the phage coat protein VIII, where X represents any amino acid. The C’s represent fixed cysteines that form a disulfide bond; this forces a loop structure, increasing surface exposure of the random peptide as displayed on the phage capsid. Phage was amplified and titered using K-91 *E. coli* (Invitrogen), and precipitated with 20% polyethylene glycol as previously described (Smith & Scott, 1993). Twenty microliters of phage stock (~1x10¹⁴) was incubated with 100 μg of BBMV preparation in total volume of 100 μL of sodium phosphate buffer pH 9.0 at room temperature for one hour. Unbound phage were then separated using a modified BRASIL method (Giordano et al., 2001). Briefly, the phage-BBMV mixture was overlaid on a single organic phase mixture of 9:1 di-butyl phthalate: cyclohexane, and spun at 10000 g for 10 minutes at 4°C. Unbound phage remains in suspension, while the bound phage pellets with the BBMV tissues. Immediately after centrifugation, the tubes were flash frozen in liquid nitrogen and then the bottom of the tube excised with a razor blade. This tube tip was placed in a sterile microcentrifuge tube with 300 μL of 50 mM glycine-HCL (pH 2.2) + BSA.
(1mg/ml), and the tissue gently resuspended by inversion and placed on a rocker for 10 minutes to elute the phage. The reaction was neutralized with 8 μL 2 M Tris pH 9.1. Fifteen microliters of this mixture was used for titration and the remainder immediately amplified as before. After amplification, this enriched pool of phage was titrated and used for the next round of bio-panning. After 3 rounds of successive binding/amplification, the eluted phage were incubated with K-91 E. coli and plated onto NZY plates containing 20 μg/ mL tetracycline. Individual bacterial clones were picked and amplified for genomic DNA isolation using standard methods (Sambrook & Russell, 2001). The sequencing primer (5’ CTGAAGAGAGTCAAAAGC-3’) was used to determine the sequence of the random peptide.

**Sequence Analysis.** The short peptides identified from the phage bio-panning were analyzed using BLASTP (Altschul *et al.*, 1997) optimized for short read sequences. The sequence of the internal 8 amino acids, as well as the complete 12 amino acid sequence (including the loop-forming cysteines at positions 2 and 11), was analyzed. Alignments were scored for identity and similarity. Following the discovery of phage similarity to the baculovirus protein ODV-E66, those peptide sequences were aligned with all available ODV-E66 sequences using ClustalW and BioEdit (Hall, 1999; Thompson *et al.*, 1994). Transmembrane predictions were calculated using several available software packages including HMMTOP (Tusnady & Simon, 2001), TMPRED (Hofmann & Stoffel, 1993), and TMHMM (Krogh *et al.*, 2001). Models with significant transmembrane domains are shown, in order of the strongest scores as calculated by the software. Hydrophilicity was examined using Protean prediction software (DNAStar Inc., vs. 5.0). ExPASy PeptideCutter was used to assess potential cleavage sites within ODV-E66 (Gasteiger *et al.*, 2003).

**Peptide synthesis.** The peptides were synthesized by Neo-Peptide, NeoBioPharma, Inc. (Cambridge, MA, USA), with N terminal 5-FAM tags with an ahx spacer. The sequences of the peptides were as follows; Aphid gut-binding peptide GBP3.1 TCSKKYPRSPCM-OH (Liu *et al.*, 2010), Negative control peptide aphid C6: FCRTADVIDACT-OH (Liu *et al.*, 2010), H. virescens gut binding peptides Hv1: SCWAVFSATLCT-OH, and HV2: HCSVWHVFAQCT-OH, AcMNPV ODV-E66 homologous peptides, AcE66B: ACDWYHFTITCT-OH, and AcE66A: TCLSYSFSQKCA.
Peptides were resuspended in PBS to a concentration of 14 mg/mL and stored in aliquots at -20°C until use.

**Cryosectioning.** *H. virescens* midguts were dissected in PBS buffer, pH 7.4, and rinsed free of the peritrophic matrix which lines the gut cavity. Whole midguts of the soybean aphid (*Aphis glycines*), the green peach aphid (*Myzus persicae*), and the pea aphid (*Acyrthosiphon pisum*) were dissected in PBS. The guts were then immediately placed in Tissue-Tek Optimal Cutting Temperature (O.C.T.) mounting medium (Ted Pella, Inc.) and frozen. Cryosections, approximately 20 µM in depth, were made using a Universal cryostat onto ProbeOn Plus slides (Fisher Scientific). Slides were air dried for two hours then washed two times with PBS for 5 minutes to remove residual O.C.T. The sections were circled with a PAP pen (Ted Pella, Inc.) to minimize the volume of reagents needed in the following steps. The sections were incubated with 71 or 142 µL of peptide solution at a concentration of 14 ng/µL in PBS (1000 ng of peptide) for 1 hour at room temperature in the dark chamber with a humidity source. Slides were again washed two times in PBS for 5 minutes. Slides were mounted using Fluoro-Gel (Electron Microscopy Services) and cover slips sealed with clear fingernail polish. Slides were stored at 4°C in the dark until viewed using a Zeiss Axioplan II fluorescence microscope using the FITC filter. Images were captured within 24 hours though still retained signal at one-week post assay. All samples were exposed for 145 ms at a magnification of 20X. For *H. virescens*, the experiment was performed using three to six cryosections from two insects in triplicate; the cryosections of the aphids were from two to three midguts in duplicate.

**Viruses.** Virus used in this study was AcIE1TV3.EGFP (Harrison *et al.*, 2010). Briefly, this virus has an additional gene, *egfp*, inserted near the polh locus, under the control of the immediate early -1 promoter. This virus is not significantly different than wildtype AcMNPV virus as indicated by bioassay and EM study (Sparks, 2010; Harrison *et al.*, 2010). Virus was amplified in vivo using proleg injection in fifth instar *H. virescens*. OB were isolated from cadavers using standard methods (O'Reilly *et al.*, 1992). ODV were isolated from the OB as described in Sparks (2010). Briefly, OB were treated with a dilute alkaline solution for ten minutes then neutralized. After one hour on an orbital shaker to release the ODV from the calyx, samples were centrifuged at low speed to pellet the calyxes, and loaded
onto sucrose step gradients for ultracentrifugation. The ODV bands were collected and washed in water, and resuspended in small volume of PBS.

**Phage peptide bioassay.** Third instar *H. virescens* showing head capsule slippage were starved overnight. Insects were droplet fed a mixture of virus (either polyhedra or purified ODV) and peptide in a 1-2 μL volume of PBS. Virus was at a dose of 2372 polyhedra (3X the LD₉₀ calculated in Sparks (2010) or 0.05-5 μg of ODV (LD₅₀ to excess lethal doses), and the peptide was at a dose of 2 μg. Only insects that consumed the entire dose of virus and phage were used for analysis. Diet was returned to the insects and the insects returned to the incubator where they were checked twice daily until pupation. Any questionable deaths were checked by light microscopy to confirm polyhedrosis.

**SDS-PAGE, western and far-western blotting.** BBMV proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN 3 cell (Bio-Rad) following the manufacturer’s instructions. Approximately 20 μg of fourth instar BBMV proteins were loaded onto a 12% SDS-PAGE gels along with ECL DualVue Western Blotting Markers (GE Healthcare) markers. The proteins were transferred to Hybond PDVF membrane (Amersham) using a BioRad Protean III Trans–Blot according to the manufacturer’s instructions. The membranes then blocked overnight in blocking buffer (GE Healthcare); and washed with TBS-0.1% Tween three times for 5 minutes with agitation. The membranes were incubated in the dark with 140 μg of each peptide in 0.1M sodium carbonate pH 9.0 at room temperature, then washed three times in TBS-0.1% Tween as before. The peptide binding was visualized using a Typhoon Imager (GE Healthcare) with an excitation of 457 nm, and emission filter of 532 nm.

**ACKNOWLEDGEMENTS**

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REFERENCES


### TABLES AND FIGURES

**Table 1.** Peptide sequences selected for *H. virescens* midgut binding by screening a phage display library. A. The 12 amino acid phage peptides. * indicates 2 clones were isolated with identical sequences. # indicated clones with high levels of homology. B. Alignments using BLAST. + indicates an amino acid mismatch of the same classification of amino acid, _ indicates a mismatch with a different classification amino acid. C. Baculovirus species abbreviations: *Mamestra configurata* NPV-A&B (*Maco* NPV), *Hyphantria cunea* NPV (*Hycu* NPV), *Agrotis segetum* NPV (*Agse* NPV), *Spodoptera exigua* NPV (*Se* NPV), *Adoxophyes orana* (AdorGV), *Choristoneura occidentalis* (ChorGV), *Cadra cautella* (CacaNPV), *Autographa californica* NPV (*Ac* NPV), *Neodiprion lecontei* NPV (*Nele* NPV), and *Bombyx mori* NPV (*Bm* NPV).

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Phage sequence</th>
<th>BLAST alignment to baculovirus proteins</th>
<th>Baculovirus Protein</th>
<th>BLAST alignments to other organisms</th>
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<tr>
<td>HV1</td>
<td>SCWAVFSATLCTA</td>
<td>W+VFS+TL</td>
<td>ODV-E66 Maco NPV A&amp;B</td>
<td>Bacteriophage, Leishmania, Trypanosoma</td>
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<tr>
<td>HV2</td>
<td>HCSVWHVFAQCT</td>
<td>VWHVF, HVFAQ, WHVF</td>
<td>ODV-E66 Maco NPV A&amp;B</td>
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<td>HV3</td>
<td>HCAYFPFWFPMC</td>
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<td>HV4</td>
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Table 2. Peptide bioassay with fourth instar *H. virescens*. Insects were starved overnight then droplet fed 1 μg ODV (AcIE1TV3.EGFP) and 2 μg peptide. These results are from 2-3 independent replicates of 10-15 insects each. *a* – only two replicates for this treatment. A Chi-square test was used to determine significant differences in survival time.

<table>
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**Figure 1.** AcMNPV ODV-E66 alignment of selected *H. virescens* gut binding peptides to AcMNPV ODV-E66. **A.** Phage peptides aligned to AcMNPV ODV-E66. The INM sorting motif is boxed, the hydrophobic domain shaded, charged amino acids bolded; the conserved N-terminal alpha helical domain of the polysaccharide lyase family protein 8 is underlined. The HV2 peptide aligns to two regions within ODV-E66 in two different species of baculovirus. **B.** Alignment of peptides and ODV-E66 sequences. The model species, AcMNPV, ODV-E66 sequence aligned with the phage peptides and the baculovirus species identified via BLAST. The line represents the division between Group I and Group II baculoviruses (an ancient evolutionary split). Both the A and B strains of MacoNPV contain two copies of ODV-E66 (open reading frames (ORF)78/77 and 144/143, respectively); SpexNPV also has two copies (ORF-57 and -114), while the single copy of AcMNPV ODV-E66 is encoded by ORF-43. The HV1 peptide has greater identity to ODV-E66B. The AcE66-A and B peptides were created based on the alignment of the phage peptides HV1 and HV2 to the AcMNPV sequence. The – represent spaces introduced for means of this figure, while – indicates a true gap inserted by ClustalW.

**A**

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</tr>
<tr>
<td>HV2 peptide</td>
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**AcMNPV ODV-E66**

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<tr>
<td>MacoNPV-B ORF-77</td>
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<td>MacoNPV-A ORF-144</td>
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<td>MacoNPV-B ODV-143</td>
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<td>SpexNPV ORF-57</td>
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<td>SpexNPV ORF-114</td>
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<td>HV1 peptide</td>
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**Figure 2.** Predicted transmembrane domains of ODV-E66. Transmembrane domains were identified using three different software packages. Only one strong transmembrane domain was predicted at the N-terminus beginning at amino acid 3; 6 putative transmembrane domains were found at positions 116, 185, 208, 294/296, 347, and 655. The four strongest models of TM topology are shown, with A favored by two programs, and B by a third. The phage peptides HV1 and HV2 are indicated by the solid and striped rectangles, respectively. Transmembrane domains are shown as white circles, and the external and internal portions of the protein shown respectively.
Figure 3. Topology predictions for *H. virescens* gut-binding peptides HV1 and HV2, their AcMNPV ODV-E66 homologs (A and B) and aphid gut-binding peptide (GBP3.1) and non-binding peptide (C6). (A) Alpha regions as predicted by Garnier-Robson and Chou-Fasman models, (B) Beta regions as predicted by Garnier-Robson and Chou-Fasman models, (T) turns as predicted by Garnier-Robson and Chou-Fasman models, (C) coils as predicted by Garnier-Robson models, (*) Alpha and Beta amphipathic regions (Eisenberg), (F) Flexible regions (Karplus-Schulz). Hydrophilicity plot (Kyte-Doolittle), antigenic index (Jameson-Wolf), and surface probability (Emini). The full 12 amino acids, including the cysteines, were used for topology predictions of the peptides.
Figure 4. Peptide binding to *H. virescens* midgut. **A.** Comparison of peptide binding to cryosections of *H. virescens* midgut epithelium. The fluorescently labeled peptides were incubated on cryosections of midgut tissues in sodium phosphate buffer pH 9.0 for 1 hour and viewed using a FITC filter, exposure 1 second. A bright field image at the same magnification is shown for comparison. **B.** Peptide binding to *H. virescens* and aphids *M. persicae* and *A. pisum* midgut tissue, exposure 154 ms. Bar indicates 100µM.
Figure 5. Increased survival time and reduced viral infection in *H. virescens* co-fed the HV1 peptide and ODV. A. ODV lethal dose curve and competition bioassay with the HV1 peptide. Striped bars indicate the effect of ODV alone, and solid bars, the effect of HV1 and ODV (AcIE1TV3.EGFP) when co-fed to fourth instars via droplet feeding. Standard error bars were calculated from 3 independent replicates of 12-15 insects each. B. Survival to fourth and fifth instar of *H. virescens* co-fed HV1 and ODV. Insects fed only ODV in gray, ODV + HV1 in black; checkered gray indicates *H. virescens* fed only ODV surviving to fifth instar, striped black indicate ODV+HV1 surviving to fifth instar.
Figure 6. Peptide binding to *H. virescens* midgut proteins. AcE66-A, C6, and HV1 were assessed for binding to BBMV, crude homogenate, and low speed pellet of cellular debris from homogenized midguts of fourth instar *H. virescens*. Black arrows indicate the strongest binding of AcE66-A to *H. virescens* midgut proteins. No binding was detected for C6 or the HV1 peptides in three independent experiments.
Chapter 4:  
General Conclusions

The occlusion-derived virus (ODV) of the alphabaculoviruses, and ODV interaction with the brush border microvilli of the columnar epithelial cells in the model organism *Heliothis virescens*, was the focus of the studies described in this dissertation. More than forty proteins are found in these complex virions that facilitate the most critical step in the virus life cycle: entry into the cell. The path from ingestion of occlusion bodies (OB) to delivery of the nucleocapsids (NC) to the nucleus is fraught with multiple obstacles for the virion as it encounters host defenses. A family of ODV envelope proteins contributing to the oral infectivity of the OB has been described. These proteins are called the *per os* infectivity factors (PIFs). The functions of these proteins are incompletely understood, and little is known about their collective effects. On the host side, viral receptors in the midgut have not been identified, and our knowledge of host immunity in the midgut is still limited.

Our examination of the ODV envelope protein ODV-E56 in Chapter Two of this dissertation showed that ODV-E56 is PIF-5, the sixth PIF protein to be described. Bioassays showed that *odv-e56* negative OBs were significantly less infectious than *odv-e56* positive virus. No significant differences were found in the infectivity of the BV, in agreement with a role for ODV-E56 in *per os* infectivity. Examination of the role of ODV-E56 using multiple approaches provided the following information: (1) Bioassays with *odv-e56*-disrupted ODV were significantly less infectious *per os* than the control viruses, (2) and the magnitude of the difference was much lower than that for the OB: two logs less infectious for *odv-e56*-disrupted ODV compared to five logs less infectious for the *odv-e56* disrupted OB. This difference suggests that ODV-E56 may function in the midgut as the ODV are released from the OB, move and cross the peritrophic matrix (PM), and come into contact with the brush border microvilli of the midgut columnar epithelial cells. Purified expressed ODV-E56 bound to a ~97 kDa protein in midgut brush border membrane vesicles (BBMV) from fourth instar *H. virescens*, suggesting an additional role for ODV-E56 at the cell surface. The *odv-e56* disrupted virus, however, showed no decrease in binding or fusion compared to the *odv-
e56 positive virus. Thus, binding of recombinant ODV-E56 to a midgut BBMV protein could represent an interaction with a host protein after binding and fusion of the virion, potentially one involved in cell signaling events. Fluorescence microscopy showed no eGFP expression for the odv-e56 disrupted viruses. Despite wild type levels of binding and fusion, the viral genome was not transcribed in the odv-e56 disrupted viruses. This result suggests a role for ODV-E56 in downstream events after viral fusion, which may include cell signaling to rearrange the actin filaments in the microvilli to transport the large nucleocapsid (NC) to the basal part of the cell (Charlton & Volkman, 1993). Hence, ODV-E56 appears to function both upstream and downstream of ODV binding and fusion with the midgut epithelial cell. Further study is needed to elucidate the role(s) of ODV-E56 in these aspects of ODV entry.

Chapter Three describes screening of fourth instar *H. virescens* midgut BBMV for binding partners using a random phage display library. Seventeen phage clones were isolated after three rounds of bio-panning against midgut BBMV. Two pairs of clones displaying identical peptides, as well as one pair displaying peptides of high similarity, were noted; the remaining clones displayed dissimilar peptide sequences. When compared to the known genome sequence repositories, these peptide sequences shared high similarity to several known pathogens, many of which are associated with insects, either as hosts or vectors for the pathogen. These pathogens included *Trypanosoma, Leishmania, Plasmodium*, as well as a gut specific virus of *Drosophila*.

Several phage clones displayed peptides that had high similarity to baculovirus proteins, and two peptide sequences, HV1 and HV2, had five hits to the ODV envelope protein ODV-E66. The function of ODV-E66 is unknown. The two phage peptide clones, HV1 and HV2, aligned to two different regions within the ODV-E66 protein (region 1 and region 2- Fig. 1B, Chapter 2), across five species of baculoviruses. Interestingly, the baculoviruses with peptide sequence matches were not species known to be highly infectious to our model host *H. virescens*. Alignment of ODV-E66 from the baculovirus species with hits with AcMNPV (the type species of the alphabaculoviruses that is highly infectious to *H. virescens*) showed the viral sequences in region 1 of ODV-E66 was more variable than region 2. Furthermore, both regions showed a distinct difference between the Group I and Group II alphabaculoviruses. The division of these two viral groups catalogs an ancient
evolutionary split, and variation in ODV-E66 is indicative of the divergence of these two groups. Also, some group II alphabaculoviruses contain two copies of ODV-E66 (thought to be acquired independently, and not the result of gene duplication), and this increased variability may be reflective of diversifying selection on the extra gene.

Synthesized versions of the peptides selected for *H. virescens* midgut BBMV binding were examined for their ability to bind cryosections of midgut from *H. virescens* and the midgut from two species of aphids. Binding of the peptides identified by the phage biopanning varied. The HV1 peptide exhibited a strong signal showing binding to *H. virescens* midgut, as well as, the guts derived from both aphid species. The HV2 peptide, exhibited much weaker binding to the *H. virescens* gut. This peptide was not tested for binding to the aphid gut. Peptides with homology to *AcMNPV* ODV-E66, AcE66-A and AcE66B (of regions 1 and 2, respectively), also showed variable binding, in a manner inverse to their phage peptide homologs: the homolog to HV1, AcE66-B, exhibited minimal binding to *H. virescens* midgut, whereas the HV2 homolog, AcE66-A, exhibited binding that was as strong as that of HV1. The aphid gut binding peptide, GBP3.1 bound to both aphid species as described previously (Liu et al., 2010), as well as to *H. virescens*. This result suggests that GBP3.1 may bind to a gut component that is common to the two orders of insects. A potential target could be a specific sugar present on the gut of both *H. virescens* and the aphid species. Several viruses target glycans or proteoglycans for use as receptors to mediate viral entry, including sialic acid used by influenza and heparin sulfate used by respiratory syncytial virus (Hallak et al., 2000; Skehel & Wiley, 2000). Other viruses utilize surface glycans for initial attachment to the cells, followed by more specific receptor interactions (Schneider-Schaudios, 2000). D-mannose is a sugar found in several hematophagous insects midguts, and monoclonal antibody specific for D-mannose was able to bind to mosquito midgut microvilli and block transmission of the malaria parasite *Plasmodium yoelii* (Dinglasan et al., 2003; Dinglasan et al., 2005). Mannose is the most abundant glycan in the aphid gut and has been implicated in the binding of luteoviruses (Banerjee et al., 2004; Rahbe et al., 1995). These five peptides were unable to bind to formalin fixed midgut tissues from *H. virescens*. A negative control peptide, C6, exhibited low levels of binding that was unaffected by the formalin fixation. Formalin results in the cross-linking of proteins,
suggesting the target ligand of the peptides in the \textit{H. virescens} midgut involved a protein. Much of what is known about the lepidopteran midgut comes from \textit{Bacillus thuringiensis} (Bt) toxin studies which show different species utilize different proteins as receptors and co-receptors. The \textit{H. virescens} midgut epithelial cells are rich in lipid rafts which are further enriched with cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI) anchored proteins, including aminopeptidases (APN) and alkaline phosphatases (ALP). Both a 120 kDa and 170 kDa APN in lipid rafts are putative Cry1a receptors, as well as GPI-anchored cadherin, and GPI-anchored ALP (Fernandez \textit{et al.}, 2006; Jurat-Fuentes & Adang, 2004; Zhang \textit{et al.}, 2008). Intracellular proteins, including actin and intracellular phosphatase, respond to aggregation of GPI-anchored proteins on lipid rafts (Krishnamoorthy \textit{et al.}, 2007; Magee \textit{et al.}, 2002; McNall & Adang, 2003). Whether baculovirus and Bt toxins utilize a common receptor is unclear; studies have shown increased susceptibility to baculovirus when co-inoculated with Bt (Schmidt \textit{et al.}, 2009). This may imply they do not share receptors, or that the Bt destroys the gut in a manner that allows the baculovirus to infect more readily or at lower doses.

When tested for their ability to compete with wild type \textit{AcMNPV} virus for binding to the gut epithelium, the HV1 peptide reduced mortality of \textit{H. virescens} fourth instar larvae at an LD\textsubscript{50} dose, and increased the survival times of infected larvae at higher doses of virus. These results suggest that this peptide is able to compete with wild type virus for cellular entry, although the effect is modest and/or may be short lived. ODV-E66 exhibits hyaluronidase activity and contains a conserved N-terminal alpha helical domain of the bacterial lyases (Vigdorovich \textit{et al.}, 2007). The HV1 peptide aligns within this domain which is also predicted to be on the exterior of the ODV based on transmembrane predictions. The implications of this are unclear; one possibility is that the HV1 peptide could be binding hyaluronan in the extracellular matrix or on the plasma membrane of the cell and impeding the movement or binding of the wild type virions. When tested for binding to fourth instar \textit{H. virescens} midgut BBMV proteins by far-western blot, the AcE66-A peptide bound to several midgut proteins but the HV1 peptide did not bind. Hyaluronan is secreted from synthetases on the plasma membrane surface, and the peptide could be binding to this protein, an unrelated protein, or to other sugars on the gut proteins in a non-specific
manner. Binding assays with these peptides and hyaluronan in its native form would address this hypothesis.

The studies of ODV-E56 and ODV-E66 suggest these proteins are involved in events of early viral infection both with the PM or movement of the ODV from the OB to the cells, and with direct interaction with the midgut cells. These studies, and earlier work, represent only the tip of the iceberg toward unraveling the mysteries of this virus. To date, only single deletion mutants of ODV-envelope proteins have been used to assess the role of a particular protein in oral infectivity. Each of the PIFs and ODV-E66 are conserved across the alpha and beta-baculoviruses. Not surprisingly, given this conservation and the critical role of ODV in the baculovirus life cycle, each deletion has resulted in a significant effect in at least one host species. These deletion mutants provide an appropriate starting point, but are limited in their scope. Likewise, proteomic analyses that have identified the components of the ODV envelope are also one-dimensional; they identify the presence or absence of a protein in a certain location in a certain species of virus. Future studies must begin to examine how these proteins work in concert with one another. Surface plasmon resonance will be invaluable toward this end, as will yeast two-hybrid systems. The greatest drawback to the study of deletion mutants is the inability to determine if the deletion phenotypes result from the lack of the protein that was deleted, a change in conformation of partner proteins, or both. Site-directed mutagenesis will be a valuable strategy for determining active sites for protein and receptor interaction, as well as, the residues that are critical to maintaining proper conformation. With this knowledge, the field can turn toward developing more precise assays of binding and fusion for the ODV, most likely requiring study in stable midgut cell lines.

Another point for consideration is the necessity to minimize host variation in the initial studies of ODV protein function. The host caterpillars exhibit increasing resistance both within an instar and throughout instars. Researchers should strive to use the same instars for consecutive studies combined with precise staging of insects. Once a standard has been set, an entire field opens up for comparison to the standard using different instars, different insects, and different viruses. Likewise, knowledge of the insect gut composition is
limited, as it too, is a very complex system that changes radically throughout the insect’s lifetime, and in response to different diets.

The phage bio-panning study, and the binding of the synthesized proteins across insect orders, highlights the need to look for commonalities in binding properties between pathogens of insects, and pathogens vectored by insects including those of medical importance. These similarities could prove to be very useful or very detrimental in the development of insecticides or medical tools, as both will require versatility and precision for practical applications. The next generation of insecticides needs to be environmentally friendly, both in terms of chemical by-products and targeting only the insect pests, and not non-target species such as the beneficial insects and other organisms present in the ecosystem. Midgut ligands will need to be assessed for their cross-species effects. If ligands for these peptides are conserved across multiple species of insects this could be exploited for developing broad acting insecticides, either through genetically modified viruses or conjugation with toxins. Using a peptide with a ligand found in all species of insects coupled with an insect specific toxin or virus would provide a means to using a ligand that is not pest specific. The peptides could be used to explore conserved molecular systems across species as well. Using a similar approach in mosquitoes has resulted in the engineering of a transgenic mosquito that expresses a gut binding peptide that inhibits malaria transmission (Ghosh et al., 2001). The gut binding peptides identified in Chapter 3 may also prove useful in helping thwart arthropod vectored diseases.

Baculovirus research has already made a significant contribution to greater scientific understanding, contributing to our understanding of apoptosis, the recognition of a novel virus fusion protein mechanism in the BV, and the co-evolution of viruses with their hosts, just to name a few. Commercially, baculovirus have been highly successful in limiting pestilence of caterpillars in three different crops of high value (Moscardi, 1999) and have been exploited for their high levels of protein expression, as well (Kost et al., 2005). The ability of these viruses to enter mammalian cells without initiating virus replication has placed them on a list of potential candidates for gene therapy (Kost et al., 2005). Currently, the development of baculoviruses as insecticides has expanded in China, where resistance to chemical insecticides is very high (Rohrmann, 2008). Recombinant viruses encoding fast
acting toxins have been used to overcome the largest impediment to effective use of baculoviruses, their slow speed to kill the insect which results in decreased crop yield (Sun et al., 2002 2005). Genetically engineered baculoviruses are as effective under field conditions as pyrethroid insecticides (Chen et al., 2000; Treacy et al., 2000), but have not been marketed due to competing natural product chemistries, and in some parts due to anti-GM sentiment. The contributions of the baculoviruses to scientific understanding and practical applications for both agriculture and medicine is far from over. Rather, future study of these viruses is warranted precisely for all the unknowns surrounding this large complex virus.

REFERENCES


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