The baculovirus *per os* infectivity complex

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ABSTRACT

Insect larvae are orally infected by baculoviruses through ingestion of proteinaceous occlusion bodies (OB) containing the so-called occlusion derived viruses (ODV). OBs disintegrate in the alkaline environment of the insect midgut releasing the ODV, which then bind and fuse with the microvillar membrane of epithelial cells thereby initiating infection. After replication and spread through the larval body, ODVs are assembled and occluded into OBs. In this thesis the protein structure of ODVs and their entry into microvillar cells were studied from the perspective of protein-protein interactions. A number of novel interactions were identified among ODV structural proteins shedding light on the spatial and temporal mechanism of ODV assembly. Furthermore, a group of highly conserved viral *per os* infectivity factors (PIF) was shown to form a complex on the ODV envelope. These PIF proteins are essential for oral infectivity of ODVs and the complex may play a pivotal role in binding and fusion of ODV with the microvillar membrane. It was further found that in the OB structure a host derived alkaline protease was tightly associated with ODVs and cleaved one of the PIF proteins (P74). Proteolytic processing of PIF proteins may be necessary to trigger conformational changes in the complex to facilitate its function in binding and fusion with the host cell membrane. This thesis provided not only novel insights on the mechanism of ODV entry and assembly and the role of individual ODV proteins, but also triggered new questions to direct future investigations.

CHAPTER 1

General Introduction

A distinctive feature of viruses is that they are obligate intracellular parasites meaning that their replication entirely relies on cellular machineries (132). The first step in the virus replication cycle is entry of virions into host cells. Entry is initiated through attachment of a virion to a host cell by interacting with molecules on the cell surface named receptors, which prepares the virus to proceed to the next step: penetration into the cell. For enveloped viruses penetration is achieved by fusion of the viral and host cellular membranes. Virus fusion can occur in two ways: 1) the viral envelope directly fuses with the plasma membrane of the host cell or 2) the enveloped virus enters the cell by endocytosis and forms vesicles in the cytoplasm. Subsequently the viral envelope fuses with the intracellular vesicle membrane to release its genetic load (141). For most enveloped viruses the latter fusion event occurs upon acidification. Both pathways result in the release of the viral genome into the cell, usually in the form of nucleocapsids. Via these forms viral genomes are then transported along the intracellular transport pathways to subcellular compartments in the cytoplasm (for most RNA viruses) or the nucleus (for most DNA viruses) to allow genome replication to occur (132).

After viral genome replication virus structural proteins are synthesized followed by assembly of progeny virus particles. Virus assembly is a sequence of events including formation of structural units of the nucleocapsid (the protective protein coat encapsulating the viral genome) from individual protein molecules, assembly of the nucleocapsid by interaction among the structural units, selection and incorporation of the virus genome, and, for enveloped viruses, acquisition of an envelope from host membranes (132). All these processes require considerable specificity, efficiency and coordination. Like in the process of virus entry, cellular machineries play diverse roles during virus assembly, assisting in folding of viral proteins, transporting viral components to specific sub-cellular compartments etc.

In this thesis the mechanisms of virus entry and assembly of an invertebrate DNA virus (baculovirus) is studied from the perspective of protein-protein interactions.

Introduction to baculoviruses

The family *Baculoviridae* comprises insect-specific rod-shaped viruses with closed, circular, double-stranded DNA genomes ranging from 80 to 180 kbp in size (137). The genomes are packaged in rod-shaped nucleocapsids, and the name "baculovirus" is in reference to the rodded shape. Baculoviruses have been isolated from more than 600 insect species belonging to the orders of Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanera, and Trichoptera (137). Baculovirus virions appear in two phenotypes (Fig. 1.1), the budded viruses (BVs) for systemic infection within the host and occlusion derived viruses (ODVs) for primary infection of larval hosts (127). The latter are occluded in a proteinaceous crystal structure to form virus occlusion bodies (OBs). Based on the morphology of OBs baculoviruses are divided into two groups: nucleopolyhedrovirus (NPV) and granulovirus (GV) (127). With the development of DNA sequencing techniques more baculovirus genome sequences became available and based on these sequences the family was divided into four genera: Alphabaculovirus (lepidopteran-specific NPV), Betabaculovirus (lepidopteran-specific GV), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV) (70).The genus Alphabaculovirus is further divided into two groups based on phylogenetic analysis: Group I NPV and Group II NPV (170). These two groups differ significantly in gene content, most notably Group I NPVs use the viral envelope glycoprotein GP64 as BV fusion protein, whereas Group II NPVs lack GP64 and utilize the F-protein instead, like the Alpha-, Beta- and Gammabaculoviruses (115).

Structure and infection cycle

The proteinaceous crystal structure of baculovirus OBs is composed of either polyhedrin for NPVs or granulin for GVs. OBs are highly stable and protect the embedded ODVs from the harsh conditions in the natural environment to retain their infectivity for a long time (127).

Only larvae are susceptible to baculovirus infection, moth is not. Baculovirus infection of insect hosts is initiated through OBs through the oral pathway by ingestion of food (e.g. leaves or fruits) contaminated with OBs (see Fig. 1.2 for baculovirus infection cycle). The latter then migrate into the insect midgut where the pH is alkaline, pH 9-11. Under this alkaline condition, the proteinaceous crystal structure of the OB dissolves and the embedded ODVs are released. They then penetrate the peritrophic membrane, a network of proteins and chitin that protects the midgut epithelium (62) against physical damage, bind and fuse with

the microvilli of midgut epithelium cells (more particularly the columnar cells) and initiate the first round of infection (137). In the infected epithelium cells BVs are produced, which then infect adjacent tissues or hemocytes to cause systemic infection of the host. Infected cells first produce BVs to further spread viral infection throughout the insect host. In later stages, cells produce ODVs, which are occluded into OBs that accumulate in large numbers in the nucleus of infected cells. When infected larvae die and liquefy huge amounts of OBs (up to 10^8 per larva) are released into the environment to spread the virus to allow a next round of larval infection.

Both ODV and BV are enveloped virus and they encapsulate nucleocapsids with identical compositions (137). However, the origin of the envelopes of ODV and BV is different due to differences in their assembly pathways. BV obtains its envelope from the plasma membrane of infected cells, when nucleocapsids bud out of the cells. ODV obtains the envelope from the inner nuclear membrane (INM) of the infected cells (18). Consequently, the lipid and protein composition (Fig. 1.1) of the envelopes of BVs and ODVs are different, and these differences are important in view of their distinct infection routes.

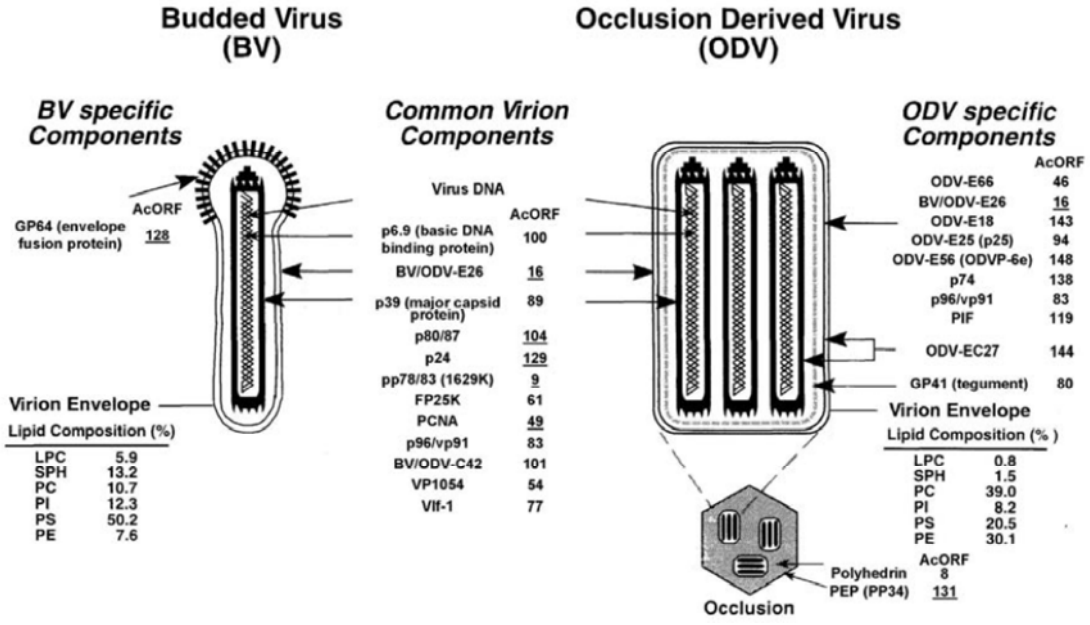

Figure 1.1. The two viral phenotypes of baculovirus. Schematic composition of the budded virus (BV) and occlusion derived virus (ODV) (adapted from (10, 16)). In this figure the *Autographa californica* (Ac) MNPV BV and ODV structures are presented. BV and ODV specific proteins are indicated on the left and the right, respectively. Proteins common for both phenotypes are shown in the center. The corresponding AcMNPV open reading frame (ORF) numbers are indicated (2), those underlined are not baculovirus conserved genes (63). Lipid composition of the BVs and the ODVs is derived from isolated virions of AcMNPV-infected Sf9 cells (17). LPC (lysophoshatidylcholine), SPH (sphingomyelin), PC (phosphatidylcholine), PI (phosphatidylinositol), PS (phosphatidylserine), PE (phosphatidylethanolamine).

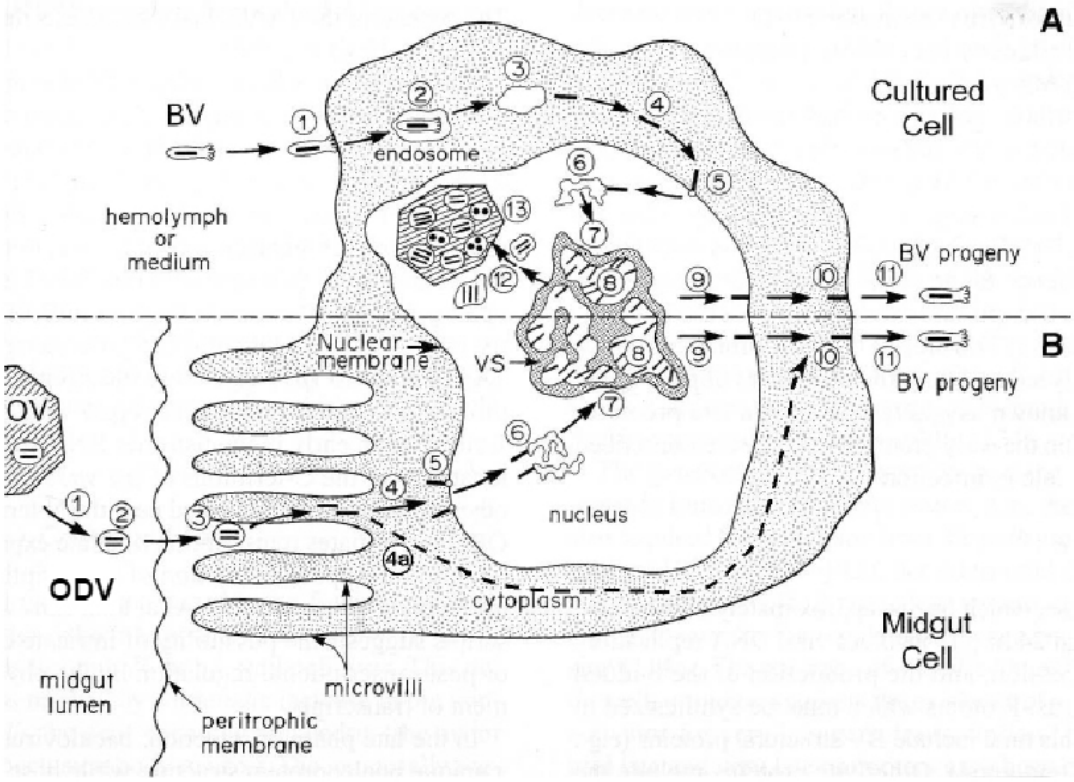

Figure 1.2. The baculovirus infection cycle (adapted from (103)).
(A) Infection route of BV. Infection is initiated by BV binding to the cell surface of cultured cells *in vitro* or body cells other than midgut epithelium cells *in vivo* (1), and virus enters through absorptive endocytosis (2). The endosomal and viral membrane fuse upon acidification of the late endosome, releasing the nucleocapsid (NC) into the cytoplasm (3).The NC is then transported to the nucleus in a cytoskeleton dependent manner (4), and enters the nucleus through nuclear pore (5). After entering the nucleus the core is released (6), and the viral DNA is transcribed (7), replicated and then packaged into newly formed NCs (8) within the virogenic stroma (VS). During the late phase of infection the newly formed NCs leave the nucleus (9) and travel to the plasma membrane (10) where the virus budding occurs and progeny BVs are produced to infect adjacent cells (11). During the late phase, the NCs are enveloped (12) and embedded in polyhedrin protein matrix to form OBs (13).
(B) Primary infection of insect midgut epithelium cells. Infection of the epithelium cells starts with the uptake of OBs (1), which dissolve in the alkaline environment of the midgut lumen, to liberate the occlusion derived viruses (ODVs). The ODVs pass the peritrophic membrane (2) and bind to the microvilli of the midgut epithelial cells (3). The NCs enter the cytoplasm through direct fusion (4). The remaining events are similar to that of the BV infection described above, except that little OBs are formed in the columnar cells of the midgut epithelium. Part of the incoming NCs may bypass the nucleus and transfer directly to the basolateral membrane to bud from the cell (4a).

Membrane proteins of BV and ODV

The major viral proteins of BV membranes are proteins that mediate binding and fusion. They differ in the two groups of alphabaculoviruses (127). In BV of Group I NPVs, GP64 is the major membrane protein. GP64 is a member of class III fusion proteins, which are characterized by the fact that they do not require proteolytic cleavage to be activated (127). Deletion of GP64 is lethal to the virus. Upon DNA transfection only single cell infections are

observed and no virus buds out to infect surrounding cells (104). GP64 is believed to mediate both receptor binding and fusion, but it was also suggested that GP64 can insert hydrophobic side chains into the cell membrane thus triggering endocytosis in the absence of specific cellular receptors (127). The lack of specific receptors and the affinity of GP64 for cell membranes could explain its ability to facilitate the entry of AcMNPV into a wide variety of cell types including mammalian cells (65). Such a feature seems to be consistent with the role of BVs in systemic infection since BVs should spread the infection to many different cell types. GP64 of group I NPVs is also important for egress from the cell envelope, where it accumulates as trimers connected by disulfide bonds. In Group II NPVs, the Fusion (F) protein is the major component of the BV membrane. F protein belongs to class I fusion proteins and its activation needs a cleavage event mediated by a furin-like protease or proprotein convertase (160). F protein also mediates both receptor binding and virus fusion. After synthesis both GP64 and F proteins are transported to the plasma membrane where nucleocapsids bud out to acquire an envelope. Unlike GP64, F protein is also found to be present on the ODV, at least in AcMNPV and *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) (16, 119), indicating that these proteins are also translocated to inner nuclear membrane during infection. Whether the presence of F-protein on the ODV envelope has a functional significance is still unknown.

Compared to BV, the protein composition of the ODV membrane is much more complex (Fig. 1.3). Several recent proteomic analyses from different baculoviruses revealed that there are more than 10 different virus-encoded membrane proteins residing in the ODV membrane (16, 33, 119). For AcMNPV ODV these proteins are AC16 (BV/ODV-E26), AC46 (ODV-E66), AC94 (ODV-E25), AC109 (ODV-EC43), AC143 (ODV-E18), AC22 (PIF-2), AC96 (PIF-4), AC115 (PIF-3), AC119 (PIF-1), AC138 (P74), AC145, AC148 (ODV-E56, PIF-5) and AC150. Since ODV envelopment takes place inside the nucleus by membranes derived from the INM these proteins need to be translocated to the INM (18). It was suggested that the continuous membrane system formed by the endoplasmic reticulum (ER), the outer nuclear membrane and the INM functions in the trafficking of these ODV membrane proteins (18). Upon accumulation of ODV membrane proteins in discrete regions in the inner nuclear membrane these viral protein enriched regions bud out into the nucleoplasm to form intranuclear membrane microvesicles for ODV envelopment (18).

Since ODV is the viral form specifically adapted for primary infection of midgut epithelium cells, these membrane proteins are expected to play important roles in initiating

virus entry and infection, and in host range determination (137). Several of these ODV membrane proteins were shown to be essential for oral infectivity and were named *per os* infectivity factors (PIF) (see below). Further information on the function of these proteins in virus entry is lacking, partly due to the fact that research on ODV entry is severely hampered by the lack of a cell line that allows ODV infection.

The PIF proteins

Among the ODV membrane proteins six PIFs have been identified. These PIF proteins are P74 (PIF0), PIF1, PIF2, PIF3, PIF4 and PIF5 (42, 45, 58, 77, 107, 120). Deletion of any of these genes encoding these proteins has no effect on virus replication *in vitro*, but dramatically impairs the ability to infect host larvae. P74, PIF1 and PIF2 were found to function in ODV binding, and individual deletion of the corresponding genes reduced the binding ability of ODVs to about 1/3 of the wild type virus (57, 107). However, infectivity of these deletion mutants was much more severely impaired, indicating that these proteins have additional functions besides mediating binding. The function of PIF3, PIF4 and PIF5 are unknown. Earlier EM analysis suggested that ODV fuses directly with the microvilli of

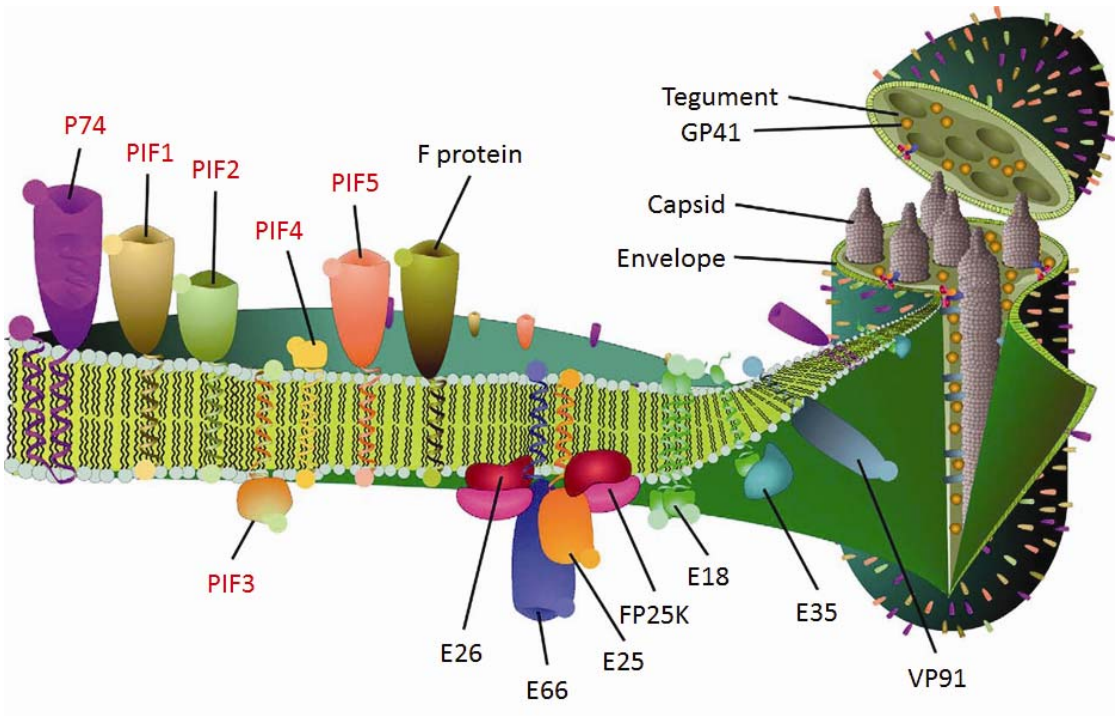

Figure 1.3. Structural model of AcMNPV ODV (adapted from (137)). AcMNPV ODV is an enveloped virus containing multiple nucleocapsids and there is a tegument structure between nucleocapsids and envelope. The integral or associated membrane proteins of ODV are indicated and the identified PIFs are shown in red.

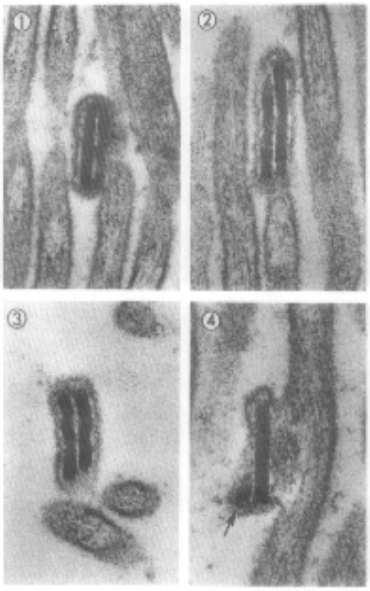

Figure 1.4. EM images showing ODV entry as published in (74). (1) A *Rachiplusia ou* nuclear polyhedrovirus (RoNPV) ODV particle adjacent to the microvilli of the midgut epithelium cells of *Trichoplusia ni*larvae. (2) A diffuse juncture between the RoNPV ODV envelope and the microvillus membrane resulting from a putative tip-to-tip interaction. (3)Electron micrograph suggestive of confluence between viral envelope and microvillus membrane. (4) RoNPV ODV nucleocapsids in the process of entering the microvillus after side-to-side fusion of the viral envelope and the microvillus membrane. The arrow indicates part of a possible second nucleocapsid present in this section.

midgut epithelium cells (Fig. 1.4) (54). But so far none of these PIFs was found to function in ODV fusion. In view of this it was suggested that certain host proteins may mediate the ODV fusion process (107). Considering that at least three PIFs function in binding, it was speculated here that PIFs form a complex and work in concert to facilitate ODV entry. At the start of this thesis research, such a macromolecular complex on the ODV membrane has not been identified. Nevertheless, it is clear that the ODV entry mechanism is complicated involving a number of protein-protein interactions among viral and cellular proteins.

A distinct characteristic for all 6 PIFs is that they are encoded by genes conserved in all sequenced baculovirus genomes so far. They belong to the so-called baculovirus core genes (151). More interestingly, it was recently revealed that all PIFs have homologs in nudiviruses (156) and polydnaviruses of braconid wasps (8). P74, PIF1, PIF2 and PIF3 are also conserved in salivary gland hypertrophy viruses (proposed family *Hytrosaviridae*) and in the crustacean white spot syndrome virus (Family *Nimaviridae*) (154). This high level of conservation suggests that these proteins were incorporated into the virus structure early in evolution and that their functional mechanisms are conserved among these large, double-stranded, nuclear replicating invertebrate DNA viruses.

Virus entry complexes

It is common among enveloped viruses with multiple membrane proteins functioning in binding and/or fusion that these proteins form a complex and work in concert to facilitate virus entry. These complexes have been accordingly named virus entry complexes (21, 23,

161). During virus entry these complexes undergo sequential conformational changes to expose functional domains to mediate binding and fusion in a coordinated order and fashion. There are two well-documented examples from vaccinia virus and herpes simplex virus (HSV) with entry complexes containing nine or four proteins, respectively (23, 161). Current knowledge of the entry complex of HSV and its functional properties are briefly reviewed here (see also Fig. 1.5) as the mechanism could be insightful to the function of a potential baculovirus ODV entry complex.

The entry of HSV into host cells involves a series of molecular events mediated by an entry complex composed of four virus glycoproteins: gD, gB, gH and gL (23). gD is the major receptor binding protein and mediates HSV binding with at least three different host receptors: nectin 1, herpes virus entry mediator (HVEM) and specific O-sulphated moieties on heparan sulphate (HS) (23). Upon binding with the receptors gD undergoes conformational changes and recruits gB and gH·gL to form a complex. This process is likely to be the triggering step of HSV fusion (21). gB and gH·gL together form the highly conserved core fusion machinery. Both gB and gH contain elements that are typical for fusion proteins but none of them could mediate complete fusion on its own (21). It is therefore unknown which of the two proteins is the fusogen. One possibility is that these two proteins work in concert to mediate virus fusion and this mechanism would be unique compared to other current models of virus fusion. The soluble glycoprotein gL forms a hetero-dimer with gH. This interaction is essential as it enables gH to adapt a functional conformation and to be translocated from the endoplasmic reticulum to the plasma membrane (21). Whether gL is merely a chaperone for gH or whether it also plays additional roles in HSV entry is unknown. Despite decades of

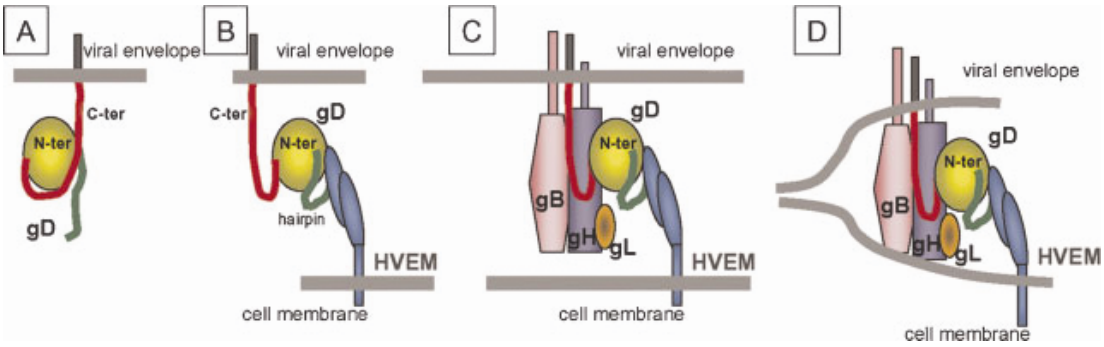

Figure 1.5. Model of HSV entry mediated by the quartet entry complex (adapted from (21)). (A) Before receptor binding gD adopts a closed conformation, where the C-terminal (C-ter) region of the ectodomain wraps around the N-terminal (N-ter) region, shown as an oval. (B) Upon HVEM binding, gD adopts an opened conformation, where the C-terminal region of the ectodomain is displaced from its binding sites on the N-terminal region. The N-terminus then forms a hairpin. (C) The receptor-bound gD recruits gB and gH·gL to form a complex. (D) gB and gH·gL execute fusion of the virion envelope with the cell membrane.

intensive investigation from the perspectives of biochemistry and structural biology, the detailed fusion mechanism of HSV is still rather elusive probably due to the complexity of this event, which involves several proteins.

Proteolytic activation

Another common feature in the entry mechanism of many enveloped viruses is proteolytic activation of the viral membrane proteins. In principle, the proteolytic cleavage converts a proprotein into an active conformation and/or exposes the functional domains, e.g. the fusion domain, to mediate virus binding and/or fusion (145). These proteolytic cleavage events fall into three major schemes (Fig. 1.6). Cleavage may happen at the very early stage of virus entry i.e. when virus is binding to host cells. For example influenza virus hemaglutinin (HA) is cleaved by human airway trypsin-like protease (HAT) present on the cell surface during virus binding (12). Alternatively, the protein is cleaved later in virus entry, i.e. during virus fusion with intracellular membranes. One example of this mechanism is the

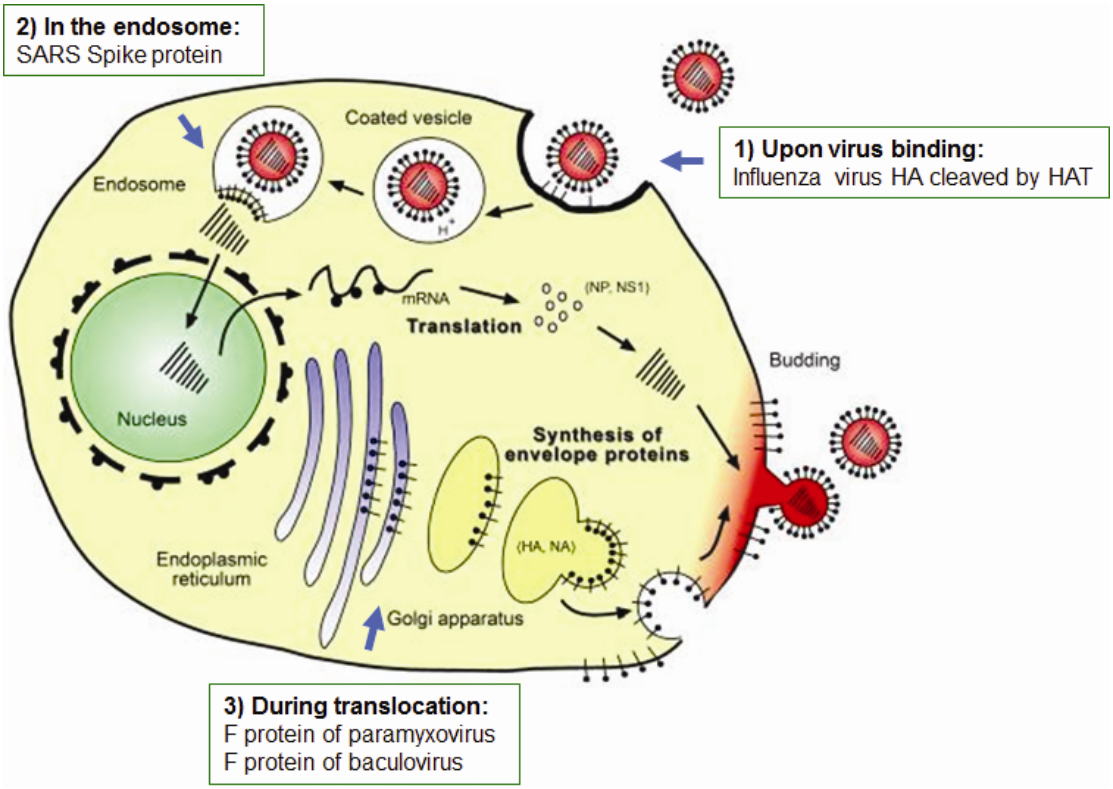

Figure 1.6. Three common mechanisms of virus membrane protein proteolytic cleavage (picture adapted from (26)). Virus membrane proteins undergo proteolytic cleavage to gain functional conformations and depending on the virus species cleavage of membrane proteins can happen either: 1) when the virus is bound to the cell surface by cell surface proteases; 2) during fusion in the endosome by endosomal proteases; or 3) during viral protein translocation from the endoplasmic reticulum to the Golgi apparatus.

cleavage of SARS-Corona virus (SARS-CoV) S protein by cathepsin L in the endosome (136). The third mechanism is post-translational cleavage of proproteins during protein transport through the *trans*-Golgi network. An example is the F protein of many paramyxoviruses, which is cleaved by the ubiquitous subtilisin-like protease furin during its transportation through the *trans*-Golgi network (141).

Baculoviruses also employ similar proteolytic processing mechanisms to activate viral membrane proteins to facilitate infection. Like the F protein of paramyxoviruses, the F protein of *Spodoptera exigua* (Se) MNPV BV is post-translationally cleaved by furin and this cleavage is essential for the infectivity of BV (159). In ODV the P74 protein of AcMNPV, a highly conserved binding protein of ODV, was reported to be cleaved by insect midgut trypsins after ODV release. Mutation of the cleavage site on P74 dramatically reduced the *per os* infectivity of the recombinant virus (139).

Besides proteolytic activation of BV and ODV membrane proteins, baculovirus may contain another mechanism of proteolytic processing, which is associated with OB. For a number of baculoviruses, including AcMNPV, an alkaline protease was found to be associated with larvae-derived OBs (L-OBs) (27, 36, 79, 80, 114, 146, 162). This protease was suggested to function in the degradation of the major matrix protein of OBs (polyhedrin or granulin) and/or to assist in the release of ODVs (114, 146). However, the functional significance of this alkaline protease to virus infection is not fully understood yet. Recently, Slack and Arif (2007)(137) envisioned that this OB-associated alkaline protease could play a synergistic role in ODV infection through proteolytic activation of released ODVs, but supporting experimental evidence is lacking.

OUTLINE OF THE THESIS

Commensurate with the relatively large genome, baculovirus ODV has a multicomponent structure containing more than ten different membrane proteins. At the same time ODV entry most likely occurs under a highly alkaline condition as it occurs in the larval midgut, while the current models of virus entry, mainly from vertebrates, are documented under neutral or low pH conditions. This thesis aims to gain novel insight in the molecular mechanism of ODV entry in the alkaline environment of the insect midgut and in ODV assembly from the perspective of protein-protein interactions.

To gain more insight in the ODV assembly mechanism, protein-protein interactions between known ODV components of *Helicoverpa armigera* nucleopolyhedrovirus

(HearNPV), a group II alphabaculovirus are analyzed in **Chapter 2**. To this aim, a matrix yeast-two hybrid (Y2H) screen is performed with 39 proteins leading to identification of a number of novel interactions. Interactions among conserved proteins are likely to be important for the formation of conserved structural subunits, such as the nucleocapsid during ODV assembly while interactions among group II specific ODV components might be important for assembly mechanisms specific to this group of baculoviruses or may not be required for assembly *per se*, but may have roles at subsequent steps such as OB formation or virus entry.

Due to limitations of the chosen Y2H system only truncated forms of membrane proteins (without hydrophobic domains) could be used for the protein interaction detection. Nevertheless, a number of interactions were identified between truncated ODV membrane proteins, however, with limited fidelity. Knowledge of ODV membrane protein interactions is essential not only to understand ODV assembly but also to unravel the ODV entry mechanism, which is mediated by the coordinated actions of a set of ODV membrane proteins, more specifically the PIF proteins. Investigation of the interactions between these PIF proteins is the subject of **Chapter 3.** Multiple strategies are used including cross-linking, co-immunoprecipitation, and SDS-PAGE with differential denaturing conditions, to analyze whether the four highly conserved PIF proteins, P74, PIF1, PIF2 and PIF3, form a complex on the AcMNPV ODV surface.

During this PhD study it became apparent that the ODV entry mechanism is probably more complicated than previously thought as new PIFs were being identified in recent years. In **Chapter 4** the aim therefore is to identify the components of the complete PIF complex. Co-immunoprecipitation analysis coupled with proteomic analysis is used to identify components of the complete PIF complex. Results are verified by deletion mutagenesis, Co-immunoprecipitation analysis and Blue-native PAGE electrophoresis.

Besides dynamic interactions within the virus entry complex, conformational changes in virus binding and/or fusion proteins may also be essential for these proteins to function in virus entry. In many cases these conformational changes are triggered by proteolytic cleavages, which may expose functional domains embedded within these membrane proteins. It was noticed in this thesis that P74 was often present in a cleaved form when ODVs were released from OBs derived from larvae, and in **Chapter 5** the mechanism behind this P74 cleavage was investigated.

In **Chapter 6** the overall results from the above chapters are discussed in relation to the mechanisms of baculovirus ODV assembly and entry. A model of the ODV entry complex and the sequential events during ODV entry is proposed. Suggestions are provided for future work to further improve the understanding of these mechanisms.

CHAPTER 2

Identification of protein-protein interactions of the ODV associated proteins of HearNPV

Abstract

The purpose of this study is to identify protein-protein interactions among the components of the occlusion-derived virus (ODV) of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV), a group II *Alphabaculovirus* of the family *Baculoviridae*. To achieve this, 39 selected genes of potential ODV structural proteins were cloned and expressed in the Gal4 yeast two-hybrid (Y2H) system. The direct cross Y2H assays identified 22 interactions. These included 13 binary interactions that of HA9-ODV-EC43, ODV-E56-38K, ODV-E56-PIF3, LEF3-Helicase, LEF3-alkaline nuclease (AN), GP41-38K, GP41-HA90, 38K-PIF3, 38K-PIF2, VP80-HA100, ODV-E66-PIF3, ODV-E66-PIF2 and PIF3-PIF2, and 9 self-associations including that of IE1, HA44, LEF3, HA66, GP41, CG30, 38K, PIF3 and P24. Five of these interactions, the interactions of LEF3-Helicase and LEF3-AN, and the self-associations of IE1, LEF3 and 38K, had been previously reported in *Autographa californica* MNPV (AcMNPV). Since HA44 and HA100 are the two newly identified ODV proteins of group II viruses, their interactions were further confirmed. The self-association of HA44 was verified with His pull-down assay and the interaction of VP80-HA100 was confirmed by co-immunoprecipitation assay. A summary of so far reported protein-protein interactions of baculovirus including 68 interactions with 45 viral proteins and 5 host proteins is presented, which will facilitate our understanding of the molecular mechanisms of baculovirus infection.

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Introduction

Baculoviruses are members of the *Baculoviridae*, a family of double-stranded DNA viruses that specifically infect arthropods of the insect orders *Lepidoptera*, *Hymenoptera* and *Diptera* and are divided into four genera, the *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* as first suggested by Jehle et al (70). The Alphabaculoviruses are subdivided into group I and group II based on phylogenetic analyses (63). A characteristic feature of the *Baculoviridae* is that they produce two viral phenotypes: the occlusion-derived virus (ODV) and the budded virus (BV). Packaged in a protein crystal-like structure called an occlusion body (OB), ODV are responsible for oral infection. From the structural point of view ODV is quite different with BV. Although BV and ODV appear to share a similar nucleocapsid structure, ODV obtains its envelope from membranes within the nucleus (18) while BV obtains its envelope when it buds through the modified plasma membrane (17). Consequently, the composition of envelope proteins of ODV are very different from that of BV (16, 33, 119, 137). In addition to the nucleocapsid and envelope, ODV has a tegument layer between them (137). The structures of ODV and BV are correlated to their functions; therefore, it is important to identify the structural components of ODV and BV to elucidate the molecular mechanisms of baculovirus infection.

Recently, the ODV protein composition of four baculoviruses have been determined by proteomic studies, that of *Autographa californica* nucleopolyhedrovirus (AcMNPV) (16), *Culex nigripalpus* NPV (CuniNPV) (119), *Helicoverpa armigera* NPV (HearNPV) (33) and *Bombyx mori* NPV (BmNPV) (165). These studies showed that ODV contains a number of proteins ranging from 20 to 41 and provided useful information for prediction of ODV assembly and structure. ODV assembly is presumed to be a complex and precisely organized process including viral DNA packaging, nucleocapsid assembly, and ODV envelopment which involves a number of protein-DNA and protein-protein interactions. Therefore identifying interactions among viral proteins would be very beneficial for interpreting the molecular mechanisms of viral assembly and virus infection.

In this report we describe the identification of interactions among ODV proteins by using HearNPV, a group II alphabaculovirus, as a model. To achieve this, the proteins that have been shown to be likely HearNPV ODV components (33, 89, 110) or HearNPV homologs of AcMNPV ODV components (16, 165) were cloned into yeast two-hybrid (Y2H) vectors and screened for protein-protein interactions. Two of the identified interactions were further verified by pull-down or co-immunoprecipitation analyses. The results were combined

with baculovirus protein-protein interactions reported by others in order to summarize our understanding of the protein interactome of baculovirus.

Materials and methods

Virus, cells and antibodies

An *in vivo* cloned strain of HearNPV (HearNPV G4) (63) was used as the wild-type virus. HzAM1 cell line derived from *Heliothis zea* (98) was used in the pull-down and co-immunoprecipitation assay. Antibodies of anti-HA44, anti-HA100 and anti-VP80 used in this study had been previously described (33).

Yeast two-hybrid assays

Thirty-nine proteins used in the Y2H study were listed in Supplemental Table 2.1. The TMHMM software (http://www.cbs.dtu.dk/services/TMHMM) was used to screen the potential TM domains as well as the inside and outside orientation of the ORFs. The entire ORFs of the twenty-nine proteins which do not contain predicted transmembrane domain (TM) were amplified from HearNPV G4 genome (63) using primers listed in Supplemental Table 2.1. For the 10 proteins which contain potential TM, primers were designed to generate the truncated ORFs without TM (Table 2.1 and Fig. 2.1). PCR products were purified and digested with corresponding restriction enzymes and then ligated into pGBKT7 and pGADT7 vectors (Clontech, Matchmaker Two-Hybrid System 3). Clones were verified with enzyme digestion and sequencing. The Y2H assays were performed according to the protocol of the manufacturer. In principle, the yeast strains of bait were constructed through transformation of bait clones (pGBKT7 clones) into yeast strain AH109 (Clontech) according to the described method (51) and selected on –Trp SD plates. For Y2H, each yeast strain of bait was transformed individually with each prey clone (pGADT7 clone) and selected on -4 SD (-Trp, -Leu, -Ade, -His) plates. Each pair of positive combinations was repeated twice in Y2H and the negative combinations were repeated once.

Confirmation of HA44 self-association by His pull-down assay

To confirm the HA44 self-association, *ha44* was PCR amplified from HearNPV G4 genome with following primers:

HA44-PD-F: GCG**GAATTC**ATGAGCAATCCCAGCAAACAATC and HA44-PD-R: GCG**GAATTC**TCAATAGCGCAAACGAGTTTCG (the bold and underlined nts indicate *Bam*HI digestion sites). The PCR product was purified and cloned into pET28a vector (Novagen) and the correct clone was transformed into *E. coli* BL21 strain to express His tagged HA44 (His·HA44). The soluble His·HA44 was purified through affinity chromatography using Ni-NTA resin (QIAGEN). To perform pull-down assay, 3×10^6 HzAM1 cells were infected with HearNPV G4 at an MOI of 5. At 36 h post infection, the infected cells and the control uninfected cells were washed with PBS for three times and incubated in 1 ml PD buffer (1×PBS, pH7.2 / 10% glycerol / 1% TritonX-100 / Cocktail, Sigma) on ice for 30 minutes. The swollen cells were then collected and sonicated in ice-cold water bath and centrifuged at 14,000 rpm for 15 min at 4℃. The supernatant was then mixed with 50 ug purified His·HA44 and incubated at 4℃ with gentle agitation for 3 h. Then 20 ul Ni·NTA resin was added to the mixture and incubated at 4℃ for another 1 After washing with washing buffer (20 mM Tris-HCl, pH7.9 / 300 mM NaCl / 30 mM imidazole / 10% glycerol) for three times, the protein was eluted from the resin with elution buffer (20 mM Tris-HCl, pH7.9 / 300 mM NaCl / 200 mM imidazole / 10% glycerol). Experiment was also performed in the absence of His·HA44 simultaneously to set up the negative control. Samples were then separated on SDS-PAGE followed by Western blot with anti-HA44 antiserum (33).

Confirmation of the HA100-VP80 interaction by a co-immunoprecipitation assay

For each experiment, 3×10^6 HzAM1 cells were infected with HearNPV G4 at an MOI of 5. At 36 h post infection, cells were first washed with PBS for three times and then incubated in 1 ml TEP buffer (1×PBS, pH7.2 / 2 mM EDTA / 10% glycerol /1% TritonX-100 / Cocktail, Sigma) on ice-cold water bath for 30 min. The swollen cells were collected and sonicated on ice-cold water bath. Samples were then centrifuged at 14,000 rpm for 20 min at 4℃ and the supernatant was mixed with indicated antiserum for 3 hours at 4℃ with gentle agitation. Then 20 ul protein A agarose beads (Calbiochem) were added to the mixture and incubated for another 1 hour at 4℃ with agitation. The beads were then collected through centrifugation and washed three times with TEP buffer. The bound proteins were eluted from the agarose beads with E buffer (10 mM glycine / HCl pH 3.0, and 0.01% sodium azide) and immediately neutralized with equal amount of N buffer (500 mM Tris Base, 0.01% sodium azide). Samples were then boiled in SDS-PAGE sample buffer and subjected to SDS-PAGE followed by Western blotting with anti-VP80 or anti-HA100 antiserum (33) as the primary

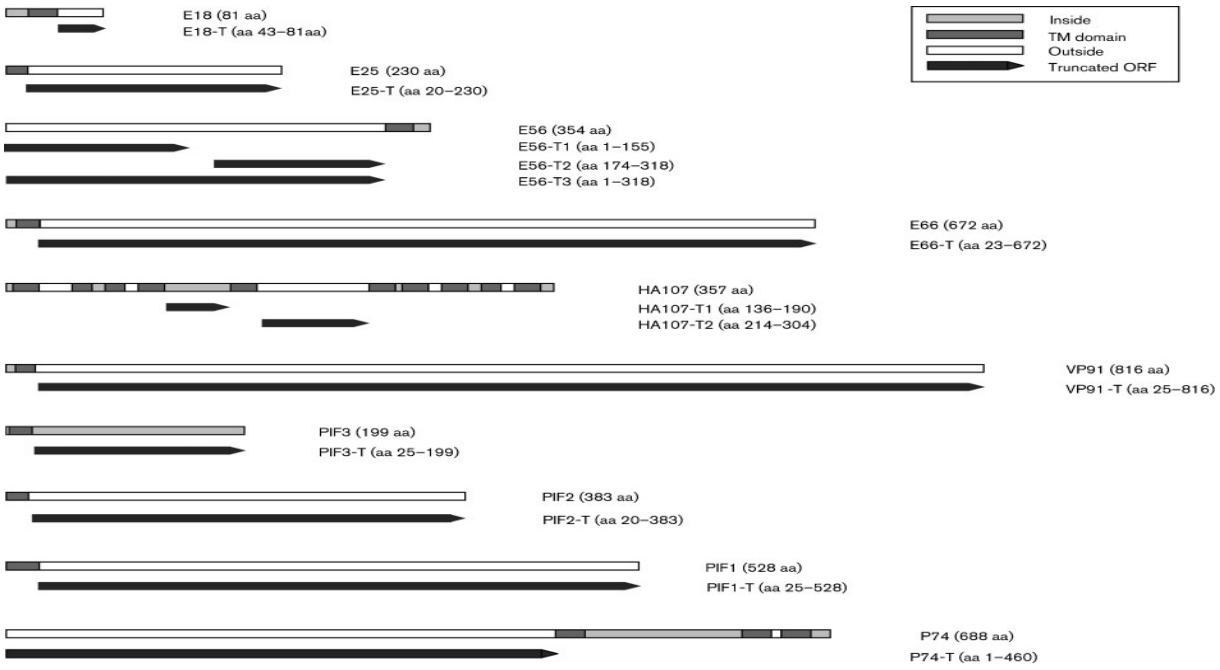

Figure 2.1. Schematic of the truncated TM domain containing ORFs in this study. The potential TM domains as well as the inside (toward tegument) and outside (on the ODV membrane surface) orientation of the HearNPV ORFs predicted by TMHMM software were presented. The dark arrows represented the truncated forms of these ORFs constructed in this study.

antibody and goat anti-rabbit IgG (JIR Laboratories Inc.) as the secondary antibody. Parallel experiments with pre-immune antiserums were simultaneously performed to set up negative controls.

Results

Construction of clones expressing HearNPV ODV associated proteins for Y2H

Thirty-nine HearNPV ODV proteins which were either determined to be either HearNPV ODV components (33, 89, 110) or HearNPV homologs of AcMNPV ODV components (16, 165) were selected in the Y2H screening Among them, 21 are encoded by conserved genes found in all baculovirus genomes thus far sequenced. The common names of these proteins were also listed in a Supplemental Table 2.S1. As the Y2H system used in this study detects the protein interactions in the yeast nucleus and thus is not optimal for proteins containing transmembrane (TM) domains, the TMHMM software was used to screen the proteins with potential TM domains. Ten of the 39 proteins were predicted to have TM

domains (Fig. 2.1). The TM containing proteins are all ODV envelope proteins including P74, PIF1, PIF2, PIF3, ODV-E18, ODV-E56, ODV-E66 and ODV-E25. For the proteins containing only one predicted TM, *e.g.* ODV-E18, ODV-E25, ODV-E66, VP91, PIF3, PIF2 and PIF1, their truncated ORFs without the TM were amplified by PCR for Y2H (Fig. 2.1). For ODV-E56, in addition to the entire ORF without TM (ODV-E56T3), two other truncated ORFs ODV-E56T1 and ODV-E56T2 were also constructed (Fig. 2.1). HA107 (which is unique to HaNPV) was reported to contain multiple TM domains (110) and two truncated ORFs, Ha107T1 and Ha107T2 were constructed (Fig. 2.1). P74 also contains three predicted TM domains, the truncated ORF containing the first 460 aa was constructed (Fig. 2.1). Therefore, there were 13 truncated ORFs representing the 10 TM containing proteins, plus 29 without a TM, making a total of 42 construct pairs. All the 42 fragments were cloned into the pGBKT7 and pGADT7 vectors and their fidelity have been confirmed by sequence analyses.

Yeast two-hybrid analysis revealed 22 protein-protein interactions

To systematically identify protein-protein interactions among HearNPV ODV proteins, direct cross Y2H assays among the 42 construct pairs were performed. Of the 42 proteins tested, 19 were involved in one or more interactions (Table 1). Of these, 9 self-associations and 13 reciprocal interactions for a total of 22 interactions were identified. These 13 binary interactions included that of HA9(homologue of AcMNPV ORF 142; =Ac142)-ODV-EC43, ODV-E56-38K, ODV-E56-PIF3, LEF3-Helicase, LEF3-alkaline nuclease (AN), GP41-38K, GP41-HA90(=Ac102), 38K-PIF3, 38K-PIF2, VP80-HA100(=Ld141/Se52), ODV-E66-PIF3, ODV-E66-PIF2 and PIF3-PIF2, and the 9 self-associations included that of IE1, HA44 (= Ld55/Se107), LEF3, HA66 (= Ac66-desmoplakin) , GP41, CG30, 38K, PIF3 and P24.

All the above interactions were reported for the first time for HearNPV. The two binary interactions, that of LEF3-Helicase (39) and LEF3-AN (100), and three self-associations, that of IE1 (108), LEF3 (38) and 38K (61, 165) have been reported in AcMNPV. Therefore, 17 interactions including 11 binary interactions and 6 self-associations are reported here for the first time.

Another 10 interactions, the interactions of HA9(=Ac142)-HA122 (unique to HearNPV) , ODV-E18-HA26(=Ac26), ODV-E56T1-ODV-E56T3, ODV-E56T1-PIF2, P74-ODV-E56T3, ODV-E66-ODV-E56T3, PIF3-ODV-E56T3, HA107T2-ODV-E56T3, PIF1-ODV-E56T3 and PIF2-ODV-E56T3, were also identified but could be detected only in one direction in Y2H (Table 2.1). Many of the interactions were detected when ODV-E56T3 was

used as the prey. HA122 (unique to HearNPV) was found to be able to auto-activate the reporter gene when expressed in the bait vector (Table 2.1). Since HA122 gives rise to false interactions, we cannot exclude the possibility that HA9-HA122 could be reciprocal interactions. The reliability, if any, of these unidirectional interactions need further confirmation and they were not included in our discussion. Fourteen proteins that of P78/83, ODV-EC27, VP1054, FP25, DNA POL, HA68(AC74), VLF-1, VP91, VP39, P33, ODV-E25, P6.9, C42 and LEF1, did not interact with any proteins in our Y2H. Among all the tested combinations, 12 previously reported interactions were not identified in our study. These include EC27-EC27, EC27-C42, C42-P78/83, FP25-ODV-E66, ODV-E66-ODV-E25, ODV-E66-VP39, VP39-IE1, VP39-VP39, VP39-ODV-E56, VP39-38K, 38K-VP1054 and 38K-VP80.

His pull-down assay confirmed the self-association of HA44

Since HA44 (= Ld55/Se107) and HA100 (= Ld141/Se52) are the two newly identified ODV proteins conserved in other group II viruses (33), their interactions were further characterized. To verify the self-association of HA44, His pull-down assays were performed. Full length HA44 with a 6×His tag at its N terminus (His·HA44) was expressed and purified. The purified protein was then incubated with lysate of HearNPV infected and uninfected cells separately. After incubation, His·HA44 and bound protein(s) were purified through affinity chromatography. Samples were then separated on SDS-PAGE and probed with anti-HA44 antiserum. Another negative control with the Ni-NTA beads was set up to exclude the possibility that viral HA44 may interact directly with the NTA resins. The predicted molecular weight of HA44 is 42.8 kDa. As shown in Fig. 2.2, in the infected HearNPV cells, two forms of HA44 with migration rate of 44 kDa and 45 kDa were captured by the purified His-HA44 as shown in lane 1. In the His-HA44 pull-down products from the healthy cell lysate (lane 2), only the 48 kDa His-HA44 (His tag added ~ 4 kDa) was detected. Almost nothing was detected in the Ni-NTA pull-down products from either infected or healthy cell lysate (lane 3 and 4). Therefore, the His-HA44 pull-down assay confirmed the HA44-HA44 interaction.

Table 2.1 Summary of the positive results of the protein-protein interactions of the HearNPV ODV proteins by Y2H.

The orders of the bait and prey were in accordance with their locations in the HearNPV genome, which was shown on the first left column. The 2nd left column showed the baits, while the top line showed the prey. Due to the limit of the space, some of the proteins names on the top line were abbreviated. Reciprocal interactions and self-associations were indicated as dark grey blocks, while interactions that could only be detected in one direction were indicated as light grey blocks. Combinations in which HA122 served as bait were marked as NA due to the autoactivation activity. The blanks were the negative results.

HearNPV ORF	Bait	Prey																									
		HA9	E18	IE1	E56-T1	E56-T3	P74	HA26	HA44	LEF3	HA66	GP41	CG30	Hel	38K	HA90	VP80	EC43	E66	PIF3	HA-100	107-T2	PIF1	AN	P24	HA-122	PIF-2
HA9	HA9																	R								O	
HA10	ODV-E18							O																			
HA14	IE1			R																							
HA15	ODV-E56-T1					O									R					R							O
HA15	ODV-E56-T3																										
HA20	P74					O																					
HA26	Ac26																										
HA44	HA44								R																		
HA56	LEF3									R				R										R			
HA66	HA66										R																
HA73	GP41											R			R	R											
HA77	CG30												R														
HA84	Helicase									R																	
HA86	38K				R							R			R					R							R
HA90	Ac102											R															
HA92	VP80																				R						
HA94	ODV-EC43	R																									
HA96	ODV-E66					O														R							R
HA98	PIF3				R	O									R				R	R							R
HA100	HA100			R													R										
HA107	HA107-T2					O																					
HA111	PIF1					O																					
HA114	AN									R																	
HA118	P24																								R		
HA122	HA122	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
HA132	PIF2					O									R				R	R							

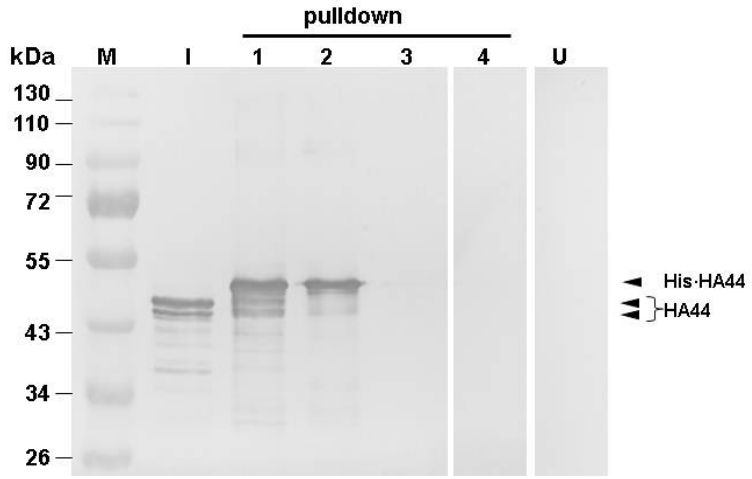

Figure 2.2. Pull-down assay of His-HA44. HzAM1 cells were infected with HearNPV at an MOI of 5 and collected at 36 h p.i. to perform pull-down assay. His-HA44 was incubated with the lysate from the infected cells or uninfected cells and pull-down proteins were purified with Ni-NTA beads. Ni-NTA beads alone were used as the negative controls. The eluted samples were separated on SDS-PAGE, blotted and probed with anti-HA44 antibody. M: molecular marker; I: lysate of the infected cells. Lane 1: proteins pull-down from the lysate of the infected cells by His-HA44. Lane 2: proteins pull-down from the lysate of the uninfected cells by His-HA44. Lane 3: proteins pull-down from the lysate of the infected cells by Ni-NTA. Lane 4: proteins pull-down from the lysate of uninfected cells by Ni-NTA. U: lysate of the uninfected cells.

In vivo co-immunoprecipitation assay verified the interaction of VP80-HA100

Co-immunoprecipitation assay was performed to verify the interaction of VP80-HA100. The antiserum anti-VP80 or anti-HA100 was added to the lysate of the HearNPV infected cells. After incubation, protein-A was added to capture the immune-complex. The precipitated proteins were then separated on SDS-PAGE and probed with anti-VP80 and anti-HA100 separately. Parallel experiments with pre-immune antiserum were performed to set up negative controls. As shown in Fig. 2.3, co-immunoprecipitation with anti-VP80 not only precipitated VP80 of 80 kDa (upper left, lane 1), but also precipitated HA100 of 60 kDa (upper right, lane 1). Likewise, co-immunoprecipitation with anti-HA100 not only precipitated HA100 (lower right, lane 3), but also precipitated VP80 (lower left, lane 3). The pre-immune antisera did not precipitate any specific proteins. The results verified the interaction of VP80-HA100 bi-directionally. In the experiments, the heavy chain of the rabbit IgG, which was used to capture target protein, was also readily detected in the Western blot due to the direct binding by goat anti-rabbit secondary IgG. A band of about 60 kDa was detected when the lysate from infected cells was probed with VP80 anti-serum (upper left and lower left, lane I). The appearance of a band migrating at a similar low molecular weight was

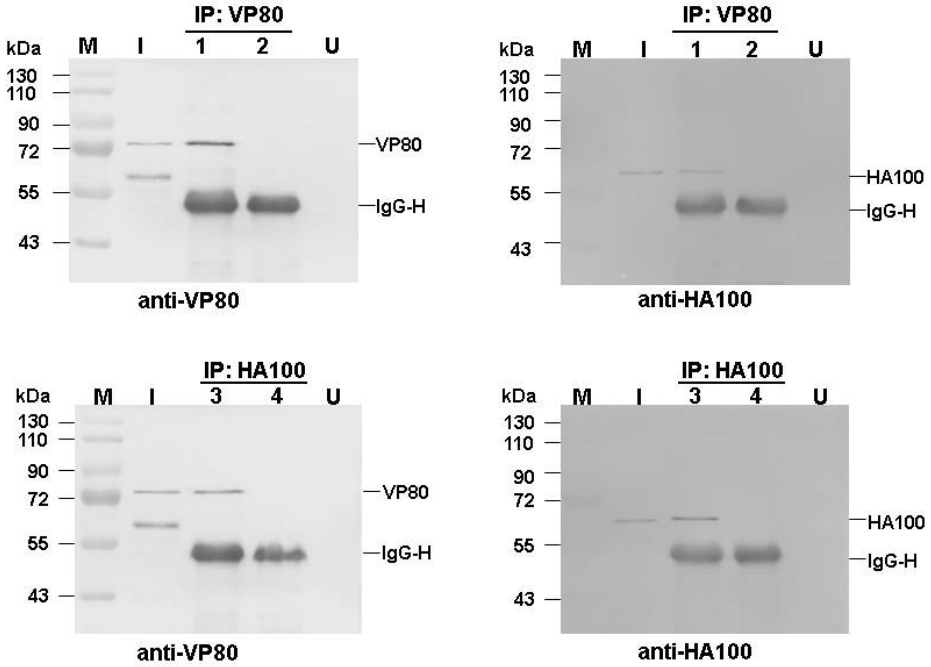

Figure 2.3. Co-immunoprecipitation assays of VP80 and HA100. HzAM1 cells were infected with HearNPV at an MOI of 5. At 36 h p.i., the lysates of the infected and uninfected cells were immunoprecipitated with the indicated antibodies and corresponding pre-immune serum. Samples were separated on SDS-PAGE, blotted and then probed with the indicated antibodies. M: molecular marker. I: lysate of the infected cells. U: lysate of the uninfected cells. Lane 1: proteins of the infected cells precipitated by anti-VP80 antibody. Lane 2: proteins of the infected cells precipitated by VP80 pre-immune serum. Lane 3: proteins of the infected cells precipitated by anti-HA100 antibody. Lane 4: proteins of infected cells precipitated by HA100 pre-immune serum. The positions positions of VP80, HA100 and the heavy chain of co-eluting rabbit IgG recognized by the goat anti-rabbit secondary IgG were indicated. The antibodies used for Western blot were indicated at the bottom of each blot.

also detected for the VP80 of CfMNPV (83) when the sample of infected cells was probed with VP80 antibody. Since this band was detected with anti-VP80 antisera, it might be due to the degradation of VP80. As shown in Fig. 2.3, this smaller protein did not interfere with the results of the co-immunoprecipitations as it was not present in the co-immunoprecipitation samples.

Discussion

This study is aimed at identifying the protein-protein interactions of potential ODV components of HearNPV. With direct cross Y2H assays covering 1764 (42 x 42) combinations, 22 interactions including 13 binary interactions and 9 self-associations were identified. To further verify the Y2H data, two pairs of interactions involving newly identified

ODV components of HearNPV, the self-association of HA44 and HA100-VP80, were verified with pull-down and co-immunoprecipitation respectively. The rest of the interactions need to be further confirmed with other methods.

To date, a number of protein interactions had been reported for baculovirus, mainly in AcMNPV, the model type of baculovirus. In a recent review, Braunagel & Summers (18) reported direct cross Y2H assays of 14 ODV associated proteins including polyhedrin (POLY), P39, P78/83, BV/ODC-C42 (C42), FP25, BV/ODV-EC27 (EC27), IE1, ODV-E66, ODV-E25, ODV-E56, BV/ODV-E26 (E26), GP41, PIF3 and Ac91. Eight interactions including VP39-POLY, VP39-ODV-E56, IE1-VP39,IE1-E26, and self-associations of POLY, VP39, EC27 and IE1 were reported. In the same review, they also presented the results of Y2H library screening. As most of the interactions identified by library screening were observed only in one direction, they were not included in our discussion. Many other interactions have been reported when individual or a few proteins were studied by Y2H, co-immunoprecipitation or other methods. With our new protein-protein interaction data, we tried to update the protein interactome of baculovirus. The so far reported protein-protein interactions of baculoviruses including our current results are summarized in Fig. 2.4. A total of 68 protein-protein interactions involving 45 viral proteins and 5 host proteins have been identified. The proteins involved in interactions are classified into three groups: DNA replication, transcription and structure associated, and are further described as following.

DNA replication: Ten have been reported to be important for AcMNPV DNA replication, among which there are seven essential proteins: DNA polymerase (DNA pol), Helicase, LEF3 (single-stranded DNA binding protein), IE1 (transcriptional factor, binds origin of replication), LEF1 (primase), LEF2 (primase associated factor), and LEF11, as well as three stimulatory proteins: IE-2 (transcriptional factor and cell cycle arrest gene), PE-38 (transcriptional factor and possible ubiquitin ligase) and P35 (apoptosis inhibitor) (88, 90, 94). In addition, DNA binding protein (DBP) has also been reported to be a replication factor for *Bombyx mori* NPV (BmNPV) (99). The interactions among these replication associated proteins have been investigated by several studies and the interactions of LEF1-LEF2 (37), LEF3-Helicase (39), the self-associations of LEF3 (38), IE1 (109, 125), IE2 (68), and DBP (101) were reported. In addition, some interactions that replication-associated proteins involved were also reported including: LEF3-AN (100), IE1-VP39 (18), IE1-E26 (71, 106) , and IE0-E26 (106). Among the proteins involved in baculovirus DNA replication, DNA pol, helicase, LEF3, IE1 and LEF1 have been reported to be ODV components and were included

in our study. Our results confirmed the interactions of LEF3-Helicase, LEF3-AN, the self-associations of IE1 and LEF3 in HearNPV (Table 2.1, Fig. 2.4).

Transcription: The late baculovirus genes are transcribed by a viral-encoded RNA polymerase with four components: LEF4, LEF8, LEF9, and P47 (55). Eight interactions among the viral RNA polymerase components were identified including 5 binary interactions (P47-LEF4, P47-LEF8, P47-LEF9, LEF4-LEF9, LEF8-LEF9) and 3 self-associations (P47, LEF8, and LEF9) (28). Apart from the above interactions, the self-associations of LEF5 (59) and host cell-specific factor 1 (HCF1) (60) were also reported. In addition, protein kinase-1 (PK1) has been shown to be involved in very late gene expression (40) and the interaction of PK1 with the protein kinase-interacting protein (PKIP) has been reported (40).

Structure associated (Structural): More than 40 proteins have been reported to be associated with baculovirus structures (ODV, BV and occlusion body) and some have been studied in greater details. When the reported interactions are summarized, it appears to be a complicated network (Fig. 2.4). We tried to categorize them into several groups:

i) EC27, C42 and P78/83. EC27 encoded by Ac144 has amino acid similarity to cellular cyclins and had been suggested to be a multifunctional cyclin (6). EC27 can interact directly with either cellular cyclin kinase cdc2 or cdk6, and the protein complex EC27-cdk6 also binds to viral proliferating cell nuclear antigen (PCNA) (6) (data not included in Fig. 2.4). Self-association of EC27 had been identified by Y2H (18). C42 of AcMNPV contains the canonical binding motif for pocket proteins and interacts with EC27 and P78/83 (128). C42 contains a nuclear localization signal at its C-terminal and the interaction of C42 and P78/83 is critical for transporting of P78/83 to the nuclei (157). P78/83 is a phosphorylated structural protein located at the basal region of the nucleocapsid (129). It is a Wiskott-Aldrich syndrome protein (WASP)-like protein which can bind to actin (81) and host cell actin-related protein 2/3 (Arp2/3) complex (53).

ii) FP25, and trafficking of BV and ODV. Normally, in a baculovirus infected cell, BVs are produced first and at the late stage of infection, the BV production is curtailed in favor of ODV formation (102). So far the mechanism of switching from BV production to ODV production is not clear, but it has been known that FP25 plays an important role. FP25 is a highly conserved baculovirus protein containing a coiled-coil region and a putative actin binding helix (13). When *fp25* is deleted, BV production increased and the ODV production decreased (163). FP25 interacts with E26 (7), ODV-E66 and GP64 (13). When it interacts with E26, cellular actin is also a component of the protein complex (7) (data not included in

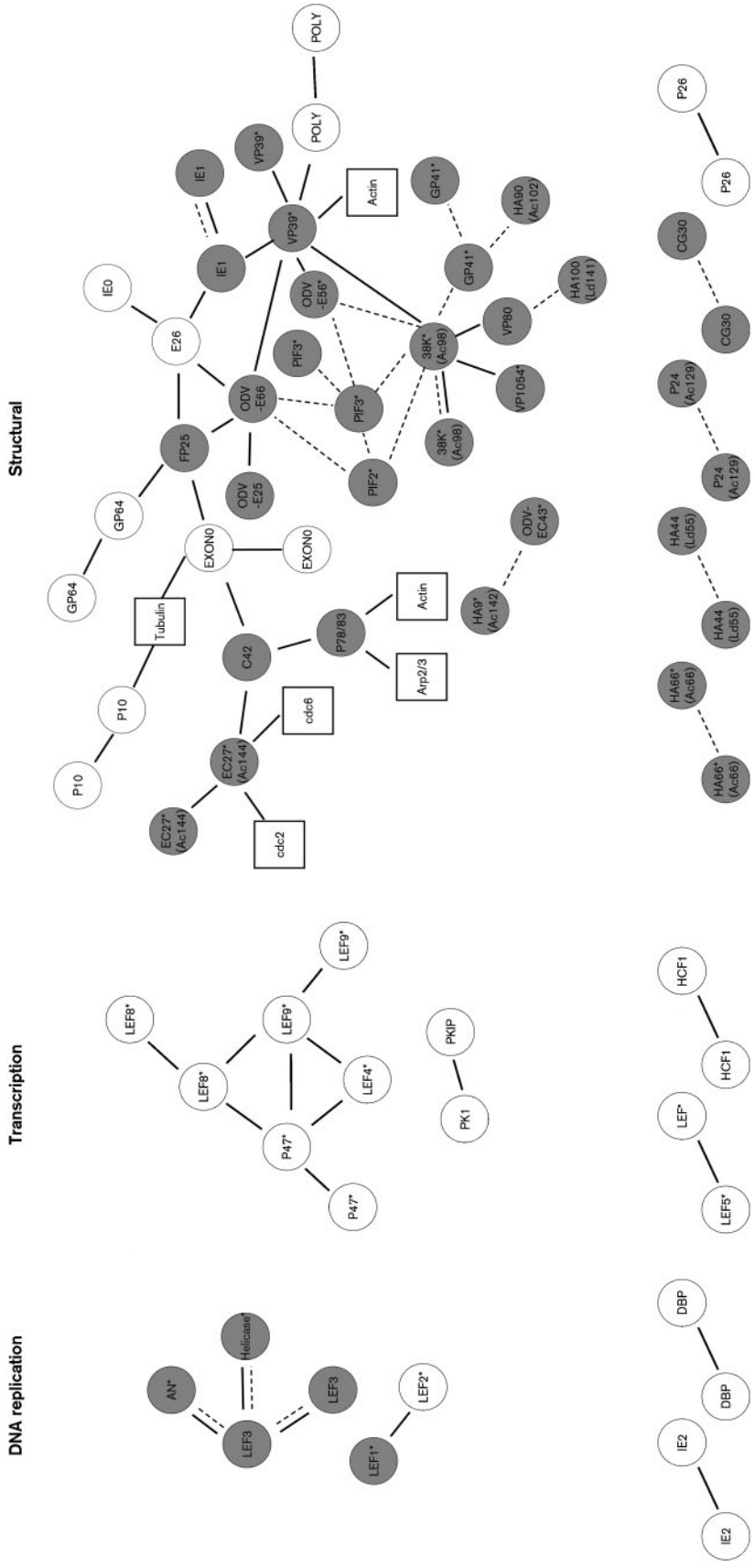

Figure 2.4. Summary of interactions of baculovirus proteins. Schematic of currently known protein-protein interactions of baculoviruses. The interactions were classified into three groups: DNA replication, Transcription and Structural. The classification is not absolute that some replication-transcription proteins were listed in the Structural because they have interactions with structural proteins. Baculoviral proteins included in the current Y2H study were presented as grey circles, and those previously published but not included in our study were presented as open circles. * indicated the conserved proteins in baculoviruses. The host proteins were presented as open squares. The interactions identified in this study were presented as dashed lines, and the interactions reported in the literatures were presented as solid

Fig. 2.4). Most ODV envelope proteins contain an inner nuclear membrane sorting motif (INM-SM) which is important for the protein to be localized into INM and eventually into ODV envelope (14). It has been shown that FP25 is involved in trafficking of ODV-E66 to the nucleus (128). Braunagel et al (2009)(14) showed that FP25 could cross-link to all the INM-SMs derived from ODV envelope proteins, suggesting it plays an important role for trafficking the ODV envelope proteins. Interestingly, in our current study, we did not detect any interactions associated with FP25 and this is consistent with the recent report by Braunagel and Summers (2007)(18) that no binary interactions were observed among FP25 and ODV envelope proteins when direct cross Y2H assays were performed (18). So far, very little is known about BV egress. GP64 is the major envelope protein of group I BVs, it forms trimer (104) and is responsible for viral entry (85) and BV budding (104). The interaction of FP25 with GP64 was reported with co-immunoprecipitation analysis (13). EXON0 is a structural protein of the nucleocapsid and is required for efficient production of BV (30, 33). EXON0 forms dimers and interacts with C42 and FP25 (41). Furthermore EXON0 was found to interact with cellular beta-tubulin (43). It is interesting to notice that both EXON0 and GP64 which are involved in BV egress were found to interact with FP25, the significance of these interactions is currently unknown. Recently, it had been shown that AC66 is required for the egress of nucleocapsid from the nucleus and for general synthesis of pre-occluded virions (76). In this study we found that HA66, the homolog of AC66, self-associated.

iii) 38K and its associations. 38K encoded by Ac98 is a striking protein which has the most extensive interactions with other proteins (Fig. 2.4). *38k* is a conserved gene in baculovirus and is essential for nucleocapsid assembly (167). Recently, the 38K was found to interact with VP39, VP80, VP1054 and itself reflecting its important role in nucleocapsid assembly (165). Our research confirmed the self-association of 38K and identified another 4 binary interactions of 38K (38K-GP41, 38K-ODV-E56, 38K-PIF2 and 38K-PIF3) in HearNPV. Domain searches revealed a haloacid dehalogenase-like (HAD) superfamily domain conserved in all 38K homologs of baculoviruses (data not shown). The majority of the enzymes in HAD superfamily are involved in phosphoryl transfer (20). If 38K is a functional phosphatase, the interactions with different structural proteins might be its interactions with multiple substrates. Whether 38K is a functional phosphatase and whether its interactions with structural proteins are essential for viral assembly are interesting questions that need to be investigated in the future.

iv) ODV envelope proteins: ODVs acquire their envelope from membranes within the nucleus and they are responsible for primary infection. Several ODV envelope proteins have been identified including P74, PIF1, PIF2, PIF3, ODV-E18, ODV-E25, ODV-E28, ODV-E56, and ODV-E66 (18, 137). P74, PIF1, PIF2 and PIF3 are essential proteins for *per os* infectivity (57, 107). P74, PIF1, PIF2 and PIF3 are conserved baculovirus genes and are also found in other invertebrate viruses such as salivary gland hypertrophy virus (SGHV) (49) and nudivirus (156). Recently, a new *per os* infectivity factor PIF4 encoded by Ac96 was also identified in AcMNPV (42). PIF4 is conserved in baculovirus and a homologue of PIF4 is found in the distantly related Hz-1 virus (42). ODV-E66 is a hyaluronan lyase and may be important for penetration of extracellular barriers during primary infection (152). ODV-E66 is conserved in lepidopteron baculoviruses (64) and is also found in SGHV (49) and nudivirus (156). The conservation of these proteins suggests a conserved mechanism of midgut infection by the invertebrate viruses. ODV-E66 was found to interact with ODV-E25, FP25, E26 and VP39 (13) and in our current study, it was found to interact with PIF2 and PIF3. Interactions of PIF2-PIF3, PIF3-ODV-E56 and self-association of PIF3 were also identified in the current study. As PIF4 is newly identified, it was not included in our study. It was suggested that the factors involved in *per os* infectivity (PIF) may function in concert as a protein complex (142) and thus it would be very interesting to further investigate the interactions among the ODV envelope proteins including the PIF proteins and their roles in primary infection.

v) other structure associated interactions. There are a few structural associated interactions that were not included into the above classification. Among them, many are self-interactions, such as that of VP39 (18), POLY (18), P10 (34), GP41, HA44, CG30 and P24 (this study). In *Spodoptera litura* NPV, it was suggested that there is either homo- or hetero-oligomerization of P24 (87). Apart from the self-associations, some binary interactions of structural proteins such as VP39-POLY and VP39-ODV-E56 (18), VP39-actin (81), P10-tubulin (113), HA9(Ac142)-ODV-EC43, VP80-HA100 and GP41-HA90 (Ac102) (this study) were also identified.

In addition to the above interactions, the self-association of P26 (=Ac136) (52), which does not fit in any of the three categories of replication, transcription or structure associated interactions, is also presented in Fig. 2.4.

Apart from the interactions mentioned above, certain interactions which were not directly confirmed, such as that of the actin rearrangement-inducing factor (ARIF-1) with F-

actin (35), IE2 with the promyelocytic leukemia protein (PML) (105), INM-directed proteins with importin-alpha-16 (14), fibroblast growth factor (FGF) with its receptor breathless (Btl) (73), *etc.*, were not included in Fig. 2.4.

Fig. 2.4 shows that many baculoviral proteins interact with more than one protein: 38K interacts with 8 proteins including itself, ODV-E66 with 7, VP39 with 7 including itself, PIF3 with 5 including itself, FP25 and E26 with 4, P47, LEF9, EC27 and EXON0 with 4 including self-association, C42, ODV-E56 and P78/83 with 3, LEF3, LEF8, IE1 and GP41 with 3 including self-association, LEF4 and VP80 with 2, and GP64, P10 and POLY with 2 including self-association. The data indicate that the interactions of baculovirus ODV proteins are complex. Therefore, further confirmation of these interactions and elucidating the corresponding functions will significantly improve our understanding of the mechanisms of baculovirus infection.

We need to point out that Fig. 2.4 is a summary of results from different baculoviruses and thus the details may vary among different viruses. For example, E26 (group I unique), HA44 (conserved in group II *Alphabaculovirus* and *Betabaculovirus*) and HA100 (group II unique) exist in only certain group(s) of baculovirus, therefore, their protein interactions would be limited to the corresponding group(s). Also, certain interactions may exist only in certain viruses but not others.

Fig. 2.4 also showed that many interactions reported previously were not identified in our Y2H study. Among all the tested combinations 12 previously reported interactions were not identified in this study. These include EC27-EC27, EC27-C42, C42-P78/83, FP25-ODV-E66, ODV-E66-ODV-E25, ODV-E66-VP39, VP39-IE1, VP39-VP39, VP39-ODV-E56, VP39-38K, 38K-VP1054 and 38K-VP80. It has been generally understood that Y2H system cannot identify all the native protein interactions and interactions identified by other methods might not be reproduced in Y2H (47). The Y2H system may have different post-translational modifications on proteins compared to the natural environment where virus proteins are produced. Consequently, for those interactions that are dependent on the modifications, the Y2H screen may not be able to identify them. Some interactions have been identified by others using Y2H system, but not identified in our study. This may due to the detailed difference in the Y2H system used. For example, the reported interaction of 38K-VP80 was detected in the less stringent selection condition but not detected in the most stringent selection condition (166), while the most stringent selection condition (lacking tryptophan, leucine, histidine, and adenine) had been used for all our combinations. In fact, employing

multiple techniques to identify protein interactions is important and indispensable to obtain a complete protein interactome.

In summary, we have identified 22 interactions among potential ODV proteins of HearNPV by Y2H. With these data, we summarized the so far reported protein-protein interactions in baculovirus which include 68 interactions with 45 viral proteins and 5 host proteins. The research will shed light on the mechanisms of ODV assembly and oral infection. On the other hand, the interactome of baculovirus is still far from complete, as viral proteins other than ODV structural proteins also need to be studied systematically. In addition, knowledge of virus-host protein interactions are critical for the understanding of viral infections, yet, very few interactions with host proteins have been identified till now. Currently, we are constructing Y2H library of host genes to further analyze the virus-host interactions of baculovirus.

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Supplementary Table 2.S1. HearNPV proteins and the primers used for the Y2H assay.
Gene names given are those commonly used in baculoviruses.

HearNPV ORF	Gene	Primer	Primer sequence (5′→3′)†
HA2	*p78/83*	ORF2-F	GCGGAATTCATGGTTCAACTGCAAAGTGTTC
		ORF2-B-R	GCGCTGCAGTTAAACTTGCGATTCAGTTGACA
		ORF2-A-R	GCGGAGCTCTTAAACTTGCGATTCAGTTGACA
HA9	*Ac142**	ORF9-F	GCGGGATCCGTATGAACTTGGACGAAAACAAAGT
		ORF9-B-R	GCGCTGCAGTTATTTAGAAGGTGGTGCTGCTA
		ORF9-A-R	GCGGAGCTCTTATTTAGAAGGTGGTGCTGCTA
HA10	*odv-e18**	ORF10-F	GCGGAATTCCAATCTAGCAGTCCGGGC
		ORF10-R	GCGGGATCCTTACATCATAGTTCTTTGGGGC
HA11	*odv-ec27* (*Ac144*)	ORF11-F	GCGGAATTCATGAAAAAGTTTAAGTGTCAAAG
		ORF11-R	GCGGGATCCTTAATCAGTATAAGTCAATATGCC
HA14	*ie1*	ORF14-F	GCGGAATTCATGGCGAATCGAATTACCA
		ORF14-B-R	GCGGTCGACTTAAGCATAATGTTTACAATCATAAC
		ORF14-A-R	GCGGAGCTCTTAAGCATAATGTTTACAATCATAAC
HA15	*odv-e56**	ORF15-T1-F	GCGGAATTCATGTCGTTTTTTAGAAATCTG
		ORF15-T1-R	GCGGGATCCTTAACCTGCTTGTTTAGCTGTTTCTAGA
		ORF15-T2-F	GCGGAATTCATTGGCGATATTGTTGAAGCTT
		ORF15-T2-A-R	GCGGAGCTCTTACAAAAGTTTCTGAAAAGATGTGCT
		ORF15-T2-B-R	GCGCTGCAGTTACAAAAGTTTCTGAAAAGATGTGCT
HA20	*p74**	ORF20-F	CGCGAATTCTGTTCTTGTCGACTTTGTTTG
		ORF20-R	GCGAAGCTTTTATTTAATTGCTATTTTAGTCATC
HA26	*Ac26*	ORF26-F	GCGGAATTCATGATCACACTGTCTCTGGC
		ORF26-R	GCGGGATCCTCATTTGTTTGTTGTTGGAA
HA44	*Ld55/Se107*	ORF44-F	GCGGAATTCATGAGCAATCCCAGCAAACAATC
		ORF44-R	GCGGGATCCTCAATAGCGCAAACGAGTTTCG
HA47	*vp1054**	ORF47-F	GCGGAATTCATGTCATCGACAAACACGG
		ORF47-R	GCGGGATCCTCAAAGAAAATATGTTCTGAAATCA
HA53	*fp25*	ORF53-F	GCGGAATTCATGGAAACTGATCTAATTAATGTAC
		ORF53-R	GCGGGATCCTCAAATGAGTCGCGTATTAA
HA65	*lef3*	ORF65-F	GCGGAATTCATGTCGAATATGGATATAAGCC
		ORF65-R	GCGGGATCCTTACAAAGGTTCGTAAGTGCC
HA66	*Ac66**	ORF66-F	GCGGAATTCATGTATCGAAACAGAGGTATTCCGT
		ORF66-R	GCGGGATCCTTATAATTTTTTTGTTATTTCTTCTTGTT
HA67	*dnapol**	ORF67-B-F	GCGGTCGACGTATGGATATTGCCTTATTGACTTG
		ORF67-A-F	GCGGAGCTCGTATGGATATTGCCTTATTGACTTG
		ORF67-B-R	GCGCTGCAGTTAACATACAGCACTCTTACAAACAA
		ORF67-A-R	GCGGAGCTCTTAACATACAGCACTCTTACAAACAA
HA68	*Ac74*	ORF68-F	GCGGAATTCATGACTTCAACCAAATGTTCTA
		ORF68-R	GCGGGATCCTTATTGTGTACAGTTTGTTTCAG
HA71	*vlf-1**	ORF71-F	GCGGAATTCATGAACAATCATCCCGC
		ORF71-R	GCGGGATCCTTAATTAAAAGATAACAAATCGG
HA73	*gp41**	ORF73-F	GCGGAATTCATGTCGCAGCCTCACGC
		ORF73-R	GCGGGATCCTCAATTCGATGTTTGTTTTAATAATT
HA76	*vp91**	ORF76-F	GCGGAATTCCTCGACGAAGCGGCG
		ORF76-R	GCGGGATCCTCATTCAGTTTGAATGTTGTGC
HA77	*cg30*	ORF77-F	GCGGGATCCATATGAATTCGATTACAGTGAATT
		ORF77-R	GCGGGATCCTTATGATTTATTTCTTTTTGCC
HA78	*vp39**	ORF78-F	GCGGAATTCATGGCCCTTGTTACCGTGC
		ORF78-R	GCGGGATCCTTATCGTCCTGTGCTGATTATCTGT

HearNPV ORF	Gene	Primer	Primer sequence (5′→3′)†
HA80	*p33**	ORF80-F	GCGGAATTCATGATTCCGTTGACACCTCTCT
		ORF80-R	GCGGGATCCTTATTTTAATTGTAATAGATTTTTGTATGTAG
HA82	*odv-e25*	ORF82-F	GCGGGATCCGTACGAATAATAAATTGAATTTT
		ORF82-A-R	GCGGAGCTCTTATTGAATTCTAAATGAAGTC
		ORF82-B-R	GCGCTGCAGTTATTGAATTCTAAATGAAGTC
HA84	*Helicase**	ORF84-F	GCGGAATTCATGGCCACCGCACCAA
		ORF84-R	GCGGGATCCTTAGCACTTAAAACTAGGTACATTTAAAT
HA86	*38k (Ac98)**	ORF86-F	GCGGGATCCGTATGCATTGCTTGTGGGTC
		ORF86-B-R	GCGCTGCAGCTAGAAAAAAACATTTTTATTATCTATAT
		ORF86-A-R	GCGGAGCTCCTAGAAAAAAACATTTTTATTATCTATAT
HA88	*p6.9**	ORF88-F	GCGGAATTCATGTACCGAAGACGTAGATCAA
		ORF88-R	GCGGGATCCTCAATAATTGCGTCGGC
HA89	*c42*	ORF89-F	GCGGAATTCATGAGTGGGGTTATGCTTTT
		ORF89-R	GCGGGATCCTTATATTCTTCTACGTTTCGACAAT
HA90	*Ac102*	ORF90-F	GCGGAATTCATGGACCTGATAGAAACCATC
		ORF90-R	GCGGGATCCTTACGCGGCATTGCCT
HA92	*vp80*	ORF92-F	GCGGAATTCATGAACCAACAATATCGCGATG
		ORF92-B-R	GCGCTGCAGTCATTCGTTGTTGCCGGCC
		ORF92-A-R	GCGGAGCTCTCATTCGTTGTTGCCGGCC
HA94	*odv-ec43**	ORF94-F	GCGGGATCCGTATGACGTGTCCTTTTAATATTAAAGTATG
		ORF94-B-R	GCGCTGCAGTTAGAAAAAGTAATTGTAACGCATTG
		ORF94-A-R	GCGGGATCCTTAGAAAAAGTAATTGTAACGCATTG
HA96	*odv-e66*	ORF96-F	GCGGGATCCGTACAAACGACAATATATTGCCATGT
		ORF96-A-R	GCGGAGCTCTTATATTTTAAATTTAAACTGATT
		ORF96-B-R	GCGCTGCAGTTATATTTTAAATTTAAACTGATT
HA98	*pif3**	ORF98-F	CCGGAATTCAAATTAAACTACACAGAG
		ORF98-R	GCGGGATCCTTATTGATAAATTTTCGAATACAAA
HA100	*Ld141/Se52*	ORF100-F	GCGGGATCCGTATGGCCGTTGAACAATTAAAAC
		ORF100-B-R	GCGCTGCAGTTAATTTAAATTGATACTAGTTTGTTTTCTG
		ORF100-A-R	GCGGAGCTCTTAATTTAAATTGATACTAGTTTGTTTTCTG
HA107	Unique to HearNPV/ HzSNPV‡	ORF107-T1-F	GCGGAATTCGATTCGGTAATTGCTTTAATGTAC
		ORF107-T1-R	GCGGGATCCTTACATTTGATTTTCATCTATCTCAAA
		ORF107-T2-F	GCGGAATTCCCAGCACATATGTTCATGGAC
		ORF107-T2-R	GCGGGATCCTTAGTCGCGAGTGGGCAACAT
HA111	*pif1**	ORF111-F	GCGCCCGGGTAATGGACATGTACCTCC
		ORF111-R	GCGGAGCTCCTGCAGCTGCAGCTGTCGCTTATGTGTACGTGG
HA114	*Alkaline nuclease**	ORF114-F	GCGGAATTCATGTCGACCACAAAAATGAC
		ORF114-R	GCGGGATCCTCATTGTTGTTGCATTAATATAG
HA118	*p24 (Ac129)*	ORF118-F	GCGGAATTCATGAACTCTAACCACACATACG
		ORF118-R	GCGGGATCCTTACTTTTTTCTGTTGATGACG
HA122	Unique to HearNPV/ HzSNPV	ORF122-F	GCGGAATTCATGGAAACGGTACGCACAT
		ORF122-R	GCGGGATCCTTATTCCTGTGCTTCTGGAATT
HA124	*lef1**	ORF124-F	GCGGGATCCGTATGAATTCAACTACACTAACGTG
		ORF124-B-R	GCGGTCGACTTACAAAATTATTTCTTGCG
		ORF124-A-R	GCGGAGCTCTTACAAAATTATTTCTTGCG
HA132	*pif2**	ORF132-F	CGGGAATTCTGGCGATTTATGCTCAAAG
		ORF132-R	GCGGGATCCTTACGACGGCAAATCCCTACGA

CHAPTER 3

Baculovirus *per os* infectivity factors form a complex on the surface of occlusion derived virus

Abstract

Five highly conserved *per os* infectivity factors, PIF1, PIF2, PIF3, PIF4 and P74, have been reported to be essential for oral infectivity of baculovirus occlusion derived virus (ODV) in insect larvae. Three of these proteins, P74, PIF1 and PIF2, were thought to function in virus binding to insect midgut cells. In this paper evidence is provided that PIF1, PIF2 and PIF3 form a stable complex on the surface of ODV particles of the baculovirus *Autographa californica* (Ac) MNPV. The complex could withstand 2% SDS, 5% β-mercaptoethanol and heating at 50°C for 5 min. The complex was not formed when any of the genes for PIF1, PIF2 or PIF3 was deleted, while reinsertion of these genes into AcMNPV restored the complex. Co-immunoprecipitation (CoIP) analysis independently confirmed the interactions of the three PIF proteins and revealed in addition that P74 is also associated with this complex. However, deletion of the *p74* gene did not affect formation of the PIF1-PIF2-PIF3 complex. Electron microscopy analysis showed that PIF1 and PIF2 are localized on the surface of ODV with a scattered distribution. This distribution did not change for PIF1 or PIF2 when the gene for PIF2 or PIF1 protein is deleted. We propose that PIF1, PIF2, PIF3 and P74 form an evolutionarily conserved complex on the ODV surface, which has an essential function in the initial stages of baculovirus oral infection.

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Introduction

The entry mechanism of enveloped viruses includes two major steps: virus binding to host receptors and subsequent fusion of the viral membrane with the cell membrane. For many viruses the processes of binding and fusion are mediated by a machinery composed of several membrane proteins working in concert with sequential events triggered by conformational changes upon interaction with host (co)receptors. Examples are herpes simplex virus (HSV) (21) and vaccinia virus (161) which have an entry machinery composed of 4 and 8 proteins, respectively. The entry of the occlusion derived virus (ODV) form of baculoviruses into insect midgut epithelial cells upon oral infection of insect larvae may involve a similar strategy, but little is known about the role of ODV membrane proteins.

Baculovirus ODVs are orally infectious, enveloped virus particles embedded in a protein crystal called occlusion body (OB) and infect midgut epithelial cells (137). After ingestion of OBs by the host, the proteinaceous OB crystal dissolves quickly due to the alkaline conditions (pH10-11) in the midgut and the ODV particles are released (reviewed by (137)). After passage through the peritrophic membrane, ODVs bind and fuse with the microvilli of columnar epithelial cells, resulting in the release of nucleocapsids into the cytosol and subsequent initiation of infection (57, 66, 137). A second type of virus particle, the budded virus (BV), is produced in these cells and infects other cells and tissues in the insect, causing a systemic infection (reviewed in (127)). While the entry mechanisms of BVs have been studied at least to a certain extent (86, 158, 171), the entry mechanism of ODVs is still rather enigmatic due to its complexity and the lack of proper cell lines supporting ODV entry.

ODVs contain more than ten different envelope proteins (18). Five of these, denoted PIF1, PIF2, PIF3, PIF4 and P74, have been identified to be essential for *per os* infection of insect larvae (42, 45, 56, 77, 107, 120). These PIF proteins function in the early stage of virus infection and deletion of any of these *pif* genes leads to a block in infection prior to viral gene expression in midgut epithelial cells (45, 57, 107). Till now three of these proteins, PIF1, PIF2 and P74, have been reported to function in virus binding (57, 107). Deletion of any of these three proteins leads to a loss of oral infectivity, while only a threefold reduction in binding is measured and no significant reduction in fusion efficiency is observed (57, 107). This suggests that the three PIF proteins, apart from binding to midgut epithelial cells, may have other unknown functions for which they may have to work together. The function of PIF3 and PIF4 is rather enigmatic although PIF3 was speculated to function in nucleocapsid

translocation along the microvilli as it seemed to be dispensable for ODV binding and fusion (107, 137).

All five proteins are highly conserved in *Baculoviridae* and are encoded by so-called core genes (18, 42, 64, 151). Recent work further revealed that these proteins have homologues in other large invertebrate DNA viruses which replicate in the nucleus such as salivary gland hypertrophy viruses (SGHVs) (49), nudiviruses (156) and White spot syndrome virus (WSSV) (*Nimaviridae*) (J.A. Jehle, personal communication). *Pif* genes are also found in polydnaviruses of braconid wasps (8). This high conservation of *pif* genes in a diverse range of large circular double stranded DNA viruses suggests that the PIF proteins are associated with a conserved and an evolutionarily ancient entry mechanism of viruses into invertebrate hosts.

The aim of the present study is to follow the ODV entry process by investigating whether the PIF proteins form a complex on the ODV membrane. Based on immunogold labeling, crosslinking, differential denaturing SDS-PAGE and coimmunoprecipitation analysis with a panel of recombinant viruses, we provide strong evidence that PIF1, PIF2, PIF3 and P74 form a complex on the ODV surface. This complex is likely to play an essential role in virus entry into midgut epithelial cells of susceptible insect larvae.

Materials and methods

Insect cells, larvae and virus

Spodoptera frugiperda Sf9 cells were propagated in flasks at 27ºC in Sf-900II medium (Invitrogen) with 5% FBS in stationary cultures. *S. exigua* (Se) larvae were reared on an artificial diet at 27ºC, 40% humidity and a 16:8 h (light:dark) photoperiod. The AcMNPV E2 strain was used as wild type (wt) virus in this study. The AcMNPV bacmid (92) is derived from the Bac-to-Bac system (Invitrogen).

Construction of recombinant viruses

AcMNPV bacmids with a deletion of the *pif1* or *pif2* ORF were constructed as previously described for the *pif*s of SeMNPV (120). To this aim, PCR products with 50 bp overhangs homologous to viral flanking regions of the *pif* genes, were generated with primer pairs pif1-del-F/pif1-del-R or pif2-del-F/pif2-del-R (Supplementary Table 3.S1) with Phusion polymerase (Finnzymes). Primers were designed to replace a fragment from nt 115 to 1489 of

the *pif1* open reading frame (ORF) and from nt 65 to 1108 of the *pif2* ORF with the chloramphenicol resistance gene (*cat*) (Fig. 3.1). The *pif3* deletion bacmid was kindly provided by Dr. Xinwen Chen of the Wuhan Institute of Virology (84). An AcMNPV *p74* deletion virus was kindly provided by Dr. Jeffrey Slack of Great Lakes Forestry Centre Canada (45).

In order to repair the *pif1*, *pif2* and *pif3* deletion bacmids, the coding sequences of these three genes plus their putative promoter region (from position -150 relative to the ATG start codon) were amplified by PCR using primer pairs pif1-rep-F/pif1-rep-R, pif-rep-F/pif2-rep-R and pif3-rep-F/pif3-rep-R. NcoI and SphI restriction sites were introduced by the primers (Table 3.S1, underlined) and used to clone the sequenced PCR products into a modified pFastBacDual vector (Invitrogen), from which the p10 promoter was deleted by fusing the Bst1107I and SmaI sites and in which the AcMNPV polyhedrin promoter and ORF were inserted between EcoRI and PstI restriction sites (pFBDΔP10-polh). The resulting plasmids were used to construct the 'repair' bacmids (Fig. 3.1) using the Bac-to-Bac transposition protocol (Invitrogen).

Proliferation and purification of virus

S. exigua 4th instars were infected with OBs of wt AcMNPV. Hemolymph was collected at 2 days post infection (p.i.) and clarified once at 3000 x *g* for 10 min at 4ºC. The supernatant was filtered through a 0.45 µm non-pyrogenic filter and used to infect Sf9 cells to generate a BV stock. To produce BV stocks from bacmids, Sf9 cells were transfected with recombinant bacmids and BVs were amplified once. OBs were produced in Sf9 cells by infection at an MOI of 5 $TCID_{50}$ units per cell and infected cells were collected at 5 days p.i. The cells were resuspended in 0.1% SDS, incubated at 37ºC for 2 h with gentle rocking and then sonicated. An OB pellet was collected through centrifugation, resuspended in deionized water and further purified by 25-65% continuous sucrose gradient centrifugation. After washing two times with deionized water OBs were stored at 4ºC until use. To liberate ODVs, OBs were suspended in DAS buffer (0.1 M Na_2CO_3, 166 mM NaCl, and 10 mM EDTA, pH 10.5) and incubated at 37ºC for 10 min. The solution was neutralized with 1/10 volume of 0.5 M Tris-HCl, pH 7.5, and incubated at room temperature for 2 min. Insoluble debris was removed by centrifugation at 800 x g for 2 min. The ODVs were collected by centrifugation at 20,600 x g for 25 min at 4ºC.

Polyclonal antibody generation

PIF1, PIF2 and PIF3, gene fragments were PCR amplified with the primer pairs pif1-ab-F/pif1-ab-R, pif2-ab-F/pif2-ab-R and pif3-ab-F/pif3-ab-R (Table 3.S1). The amplified DNAs were cloned into pET28a (Novagen) between BamHI and HindIII sites for protein expression. The resulting constructs pET28a-*pif1*, pET28a-*pif2* and pET28a-*pif3* encode PIF proteins that lack the N-terminal amino acids that correspond to putative transmembrane domains and have an N-terminal 6xHis tag. *Escherichia coli* BL21 cells were used to express the proteins after induction with IPTG (1 mM) for 4 h at 37°C. The bacteria were collected by centrifugation, washed three times with PBS and resuspended in lysis buffer (0.1% SDS, 1% Triton-X100 in PBS, [pH 7.4]). They were incubated at 37ºC under gentle rocking for 30 min and sonicated. The resulting suspension was centrifuged at 3000 x g for 5 min. The pellet was resuspended in lysis buffer as described above, and the same procedure was repeated twice. Finally the pellet was dissolved in Laemmli buffer (125 mM Tris-HCl, 2% sodium dodecyl sulfate, 5% ß-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, [pH 6.8]) and heated at 95ºC for 10 min. The proteins separated by 12% SDS-PAGE were stained with 0.25 M KCl at 0°C. Polyclonal antibodies were raised in rats against the isolated PIF proteins (Eurogentec).

Immunogold labeling analysis

Purified ODV samples were attached to carbon coated nickel grids and incubated for 30 min on drops of blocking buffer (Aurion). Grids were then incubated for 1.5 h at room temperature with antibodies diluted 300 times in 10% blocking buffer, washed 6 times 5 min on drops of 1% blocking buffer and further incubated for 45 min with 1:20 diluted goat anti-rat IgG conjugated with 10 nm gold particles. Grids were washed and negatively stained with 2% phosphotungstic acid pH 6.5. ODV were observed with a JEOL 1011 transmission electron microscope. Five squares of each grid were arbitrarily selected and the number of gold particles on 10 intact ODVs per square was counted and the average number of gold particles per virion was calculated. Experiments were repeated two times. A Mann-Whitney U-test for pair-wise comparison (SPSS Inc.) was performed to compare the data obtained with PIF1, PIF2 and PIF3 antibodies to determine the significance of the data.

Crosslinking analysis

To perform crosslinking, 1 x 10^8 OBs were treated with 2.4 ml DAS at 37ºC for 10 min with gentle rotation. Neutralization with Tris solution was skipped to keep the ODVs in alkaline condition. Insoluble debris was removed by centrifugation at 800 x g for 2 min. Formaldehyde was added to the supernatant to a final concentration of 0.5% and crosslinking was allowed for 30 min at 4ºC under rotation. ODVs were collected by centrifugation at 20,600 x g for 25 min at 4ºC and resuspended in Laemmli buffer. The samples were heated at 65ºC (for crosslinking) or 95ºC (for reversion) for 5 min and separated by 12% SDS-PAGE.

Stability of the PIF protein complex

To test the stability of the PIF complex, ODVs were purified from wt or recombinant AcMNPV viruses and heated in Laemmli buffer at 50ºC or 95ºC. Samples were separated by 12% SDS-PAGE. For non-reducing SDS-PAGE wt ODVs were purified with DAS buffer containing 40 mM iodoacetamide and treated with Laemmli buffer with or without 5% ß-mercaptoethanol at 95ºC for 5 min.

Co-immunoprecipitation analysis of ODV membrane proteins

For each CoIP analysis ODVs were purified from 2.5 × 10^8 OBs of wt or deletion mutant viruses and resuspended in 550 µl IP buffer (25 mM Tris, 150 mM NaCl, [pH 7.2]) containing 0.5% Triton-X100 and sonicated briefly. The suspension was incubated at 4ºC for 2 h. Meanwhile 20 µl of antiserum was mixed with 25 µl bed-volume of Protein G agarose (Pierce) in 500 µl IP buffer and incubated at 4ºC for 2h. The protein G agarose-antibody complex was collected by centrifugation at 500 ×g for 2 min and washed once with 1 ml IP buffer. The ODV membrane protein suspension was centrifuged at 20,600 x g for 20 min at 4ºC and 500 µl supernatant was added to the IgG-Protein G agarose and incubated at 4ºC overnight. The protein G agarose was precipitated, washed 3 times with 1 ml IP buffer and captured proteins were eluted in Laemmli buffer by heating at 95ºC for 5 min. The remaining 50 µl of the ODV membrane protein suspension was heated in Laemmli buffer and used as input control. Samples were separated by 12% SDS-PAGE.

Western blot analysis

Western blot analyses were performed with antibodies against PIF1 or PIF2 (1:2000 dilution), PIF3 (1:1000 dilution), or P74 (1:50 dilution) using standard detection methods

using alkaline phosphatase (118). P74 monoclonal antibody was kindly provided by Dr. Gary S. Blissard, the Boyce Thompson Institute at Cornell University, USA (45).

Computational analysis

The transmembrane domains and orientations of PIFs were predicted by the server: http://www.cbs.dtu.dk/services/TMHMM/. The cysteine disulfide bonding state prediction was performed with the server: http://www.predictprotein.org/. Cysteines that were predicted to form disulfide bond with a confidence >7 (0 = low, 9 = high) were indicated in a schematic representation (Fig. 3.7).

Results

Construction of recombinant viruses

To study the localization and molecular interactions of PIF proteins, recombinant viruses were constructed with deletions in the *pif* genes as well as repaired versions of these

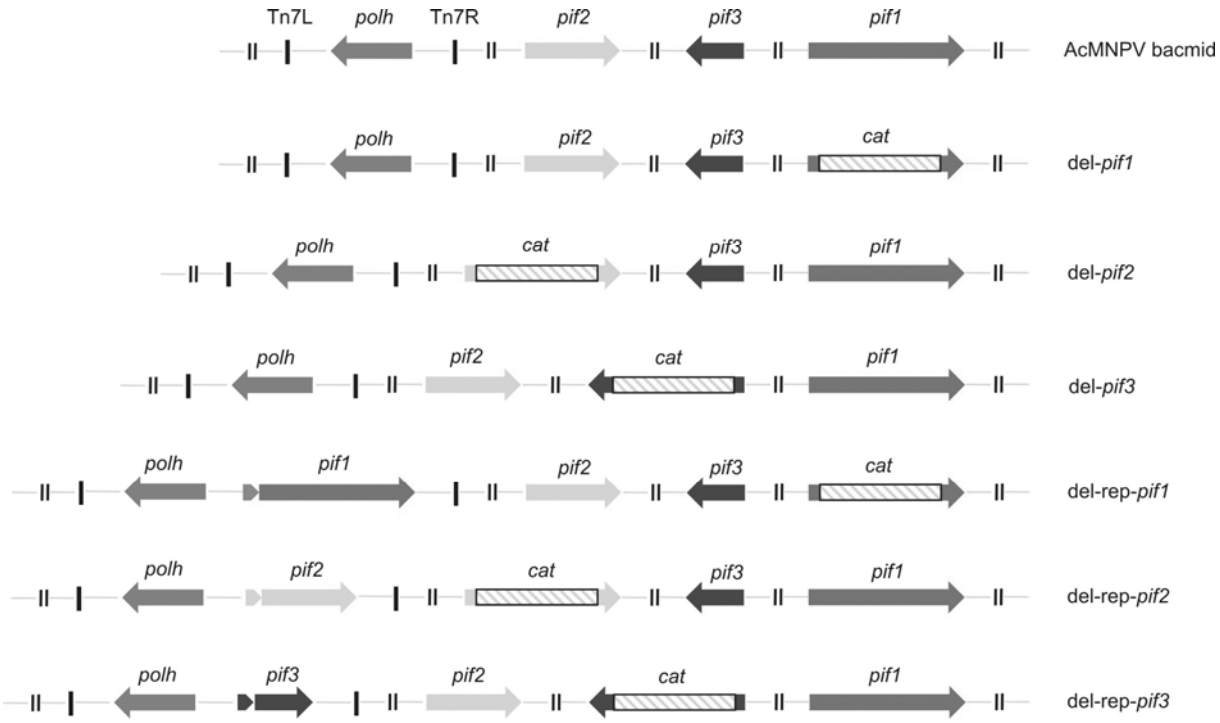

Figure 3.1. Schematic of the recombinant bacmids used in this study. The orientation of *pif1*, *pif2* and *pif3* in the bacmids is indicated. All bacmids contain the polyhedrin ORF under its own promoter. A major part of each of the three genes was replaced with the chloramphenicol resistance gene by homologous recombination. In the repaired bacmids *pif* ORFs with their putative promoter regions were included. del: deletion; rep: repair.

recombinants (Fig. 3.1). In all recombinant bacmids, the polyhedrin gene was reintroduced downstream of its native promoter so that the viruses were able to form OBs in the same temporal frame and to the same yield. The bacmid-derived del-*pif1* and del-*pif2* AcMNPV viruses were routinely tested for their infectivity for insect cells (BV) and insect larvae (OB). Consistent with previous reports on *Helicoverpa armigera* nucleopyhedrovirus (HearNPV) and *Spopdoptera exigua* MNPV (120, 142),deletion of *pif1* and *pif2* did not significantly affect AcMNPV BV infectivity but lead to a total loss of oral infectivity (data not shown). Reinsertion of *pif1* (del-rep-*pif1), pif2* (del-rep-*pif2*) and *pif3* (del-rep-*pif3*) downstream of their own putative promoter region rescued oral infectivity in all mutants.

Localization of PIF proteins on ODV

To determine the localization and orientation of PIF1, PIF2 and PIF3 in ODVs, immuno-gold labeling of purified ODVs was performed using antibodies against each of these proteins (Fig. 3.2, Table 3.1). Del-*pif1*, del-*pif2* and del-*pif3* viruses were included as negative controls. Labeling of PIF1 was also performed on del-*pif2* virus and vice versa to analyze whether these two proteins mutually affect each other's localization. The average number of gold particles per ODV virion was calculated from 150 virions (Table 3.1). With PIF1 antibody low but significant number of gold particles were found on the ODV surface of wt (2.9 ± 0.21), del-*pif2* (2.4 ± 0.26), but not on ODV of del-*pif1* AcMNPV (0.1 ± 0.03) *(P<0.05)*. Similarly, PIF2 was detected on ODVs of wt AcMNPV (2.7 ± 0.13), del-*pif1* (2.3 ± 0.31) but not on ODVs of del-*pif2* AcMNPV (0.2 ± 0.12) (P<0.05). The distribution of gold

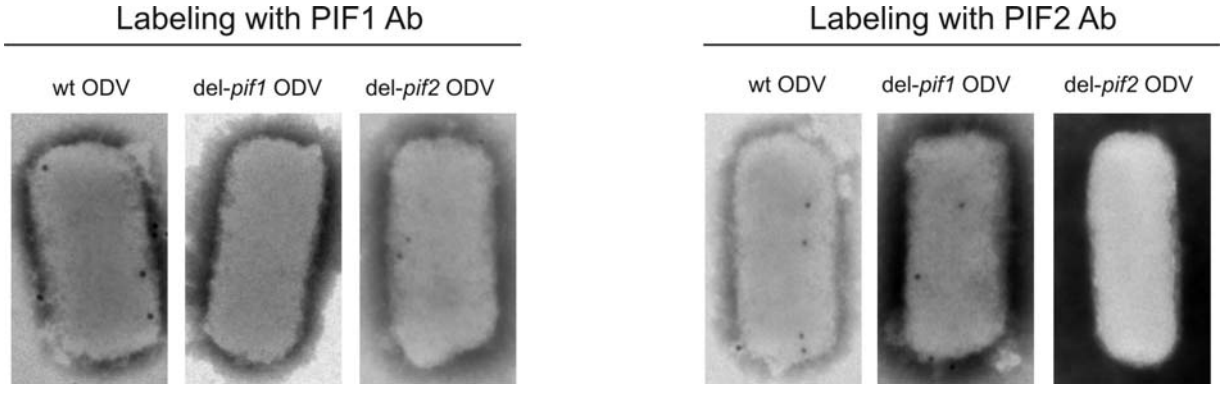

Figure 3.2. Immunogold labeling analysis of PIF1 and PIF2 on the ODV surface. ODVs were purified and probed with PIF1 or PIF2 antibodies and then with goat anti-rat IgG conjugated with 10 nm gold particles. Samples were stained with 2% PTA and studied at 15,000 times magnification. Ab: antibody; wt: wild type virus; del: deletion.

particles was scattered for both PIF1 and PIF2 over the ODV surface and did not show an apparent apical localization. The low absolute numbers of gold particles observed in these studies may reflect the low amount of PIF proteins present in the ODV membrane, which has been reported before (18, 45, 77). Alternatively, these antibodies might not be very efficient in gold labeling analysis. Only very incidental gold labeling was found on nucleocapsids with PIF1 or PIF2 antibodies (data not shown). When PIF3 antibody was used there was no significant labeling of wt ODVs (0.8 ± 0.32) when compared with labeling of del-*pif3* ODVs (0.4 ± 0.12) (Table 3.1). Labeling of PIF3 was also not observed on nucleocapsids (data not shown). Thus, PIF1 and PIF2 were shown to localize on the ODV surface with a scattered distribution, while the localization of PIF3 on the ODV is still inconclusive.

Table 3.1. Average number of gold particles on ODV

Antibody type	Avg no. of gold particles (± SD) per ODV virion			
	wt virus	del-PIF1 virus	del-PIF2 virus	del-PIF3 virus
PIF1	2.9 ± 0.21	0.1 ± 0.03	2.4 ± 0.26	ND[a]
PIF2	2.7 ± 0.13	2.3 ± 0.31	0.2 ± 0.12	ND
PIF3	0.8 ± 0.32	ND	ND	0.4 ± 0.12

[a] ND: not determined.

Crosslinking reveals a complex containing PIF1

To investigate whether PIF proteins are present in the ODV membrane in the form of a complex, protein crosslinking with formaldehyde was carried out. The value of crosslinking with formaldehyde is that it is one of the shortest crosslinkers (2.3–2.7 Å) and therefore crosslinking between two proteins can occur only when they are in very close proximity as one might expect for a native protein complex (147). After crosslinking, a major proportion of PIF1 migrated in a high molecular mass complex (>170-kDa) and the remainder migrated as monomeric PIF1 with a size of around 60-kDa (Fig. 3.3A). In some experiments a fraction of PIF1 migrated at around 150 kDa. (Fig. 3.3 A). Since the 150 kDa band was not as common as the >170 kDa band, the nature of the 150 kDa band was not investigated further in this study. When the crosslinking was reversed by heating, PIF1 migrated exclusively as monomer. The same samples were probed with PIF2, PIF3 and P74 antibodies. For PIF2 and P74 crosslinking seemed to have affected the epitopes of these proteins as their detection was significantly reduced and therefore it was difficult to interpret the outcome of the experiments.

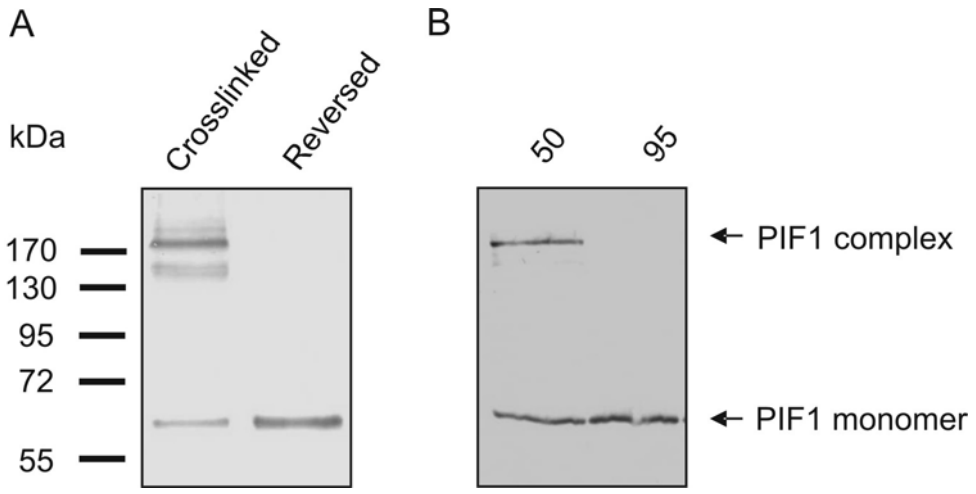

Figure 3.3. Detection of the PIF1-containing complex by western blot analysis. A: Crosslinking analysis of wt ODV. ODVs were purified and crosslinked with formaldehyde. Samples were then heated in Laemmli buffer at 65°C or 95°C for 5 min for the crosslinked and reversed sample, respectively, before analysis in SDS-PAGE. B: Detection of a PIF1 at different temperatures without crosslinking. ODVs were purified and heated in Laemmli buffer at 50°C or 95°C for 5 min and then separated by SDS-PAGE . Western blot analysis was performed with PIF1 antibody. The predicted molecular mass of PIF1 is 60 kDa.

In the case of PIF3, the cross-linked sample did not show a significant difference from the reversed sample (data not shown).

The PIF1-containing complex is stable

Subsequently it was noticed that the higher molecular mass complex (> 170 kDa) is very stable. When crosslinking was omitted and purified ODV samples were dissociated at 50°C in Laemmli buffer a complex with the same high molecular mass appeared on blots probed with PIF1 antibody, whereas at 95°C only monomeric PIF1 was found (Fig. 3.3 B). The complex apparently was stable in the presence of 2% SDS and 5% β-mercaptoethanol at 50°C, but not at 95°C.

PIF1, PIF2 and PIF3 are crucial for complex formation

To investigate whether other PIF proteins are involved in the formation of the PIF1-containing complex, recombinant viruses with deletions in the *pif* genes were analyzed. ODVs were purified from del-*pif1*, del-*pif2*, del-*pif3*, del-*p74* and wt OB and treated with Laemmli buffer at 50°C or 95°C and analyzed with PIF1 antibody on western blots. As expected, no PIF1 was detected in the del-*pif1* ODV (Fig. 3.4 A). For del-*pif2* or del-*pif3* ODV, only monomeric PIF1 was detected in both the 50°C and 95°C treated samples (Fig. 3.4 A).

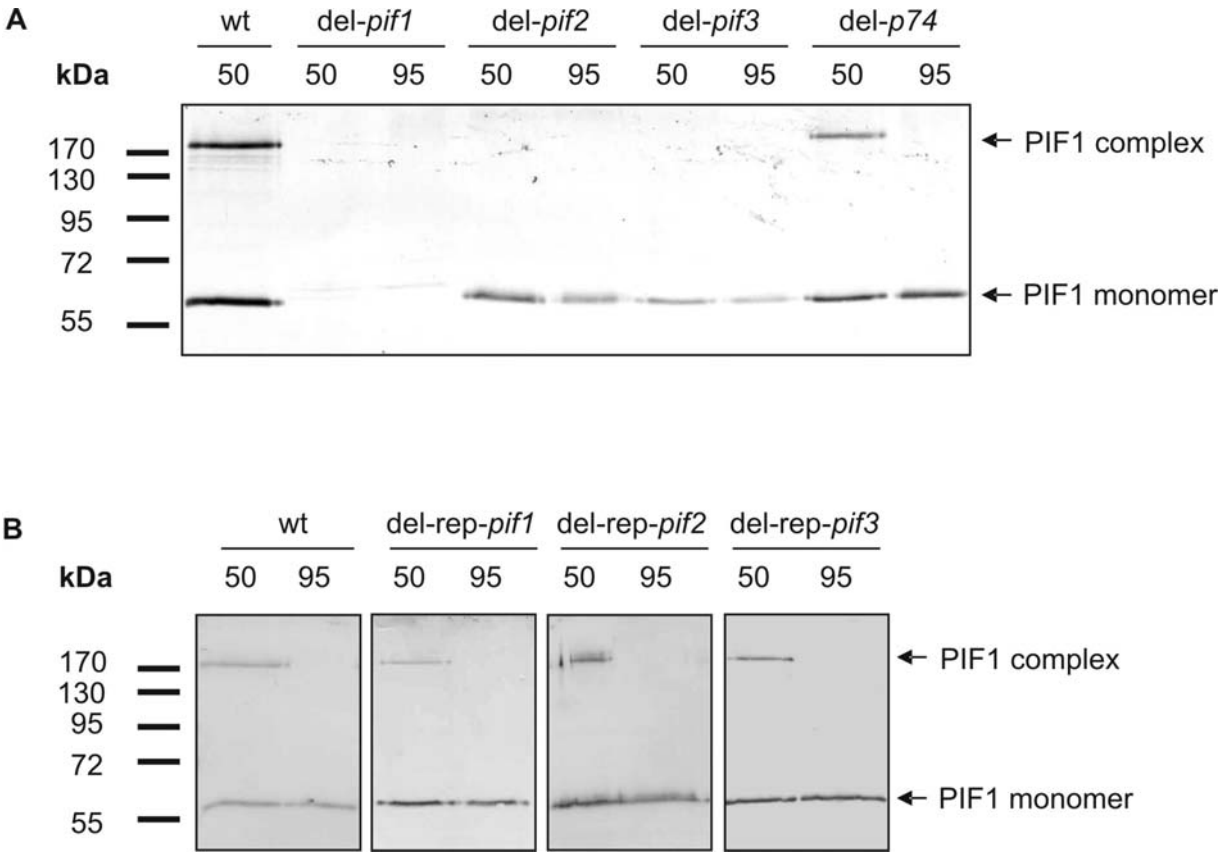

Figure 3.4. Detection of the complex by western blot analysis in deletion and repair viruses.
A: Absence of the complex in deletion viruses, del-*pif1*, del-*pif2*, del-*pif3*, but not in del-*p74*.
B: Detection of the complex in repair viruses, del-rep-*pif1*, del-rep-*pif2* and del-rep-*pif3*. Wild type (wt) virus was used as control. ODVs were purified and heated in Laemmli buffer at 50˚C or 95˚C for 5 min and then separated by SDS-PAGE. Western blot analysis was performed with PIF1 antibody. wt: wild type; del: deletion; rep: repair.

However, for del-*p74* ODV, the complex was observed in the 50°C treated sample while only monomeric PIF1 was found after heating at 95°C as was seen in wt ODVs. Apparently, PIF1, PIF2 and PIF3, but not P74, play an important role in the formation of the complex and no stable, PIF1-containing (sub-) complexes are formed in the absence of either PIF2 or PIF3.

Complex formation is restored in 'repair' viruses

To confirm that impairment of complex formation is due to the absence of PIF1, PIF2 or PIF3 and not to an unexpected change at another locus in the bacmid backbone, repaired viruses were analyzed. These repaired viruses showed comparable infectivity with wt virus (data not shown). ODVs from these 'repair' viruses (del-rep-*pif1*, del-rep-*pif2* and del-rep-*pif3*) were purified and analyzed by western analysis using PIF1 antibody. For all three

viruses, the complex was observed when samples were treated at 50°C, while at 95°C only monomeric PIF1 was detected (Fig. 3.4 B). These results confirmed that PIF1, PIF2 and PIF3 are essential for the formation of a stable complex containing PIF1 and suggested that this complex may be formed by PIF1, PIF2 and PIF3.

The complex contains PIF2 and PIF3

To determine whether PIF2 and PIF3 are associated with the PIF1-containing complex, wt ODVs were purified and treated in Laemmli buffer at 50°C or 95°C and probed with PIF1, PIF2, PIF3 and P74 antibodies. As shown in Fig. 3.5, in the samples heated at 50°C the > 170 kDa complex could be detected with PIF1, PIF2 and PIF3 antibodies. Samples heated at 95°C revealed only monomeric forms of these proteins. The predicted molecular mass of PIF3 is 23 kDa, however PIF3 migrated with a higher molecular mass of around 28 kDa. A similar result was reported before (84). Whether this is due to post-translational modification or to conformation of PIF3 is unknown yet. With P74 antibody only monomeric P74 was detected in both the 50°C and 95°C heated samples. For detection with PIF1 antibody, the signal intensity of complex is comparable to that of the PIF1 monomer but for detection with PIF2

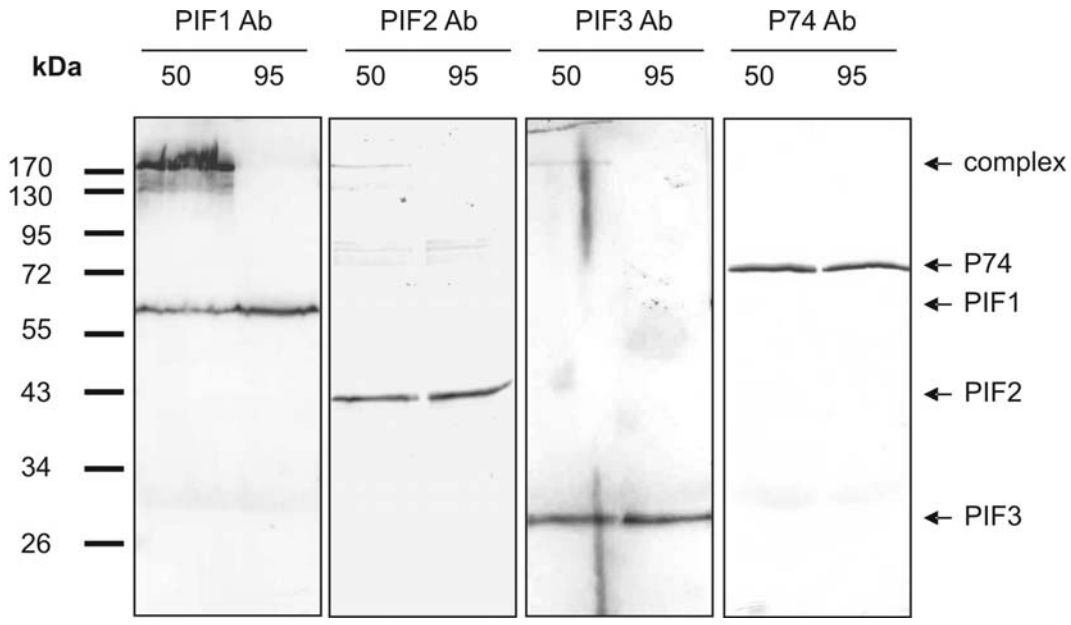

Figure 3.5. Analysis of the complex on ODVs with antibodies against PIF1, PIF2, PIF3 and P74 without crosslinking. ODVs were purified and heated in Laemmli buffer at 50°C or 95°C for 5 min and then separated in SDS-PAGE, which was followed by Western blot analysis. Western blot was performed with PIF1, PIF2, PIF3 and P74 antibodies. The location of complexed and monomeric forms of each protein is indicated. The predicted molecular mass of PIF2, PIF3 and P74 are 44, 23 and 74 kDa respectively. PIF3 migrated as a 28 kDa protein instead of the predicted 23 kDa. Each lane contained ODV liberated from 6.25×10^6 OBs.

or PIF3 antibodies the signal of the complex was weaker than that of the monomer. It is possible that the proportion of PIF2 and PIF3 involved in this complex is lower than that of PIF1. This could also explain why an apparent decrease in the monomeric form of PIF2 or PIF3 in the sample heated at 50°C was not observed. Alternatively, part of the epitopes of PIF2 and PIF3 may be shielded when they are in the complex. The complex was not detected with PIF3 antibody in the crosslinking analysis. Probably crosslinking of PIF3 in the complex either modified or shielded the epitopes of PIF3 and therefore detection was not successful. These results showed that PIF1, PIF2 and PIF3 but not P74 are part of a stable complex that can withstand treatment by 2% SDS, 5% β-mercaptoethanol and heating at 50°C.

The stability of the complex is not dependent on disulfide bonds

When the amino acid sequences of AcMNPV PIF1, PIF2 and PIF3 were compared with those from other baculoviruses, the cysteines in all three proteins are highly conserved. To test whether potential disulfide bridges between these cysteines are responsible for the

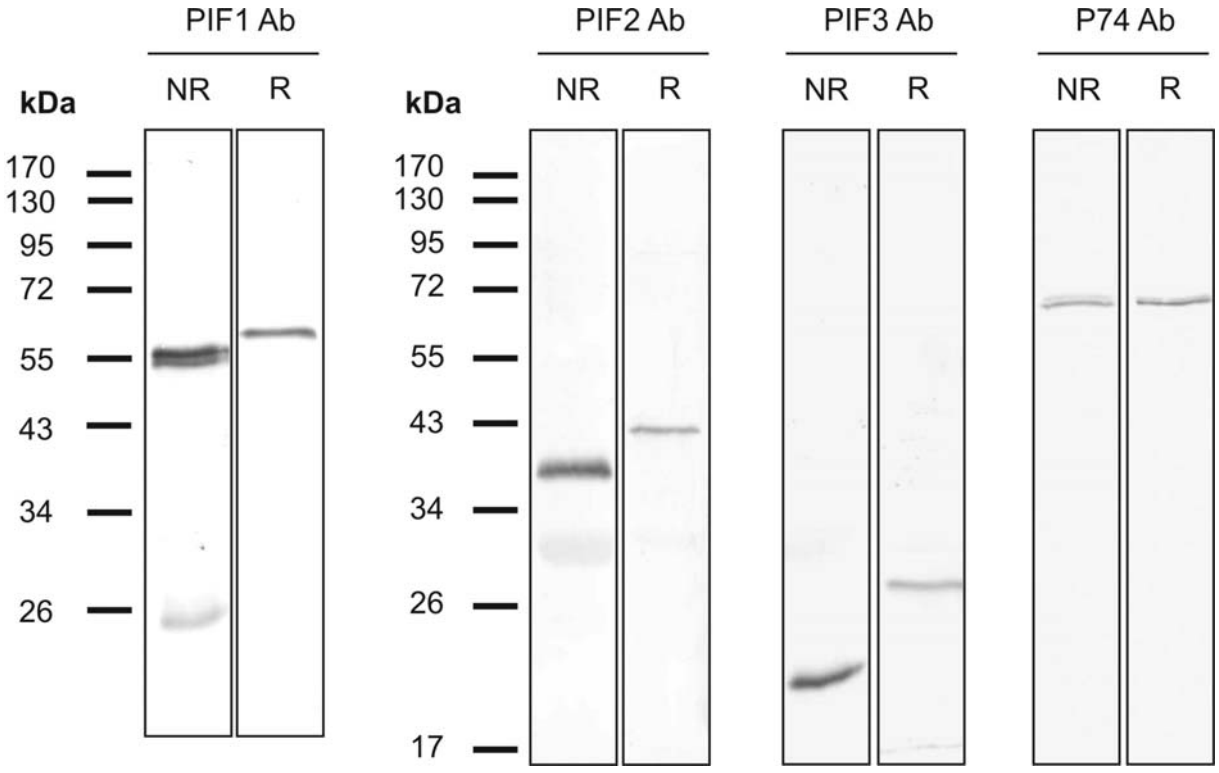

Figure 3.6. Analysis of non-reduced and reduced forms of PIF proteins. ODVs were purified in the presence of iodoacetamide in DAS solution and heated in Laemmli buffer with (reduced) or without (non-reduced) 5% ß-mercaptoethanol at 95˚C for 5 min. Samples were separated by SDS-PAGE and probed with the indicated antibodies. NR: non-reduced, R: reduced.

stability of the complex, ODVs were purified from wt AcMNPV OBs with or without iodoacetamide in the DAS solution. Iodoacetamide is an alkylating reagent for free cysteines which prevents artificial disulfide bond formation (29) e.g. during ODV purification. Samples were treated with Laemmli buffer with (reduced) or without (non-reduced) 5% ß-mercaptoethanol and probed with PIF1, PIF2, PIF3 and P74 antibodies. As shown in Fig. 3.6 for PIF1, PIF2 and PIF3 the non-reduced protein migrated slightly faster than the reduced form and no high molecular mass band representing the complex was observed, meaning that these proteins were not linked together by disulfide bridges. The higher migration rate in the non-reduced samples may be due to formation of intra-molecular disulfide bonds within these PIF proteins which keep them in a folded conformation. Indeed these three proteins were predicted to contain a number of cysteines with high potential to form intra-molecular disulfide bonds (Fig. 3.7). For PIF1 two forms, the doublet bands near 55 kDa marker (Fig. 3.6), were detected in the non-reduced sample which may be due to variation in the intra-molecular disulfide bonds. A 25 kDa band was also observed. Whether this was due to breakdown of PIF1 is unknown yet. For P74, non-reduced and reduced protein migrated at the same rate. This is consistent with the fact that P74 does not contain cysteines with the prediction confidence higher than 7 (Fig. 3.7). Notably, for PIF1, PIF2 and PIF3, detection in the non-reduced sample yielded a stronger signal than in the reduced sample although the

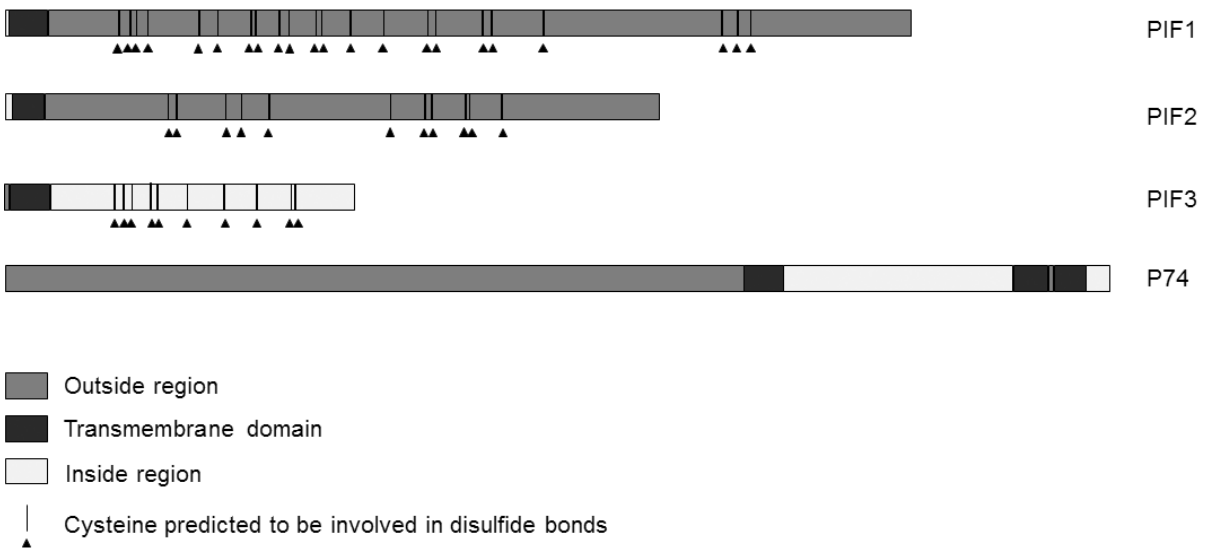

Figure 3.7. Schematic structure of PIF1, PIF2 and P74. The predicted transmembrane domains and orientations of these proteins are shown in different patterns. The cysteines in these proteins that are predicted to form disulfide bonds with high confidence are indicated.

same amount of protein was loaded. This may be due to the fact that the non-reduced proteins may contain more conformational epitopes for the polyclonal antibodies generated in this study. Comparatively, detection of P74 with monoclonal antibody did not show a difference of signal intensity between non-reduced and reduced sample. These results showed that the stable complex of PIF1, PIF2 and PIF3 is not dependent on disulfide bonds.

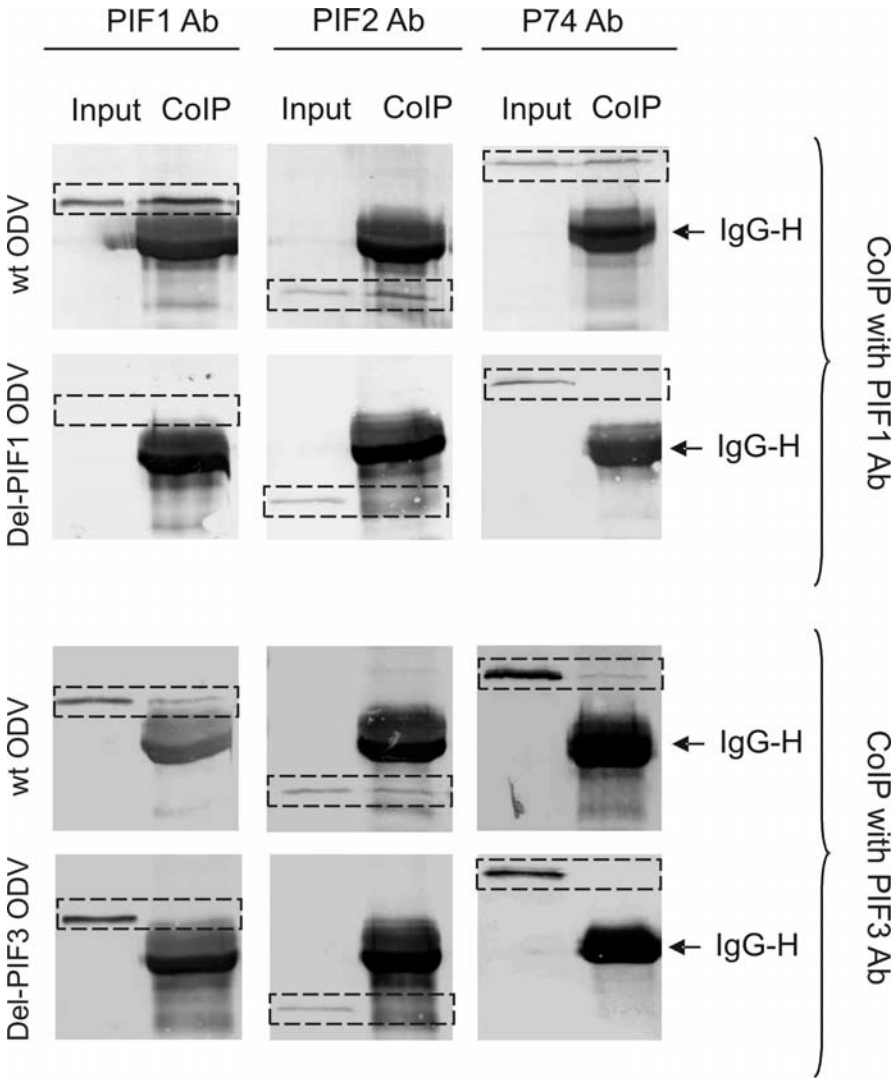

Figure 3.8. Co-immunoprecipitation of PIF proteins. Wt or corresponding deletion ODVs were purified and membrane proteins were extracted. The membrane proteins were incubated with protein G agarose bound to the indicated PIF IgGs. After washing, the captured proteins were collected. The input sample and the captured proteins were heated in Laemmli buffer at 95°C for 5 min and separated in SDS-PAGE. Western blot analysis was performed with the indicated antibodies. The position of the heavy chain of rat IgG (IgG-H) is indicated. Del-PIF1 ODVs were used as negative control for CoIP with PIF1 antibody, while del-PIF3 ODVs were used as negative control for CoIP with PIF3 antibody. The frames indicate detection of the PIF proteins.

CoIP confirmed the interaction among PIF1, PIF2 and PIF3 and revealed an additional interaction with P74

As an alternative approach to analyze the interactions of PIF proteins, ODV membrane proteins were isolated and CoIP was performed with PIF1 and PIF3 antibodies. For CoIP with each antibody, ODV of the corresponding deletion mutant, i.e. del-PIF1 for PIF1 antibody or del-PIF3 for PIF3 antibody, was used as negative control. In CoIP of wt ODV membrane proteins with PIF1 antibody, PIF1, PIF2 and P74 were detected in the eluate by Western (Fig. 3.8). In contrast, for the CoIP eluate of del-PIF1 ODV with PIF1 Ab, although PIF2 and P74 were detected in the input, they were absent from the CoIP. Likewise, when wt ODV membrane protein preparations were precipitated with PIF3 antibody, PIF1, PIF2 and P74 could be detected in the eluate, but not in the eluate from del-PIF3 virus, although all were detected in the input. In the eluate of CoIP with PIF3 antibody, detection of PIF1, PIF2 and P74 appeared weaker than that of CoIP with PIF1 antibody. Possibly the affinity of PIF3 antibody in the CoIP analysis is lower than that of PIF1 antibody resulting in reduced amount of captured protein. Alternatively, when trapped in the complex, PIF3 is less accessible to the antibody and consequently less PIF3 together with the complex was captured. CoIP analysis with PIF2 antibody was not successful as PIF2 was not efficiently captured by this antibody. Possibly, PIF2 when present in the complex is not readily accessible for antibodies. In all CoIP experiments detection of PIF3 in the eluate with PIF3 antibody was not possible as its signal would be masked by the signal of the light chain of the IgG which has a similar molecular mass (25-kDa) as PIF3. These results not only confirmed the interactions of PIF1, PIF2 and PIF3 but also revealed the association of P74 with this PIF complex.

Discussion

Baculovirus oral infection of midgut epithelial cells by ODVs involves a number of *per os* infectivity factors residing in the ODV membrane. Functional interaction between PIF1, PIF2 and P74 in cell binding has been suggested (42, 77, 120). The fact that deletion of any of these proteins leads to a threefold reduction in binding efficiency compared to wt virus (57, 107) indicated that these proteins need to work in concert to achieve efficient binding. The observation that an N-terminal protein fragment of P74, which lacks membrane anchor domains, can rescue the oral infectivity of a *p74* null virus further suggested that P74 may be in a complex with other PIFs on the ODV surface (140). In this study, a PIF1-containing complex was first identified by crosslinking. The complex was then found to be very stable

and PIF1, PIF2 and PIF3, but not P74, are essential for the formation of the stable complex. Western analysis showed that the antibodies of PIF1, PIF2 and PIF3, but not P74, can recognize the same stable complex indicating that the complex is composed as least by PIF1, PIF2 and PIF3. CoIP analysis further confirmed the association of PIF1, PIF2 and PIF3 in a complex and revealed that P74 is associated with these proteins. Based on this collective evidence, we report the identification of a stable complex composed of PIF1, PIF2 and PIF3 and offer experimental evidence of the association of P74 with this complex.

Functional binding mediated by multiple membrane proteins is not unusual among enveloped viruses but the involvement of three proteins functioning in receptor binding prior to entry is rare. An example comparable to baculovirus ODV might be HSV. In HSV, the first interaction with the cell is the attachment to cell surface glycosaminoglycans (GAGs) mediated by gC and gB. Subsequently, entry into specific target cells is mediated by the interaction of gD with its corresponding entry receptor(s) (21). However, the initial binding of gC and gB is dispensable for HSV infection as a mutant virus with a gC deletion or a gB mutation at the binding site is still infectious although with lower efficiency (123). This is different from baculovirus ODVs, where the three proteins, PIF1, PIF2 and P74 involved in binding are all indispensable for infectivity.

However, binding alone cannot account for the total functional significance of the three proteins as oral infectivity dropped completely upon deletion of any of these while binding was reduced only threefold (57, 107). In addition, these proteins do not have an apparent function in fusion as deletion of their genes had no notable effects on fusion efficiency (57, 107). Therefore, the PIF proteins may have additional functions in further downstream events. The presence of PIF3 in the complex favors this view since this protein, although dispensable for binding and fusion is essential for productive infection in midgut epithelial cells (107). PIF3 has been suggested to function in the translocation of ODV nucleocapsids along the microvilli (137). The binding events mediated by P74, PIF1 and PIF2 may trigger conformational changes to the complex which may then allow PIF3 to come into action in subsequent infection steps. Another possibility is that recognition of a host receptor by one or more of the three binding proteins will trigger certain signaling pathways, i.e. the integrin pathway (107), to facilitate subsequent steps of ODV infection. Activation of signal pathways by virus-receptor recognition to facilitate virus infection e.g. assist entry and movement within the cells or produce cellular responses that enhance virus propagation and/or affect pathogenesis have been reported for several viruses (131).

PIF1, PIF2 and PIF3 form a stable core complex, while P74 is – in comparison - loosely associated with this complex. Deletion of *p74* has no effect on the core complex (Fig. 3.4). The stability of the core complex is unusually high as it resists treatment with 2% SDS, 5% β-mercaptoethanol at 50°C for several minutes (Fig. 3.5). The first assumption was that the complex might be determined by disulfide bonding, which is a commonly observed link among stable protein complex. To investigate this possibility, ODVs were treated under non-reducing conditions and was analyzed by Western. The high molecular mass complex was not observed in the non-reduced samples, instead the non-reduced forms of PIF1, PIF2 and PIF3 migrated faster than the reduced forms indicating the presence of intra- but not inter-molecular disulfide bonds (Fig. 3.6). Disulfide bond is therefore not the determinant of the stable complex formation. It is reasonable to predict that there may be multiple large interacting interfaces, which link the three proteins by many non-covalent bonds. These non-covalent bonds may confer the complex a tight conformation which may protect internal active domains of the PIFs from unspecific protease cleavage in the potent digestive environment of the larvae midgut, until they receive certain activation signals for example conformational changes triggered by receptor binding with cellular receptors. Interestingly, P74, the comparatively loosely associated component, has been reported to undergo a protease cleavage activation event to facilitate its function (139). Whether PIF4 is also part of this complex is not known.

A common feature of PIF1, PIF2 and PIF3 is that they all contain a predicted transmembrane domain at the N terminus (Fig. 3.7). Therefore, the remaining hydrophilic fragments would be either on the outside of the ODV membrane or on the inside. Gold labeling analysis showed that PIF1 and PIF2 are localized on the ODV surface. PIF3 could not be localized with gold labeling, as there was no significant labeling neither on ODV surface nor on nucleocapsids. Till now, the major part of PIF3 was predicted to be on the inside of the ODV membrane (137), which is compatible with the observation that it is dispensable for virus binding and fusion. However, the high stability of the complex even in the presence of detergent suggests that at least some part of PIF3 is localized on the surface of ODV too, in order to be able to interact strongly with PIF1 and PIF2. A recent study reported the interaction of PIF2 and PIF3 of HearNPV in a yeast two hybrid (Y2H) screen, in which the two proteins were expressed without a transmembrane domain (118). This result supports the idea that the major fragment of PIF3 interacts with other PIFs and therefore should be exposed on the ODV surface. The failure of immunogold labeling of PIF3 could be explained

by masking of PIF3 epitopes by other components of the complex. P74 has already been shown to localize on the surface of ODVs (45).

Two models have been proposed for ODV binding and fusion with the microvilli. One model suggests that the ODVs bind and fuse with the tip of microvilli of columnar midgut epithelial cells in an 'end-to-end' apical way (1). In the other model ODVs bind and fuse with microvilli in a 'side-by-side' way (75). Electron microscopical analysis suggested that both modes might be used for ODV binding and fusion (75). Our observation that the location of the complex on the ODV shows no preference for the apical ends of the ODV, favors the side-by-side model. Binding and fusion in the side-by-side way seems to be more efficient as there may be more binding sites available for the virus to set up an efficient attachment with the host cell.

All four proteins studied here are encoded by genes conserved among all sequenced baculovirus genomes (18, 42, 64, 151). This suggests that the interactions among these proteins and the associated mechanisms are highly conserved in the *in vivo* entry process of viruses in the *Baculoviridae* family. Recent analysis showed that all four genes (*pif1-3* and *p74*) have homologues in several other large invertebrate, nuclear-replicating DNA viruses such as nudiviruses (156), SGHVs (49) and polydnaviruses (8). In the latter case it has been hypothesized that the homologues have been derived from an ancient nudivirus (8). The conservation of these four genes suggests a similar basic entry mechanism for these invertebrate viruses that arose early in evolution. This makes it highly interesting to study the initial entry mechanisms of these viruses and to gain further insights in the mechanism of baculovirus oral infectivity and the role the PIF proteins.

Acknowledgements

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Supplemental Table 3.S1: Primers used for PIF deletion, repair and antibody generation

Primer name	Function	Sequence
PIF1-del-F[a]	deletion	*AGACTTGATAGATGTGCACCATGAAGAGGTGCGTTATCCTATTACG* *G*TTTTTCCTGTGCGACGGTTAC
PIF1-del-R	deletion	*TGTTGCCCTTGGGTCATGTCGCCTGTAGTGACCGCTCTTATTGTGTT* *TC*GTTTAAGGGCACCAATAACTG
PIF2-del-F	deletion	*GATCGTGTTTTTCCTGTTCGTGTTTCTTTATATAGTGTACCAGCCCT*T TTTTCCTGTGCGACGGTTAC
PIF2-del-R	deletion	*TTATCACAATTAAGATAACTGCCCTCTGACGTCATTGTAATTAACTCG* *A*GTTTAAGGGCACCAATAACTG
PIF1-rep-F[b]	repair	GCG**CCATGG**CAAAACCACGTACATGACGTTAT
PIF1-rep-R	repair	GCG**GCATGC**TTATACAGAGTAGTTGGGGTAAGTTTC
PIF2-rep-F	repair	GCG**CCATGG**CGCGTATCTGCTGTCTATCAC
PIF2-rep-R	repair	GCG**GCATGC**TTAAGATAACTGCCCTCTGACGTC
PIF3-rep-F	repair	GCG**CCATGG**AGCGCGTTAGGAACGAG
PIF3-rep-R	repair	GCG**GCATGC**TTAGTTGTAAACGCGTCTGTACAA
PIF1-ab-F	antibody	GCG**GGATCC**CACCATGAAGAGGTGCGTT
PIF1-ab-R	antibody	GCG**AAGCTT**TTATACAGAGTAGTTGGGGTAAGTTTC
PIF2-ab-F	antibody	GCG**GGATCC**GCCCAACAAGATTACAATGAC
PIF2-ab-R	antibody	GCG**AAGCTT**TTAAGATAACTGCCCTCTGACG
PIF3-ab-F	antibody	GCG**GGATCC**AAATTTATTTTGCAAGATGCATAC
PIF3-ab-R	antibody	GCG**AAGCTT**TCAGTTGTAAACGCGTCTGTAC

[a] For the *pif* deletion primers, the viral flanking sequence used for homologous recombination are showed in italics. The 3' end of the deletion primers is used for PCR amplification of the chloramphenicol resistance gene from pBeloBAC11.
[b] For primers used for *pif* repair or antibody generation, the restriction sites are showed in bold font.

CHAPTER 4

Characterization of novel components of the baculovirus *per os* infectivity factor (PIF) complex

Abstract

Baculovirus occlusion-derived virus (ODV) infects insect midgut cells under alkaline conditions, a process mediated by highly conserved *per os* infectivity factors (PIFs): P74 (PIF0), PIF1, PIF2, PIF3, PIF4, and PIF5 (ODV-E56). Previously, a multi-molecular complex composed of PIF1, PIF2, PIF3 and P74 was identified, which was proposed to play an essential role during ODV entry. Recently, more proteins have been identified to play important roles in ODV oral infectivity including PIF4, PIF5 and SF58, which might work in concert with previously known PIFs to facilitate ODV infection. Therefore identifying components of the complete PIF complex is crucial to understand the complicated ODV entry mechanism. The aim of this study is to identify additional components of the PIF complex. Co-immunoprecipitation (CoIP) combined with proteomic analysis was used to identify components of the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) PIF complex. PIF4 and P95 (AC83) were identified as components of the PIF complex, while PIF5 was not, and this was confirmed with Blue-native PAGE and a second CoIP. Deletion of the *pif4* gene impaired complex formation, but deletion of *pif5* did not. Differentially denaturing SDS-PAGE further revealed that PIF4 forms a stable complex with PIF1, PIF2 and PIF3, with which P95, as well as P74, are more loosely associated. Three other proteins, AC5, AC68 and AC108, were also found in the proteomic analysis to be associated with the PIF complex. Finally the functional significance of the PIF protein interactions is discussed.

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Introduction

Entry of enveloped viruses, in many cases, is mediated by a number of viral envelope proteins which form a complex and function in concert during virus entry. This complex is accordingly named as virus entry complex/machinery (133). Entry of baculovirus occlusion derived virus (ODV), an enveloped virion embedded in a viral occlusion body (OB) (137), is mediated by a group of envelope proteins, which are essential only for the oral infectivity of the virus and are known as *per os* infectivity factors (PIFs). So far six PIF proteins have been identified: P74 (PIF0), PIF1, PIF2, PIF3, PIF4 and PIF5 (ODV-E56) (42, 45, 58, 77, 107, 120, 143). P74, PIF1, and PIF2 were reported to function in ODV binding, but the function of the other PIFs in the oral infection process is not known (42, 57, 107, 143). These PIFs might work in a synergistic way and it was recently found that P74, PIF1, PIF2 and PIF3 form a complex (117). This complex very likely plays an essential role during the initial steps of ODV entry. Interaction of this complex with PIF4 or PIF5 and with other known ODV membrane proteins has yet to be determined.

All six currently known PIFs are encoded by baculovirus core genes, meaning they are conserved in all baculoviruses of which the genomes have been sequenced (151). All PIFs described so far have homologs in nudiviruses (156) and in polydnaviruses of braconid wasps (8). P74, PIF1, PIF2 and PIF3 are also conserved in the unrelated salivary gland hypertrophy viruses (SGHVs, Family *Hytrosaviridae*) and in white spot syndrome virus of shrimp (Family *Nimaviridae*) (154). This high level of conservation in a diverse range of large invertebrate DNA viruses suggests that the mechanisms mediated by these proteins and their interactions are probably conserved during evolution.

ODV entry most likely takes place in the highly alkaline environment of the insect larval midgut. This is very different from the well documented viral entry events under neutral or low pH conditions in vertebrates (127, 137). Considering the high level of conservation of the PIFs among large, nuclear-replicating, invertebrate DNA viruses and the unique alkaline condition during ODV infection, baculovirus ODVs offer a unique model to investigate this potentially novel viral entry mechanism, which involves complex interactions between a group of conserved viral proteins and unknown host ligand(s).

To understand the mechanism of ODV entry it is prerequisite to know the components of the putative ODV entry complex composed of PIFs and maybe other proteins. In this study, co-immunoprecipitation (CoIP) followed by proteomic mass spectrometric analysis is used to identify components of the PIF complex. Blue-native PAGE (BN-PAGE) and another CoIP

analysis are used to verify the newly identified interactions. Differentially denaturing SDS-PAGE was further used to reveal new components of the previously identified stable complex containing PIF1, PIF2 and PIF3 (117), which appears to form the core of the PIF complex. Characterization of this multicomponent PIF complex provides fundamental information to allow investigation of the ODV entry mechanism.

Materials and methods

Virus and insect cells

The AcMNPV E2 strain was used as wild type (wt) virus in this study. The AcMNPV bacmid (bMON14272) is derived from the Bac-to-Bac system (Invitrogen). The PIF4 deletion (del-*pif4*), PIF4 repair (del-rep-*pif4*-HA), in which the deletion was repaired with PIF4 with a C-terminal influenza hemagglutinin (HA) tag, and the PIF1 deletion (del-*pif1*) viruses used in this study were described previously (42, 117). The PIF5 deletion (del-*pif5*) and PIF5 repair virus (del-rep-*pif5*-HA) were generated in this study. All recombinant viruses carry a polyhedrin (*polh*) gene for OB production. *Spodoptera frugiperda* Sf9 cells (Invitrogen) were propagated as stationary or suspension cultures in Sf-900II medium (Invitrogen) with 5% fetal bovine serum (FBS).

Recombinant virus construction

The *pif5* (*odv-e56*) deletion virus was generated using the method described by Datsenko and Wanner (32). Briefly, a zeocin resistance gene was amplified using two primers (5'- CAATTGCGGCGCGTGGACAACGTGCCAGACTTTAATTACCACACCAAGCATT CGGATCTCTGCAGCAC - 3' and 5'- TAAAAACAAGACCGCGCCTATCAACAAAATG ATAGGCATTAACTTGCCGCTCGAGGTCGACCCCCCTG - 3') using the plasmid p2ZeoKS (Invitrogen, Inc.) as the template. The PCR product was gel purified and electroporated into *Escherichia coli* BW25113-pKD46 cells, which contained the AcMNPV bacmid. Colonies resistant to zeocin (30 μg/ml) and kanamycin (50 μg/ml) were selected for further confirmation by PCR. Two different pairs of primers were used to verify that *pif5 (ac148)* had been inactivated by correct insertion of the zeocin cassette: a zeocin primer (5'- CCGATATACTATGCCGATGATT - 3') combined with a 5' *pif-5* flanking primer (5'- ACAAGCACTCCCGCCGGTTTCA - 3'); and 3' flanking primer (5'- GGGTCTGGTT CCGTTGTC-3') with a second zeocin primer (5'- CTGACCGACGCCGACCAA - 3'). The

del-*pif5* bacmid was repaired by Tn7-mediated transposition (92) of *polh* and *gfp* (as a marker) from plasmid pFAcT-GFP (30).

To tag the C-terminus of PIF5 with the influenza hemagglutinin (HA) epitope (CYPYDVPDYASL), *pif5* was amplified from bMON14272 with primers (5'- GCGGCA TGCTACACAACAAATGCGCCTT-3') and (5'- GCGGCGGCCGCTTAGGC GTA GTCGGGCACGTCGTAGGGGTATCGAGGGGCCGTTGTT-3'). The *pif5*-HA PCR product, which encompasses the native *pif5* promoter and open reading frame (ORF), was digested with *Sph*I and *Not*I and then cloned into the same sites of pFAcT-GFP-Tnie1p(A) (106). The resulting plasmid, pFActac148HA, which contains *pif5*-HA with the *Trichoplusia ni* SNPV *ie1* poly(A) signal downstream, and *polh*, as well as the marker *gfp,* was used to introduce *pif5*-HA, *polh*, and *gfp* into the pif5-del bacmid as described above, finally resulting in virus del-rep-*pif5*-HA.

SDS-PAGE and western blot analysis

Protein sample treatment in Laemmli buffer at 50ºC or 95ºC, SDS-PAGE and western blot analysis were performed as previously described (117). HA-tagged proteins were detected with anti-HA rat monoclonal antibody (Roche clone 3F10) at a concentration of 100 ng IgG/ml. Dilutions of other primary antibodies were: anti-ODV-E25 rabbit antiserum 1:5000 (128), anti-ODV-E56 rabbit antiserum 1:2000 (15) and anti-P95 rabbit antiserum 1:4000 (130). Dilutions of antibodies against PIF1, PIF2, PIF3 (all produced in rat) and P74 (mouse monoclonal antiserum), and secondary antibodies conjugated with alkaline phosphatase were as previously described (117).

Blue-native PAGE

OBs of del-*pif4*, del-rep-*pif4*-HA, del-*pif5* and del-rep-*pif5*-HA viruses were produced and purified from infected Sf9 cells as described previously (117). For this experiment 7×10^8 OBs of each virus type were incubated with 6 ml DAS solution (0.1 M Na_2CO_3, 166 mM NaCl, and 10 mM EDTA, pH 10.5) with gentle rotation for 10 min at room temperature. Non-dissolved debris was removed by centrifugation at 1,500×g for 2 min. The supernatant was collected and ODVs were pelleted by centrifugation at 20,600×g for 25 min at 4°C. To extract the membrane proteins, the ODV pellets were resuspended in 180 µl extraction buffer (6.25 mM Tris, 37.5 mM NaCl, 0.5% Triton-X100, pH 7.2), and sonicated briefly. The suspension was incubated at 4°C with gentle rotation for 2 h and centrifuged at 20,600×g for 20 min at

4°C. The supernatant was collected and mixed with 4× BN-PAGE sample buffer (200 mM Bis-Tris, 64 mM HCl, 200 mM NaCl, 40% w/v glycerol, 0.004% Ponceau S, pH 7.2). The protein samples were then supplemented with Coomassie G-250 to a final concentration of 0.1%. Electrophoresis, staining with colloidal blue and western blotting were performed according to the manual provided by Invitrogen for the NativePAGE™ Novex® Bis-Tris Gel System. Each lane contained ODV membrane protein purified from approximately 6×10^7 OBs. NativeMark™ unstained protein standard from Invitrogen (LC0725) was used as marker.

Co-immunoprecipiation

Co-immunoprecipitations (CoIP) of ODV membrane proteins was performed as previously described (117) with some modifications. In CoIP for mass spectrometry analysis, ODVs were purified from 4×10^8 OBs of wt or del-*pif1* viruses. In this case, the ODV membrane proteins were extracted in 550 µl IP buffer (25 mM Tris-HCl, 150 mM NaCl, pH7.2) containing 0.5% Triton-X100. The ODV membrane protein suspension was centrifuged at 20,600×g for 20 min at 4ºC. Simultaneously, 30 µl of PIF1 antiserum was incubated with 40 µl bed-volume of Protein G agarose in 1000 µl IP buffer at 4ºC for 2 h. The Protein G agarose with captured anti-PIF1 IgG was collected and washed as described previously, and divided between two Eppendorf tubes, mixed with either the wt or the del-*pif1* samples, and further processed as described previously (117). A portion of each of the supernatants was reserved as input sample. The captured proteins were either analyzed with SDS-PAGE followed by western blot analysis or treated further for mass spectrometry analysis as described below. To be able to perform stringent statistical analysis, 4 sample preparation replicates (4 separate CoIPs with the same protein input) were performed.

For CoIP verification of the identified interactions using PIF1 antiserum or PIF1 pre-immune serum, del-rep-*pif4*-HA ODVs were purified from 8×10^8 OBs and the ODVs were incubated in 1000 µl IP/Triton-X100 and processed as described above. Meanwhile 15 µl of PIF1 antiserum or PIF1 pre-immune serum was incubated with a 20 µl bed-volume of Protein G agarose (Pierce) in 500 µl IP buffer. The ODV membrane proteins (450 µl) was added to the collected IgG-Protein G agarose. Further processing and sample preparation in Laemmli buffer were performed as reported previously (117). Samples were analyzed with SDS-PAGE followed by western blot analysis.

Mass spectrometric analysis

To prepare samples for proteomic analysis after CoIP, Protein-G agarose beads were washed twice with 200 µl 50 mM ammonium bicarbonate (ABC, pH 7.8). The beads were supplemented with 15 µl 50 mM ABC (pH 7.8) containing 50 mM dithiotreitol and incubated at 60°C for 1 h. Afterwards, 18 µl of 50 mM iodoacetomide in 50 mM ABC (pH 8) was added, and the sample was incubated at room temperature in the dark for 1 h followed by addition of 21 µl of 50 mM cysteine. The proteins were digested with sequencing grade bovine trypsin (Roche) at 20°C for 15 h. The beads were then centrifuged at 20,600×g for 2 min. The supernatants were collected and supplemented with 10% trifluoroacetic acid (TFA) to adjust to pH3. The peptides resulting from this digestion were analyzed by LC-MS/MS as described before (72).

The raw data files from the LTQ-Orbitrap were analyzed with MaxQuant software version 1.1.1.36 (24, 25), which also includes label-free relative quantitation. Default MaxQuant 1.1.1.36 settings were used except that asparagine and glutamine de-amidations were added as variable modifications and the label-free quantitation as well as the "match between runs" options were enabled (with a default time window of 2 min). To identify the viral proteins, the MS/MS spectra obtained from the LC-MS/MS were searched against an AcMNPV database using the Maxquant search engine Andromeda. The AcMNPV protein database used for the analysis was downloaded from http://www.ncbi.nlm.nih.gov at December 2010. Protein sequences of rat IgG and Streptococcus protein G were included. Next to those, a standard contaminants database was used, which included e.g. BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovine), trypsin (P00761, porcine), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human). Also decoy handling was done by MaxQuant. Bioinformatics analysis of the MaxQuant workflow output and the statistical analysis of the abundances of the identified proteins were performed with the Perseus module (available at www.MaxQuant.org) (67).

Results

Construction of a *pif5* deletion and repair virus

Except for the *pif5* knockout and repair viruses all other viruses used in this study have been previously described (117). The del-*pif5* and del-rep-*pif5*-HA viruses were

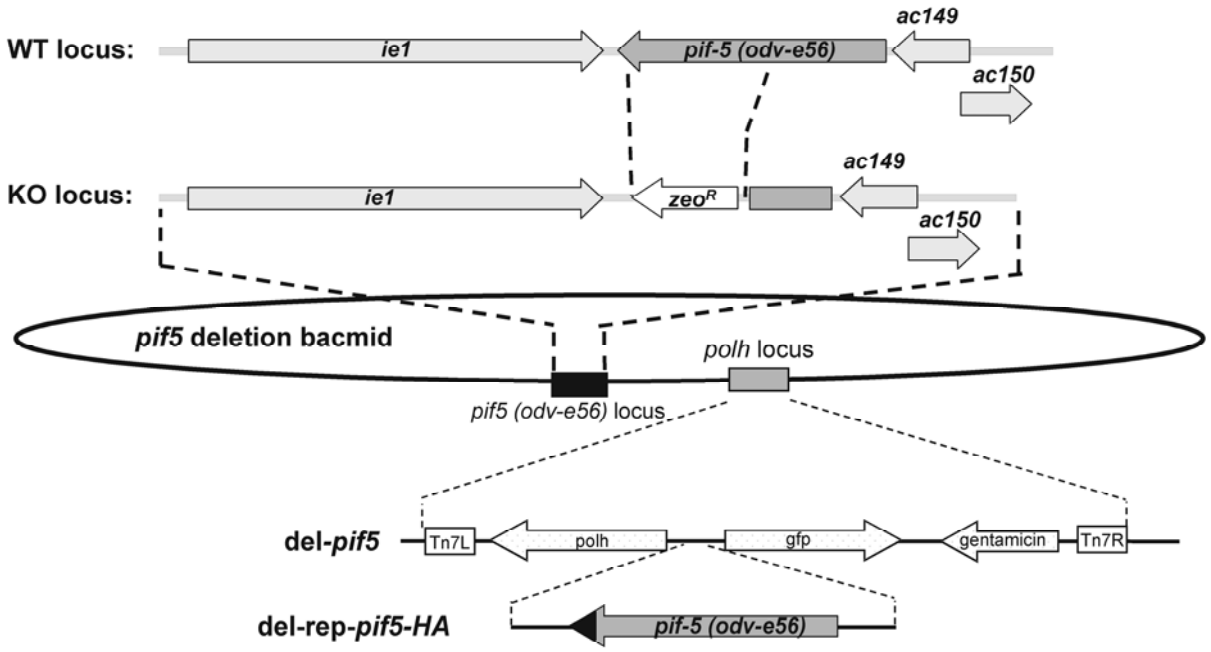

Figure 4.1. Schematic of the construction of the del-*pif5* and del-rep-*pif5*-HA bacmids. A major part of the *pif5* ORF was replaced by a *zeocin* resistance gene cassette via homologous recombination in *E. coli* to generate a *pif5* knockout bacmid. The lower part of the figure shows the genes inserted into the *polyhedrin (polh)* locus of the *pif5* knockout bacmid by Tn*7*-mediated transposition to generate del-*pif5* and del-rep-*pif5*-HA bacmids. WT, wild type; KO, knockout.

generated by first deleting the majority of *pif5* ORF (*ac148*) from the AcMNPV bacmid by homologous recombination in bacteria and replacing it with a cassette expressing the zeocin antibiotic resistant gene (see Materials and Methods; Fig. 4.1). The *pif5* knockout bacmid was supplemented with *polh* and *gfp* to generate del-*pif5* virus, or with *polh*, *pif5*-HA, and *gfp* to generate the *pif5* repair virus, del-rep-*pif5*-HA, respectively. The del-*pif5* OBs lack oral infectivity while the OBs of *pif5* repair virus have oral infectivity comparable with wild type virus (data not shown).

Mass spectrometric analysis of the PIF complex

ODV membrane fractions of AcMNPV wt and del-*pif1* were subjected to CoIP using PIF1 antiserum and analysed by LC-MS/MS (Fig. 4.2). The proteins that were significantly enriched ($P<0.01$) by the PIF1 antibody in the wt AcMNPV sample are marked in purple, proteins that were not enriched are marked in blue, and contaminants in yellow. For P74, PIF2, PIF3, PIF4 enrichment factors of more than 30 (or log10 of 1.5) were observed. Four other viral proteins (AC5, AC68, P95, and AC108) were also found to be significantly enriched

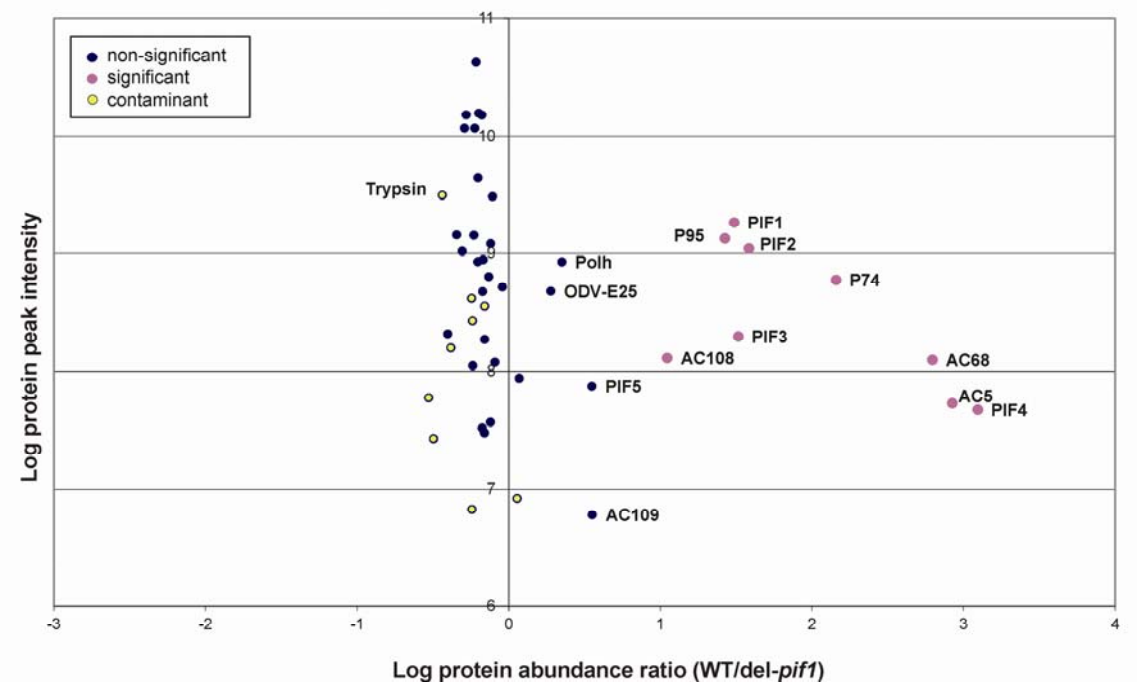

Figure 4.2. Mass spectrometric proteomic analysis of PIF complex. ODV membrane proteins were prepared from wild type or del-*pif1* virus and precipitated by PIF1 antiserum. The precipitation experiments were repeated 4 times from the same starting material and precipitated proteins were analyzed with LC-MS/MS analysis using MaxQuant software and the statistical analysis was performed with the Perseus module. The Y-axis shows the relative abundance (peak height) as log(10) values, and the X-axis shows the log(10) of the abundance ratio of proteins precipitated in the wild type sample over proteins precipitated in the del-*pif1* sample. Proteins that are significantly enriched in the wild type sample over the control are shown in purple ($P<0.01$), indicating their presence in the PIF complex. Proteins that are not significantly enriched are shown in blue. Contaminants such as IgG, human keratins and trypsin are shown in yellow.

with log(10) increase factors of more than 1. Other viral proteins such as POLH, PIF5, ODV-E25 and AC109 were found within the range of 0 to +1 on the X-axis. This indicates that these proteins are not significantly enriched in the wild type sample after precipitation by PIF1 antiserum and suggests that they are not associated with the PIF complex. The proteomic data are based on 4 replicates with stringent statistical analysis, therefore the detected enrichment of these proteins reflects their presence in the PIF complex, while proteins that are not enriched are not part of the complex. It should be noted that, unexpectedly, PIF1 peptides were also identified in del-*pif1* samples in the proteomic analysis. The source of the PIF1 peptides in the del-*pif1* sample is unknown. It is possible that the trace amount of PIF1 peptides (approximately 3% of the wild type sample) may result from contamination from wt virus sample during sample preparation. This contamination could not be detected by less sensitive methods such as western blotting analysis (data not shown), but

apparently can be recognized by the highly sensitive LC-MS/MS analysis. Nevertheless the significant enrichment of other proteins in the wt sample clearly suggests their interactions with PIF1. To further verify the proteomic analysis several of these identified protein interactions were analyzed by a BN-PAGE and CoIP analysis as described below.

PIF4 and P95 are components of the PIF complex

BN-PAGE is a technique with which membrane protein complexes are separated in native gel and therefore complexes can be analysed under native conditions. It has proven to be a powerful technique to analyse membrane protein complexes in their native state and has been successfully used, for example, to identify mitochondrial and chloroplastic membrane

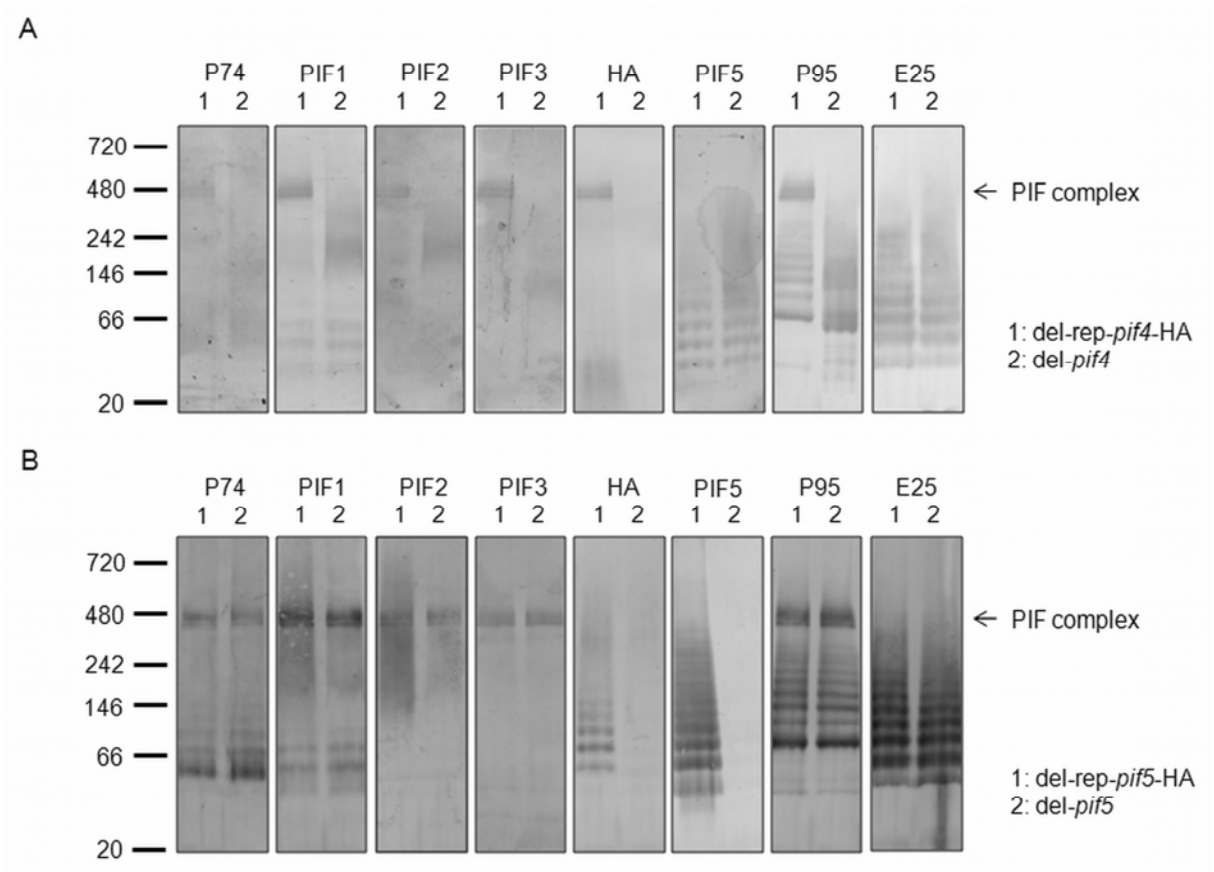

Figure 4.3. Blue native-PAGE analysis of the PIF complex.
(A) Analysis of PIF4. ODV membrane proteins were purified from del-rep-*pif4*-HA (lane 1) or del-*pif4* (lane 2) viruses and separated in BN-PAGE followed by western blot analysis with antibodies against P74, PIF1, PIF2, PIF3, HA (detecting PIF4-HA), PIF5, P95 or ODV-E25 as indicated above the panels. (B) Analysis of PIF5. ODV membrane proteins from del-rep-*pif5*-HA (lane 1) or del-*pif5* (lane 2) viruses were analyzed in the same way. In this experiment the HA antibody detects PIF5-HA. The arrows indicate the position of the PIF complex and the migration pattern of the marker is indicated on the left.

protein complexes (122). Therefore this method is chosen to analyse the PIF complex in the current study. ODV membrane proteins prepared from del-*pif4*, del-rep-*pif4*-HA, del-*pif5* and del-rep-*pif5*-HA viruses were separated in BN-PAGE and analysed by western blotting with a panel of antibodies against P74, PIF1, PIF2, PIF3, HA (for PIF4 or PIF5), PIF5, P95 and ODV-E25.

When proteins from del-rep-*pif4*-HA virus were analysed, antibodies against P74, PIF1, PIF2, PIF3, HA (detecting PIF4-HA) or P95 recognized one or more protein complex(es) with molecular masses of approximately 480 kDa (Fig. 4.3A lanes 1). Antibodies to PIF5 and ODV-E25 did not recognize the 480 kDa complexes, but showed a "ladder pattern" in the lower molecular range. In contrast, when proteins from del-*pif4* virus were analysed (lanes 2) the ~480 kDa complexes were not present and the location of P74, PIF1, PIF2, PIF3 and P95 became indefinable. As expected, no specific signal was detected in the del-*pif4* sample with HA antibody. The migration patterns of PIF5 and ODV-E25 were identical whether PIF4 was present or not (del-rep-*pif4*-HA versus del-*pif4* sample). These results suggested that PIF4 together with P95, P74, PIF1, PIF2 and PIF3 form large multi-molecular complexes and that deletion of the *pif4* gene impaired complex formation. In contrast, PIF5 and ODV-E25 are not associated with the PIF complex. Why the PIF complex seems to be present in multiple forms in the BN-PAGE is not known. Whether this is due to the BN-PAGE system or to the fact that the complex is indeed present in various stoichiometric configurations needs to be verified in the future.

PIF5 is not required for PIF complex formation

BN-PAGE analysis was also applied with PIF5 recombinant viruses. When proteins from del-rep-*pif5*-HA (Fig. 4.3B, lanes 1) and del-*pif5* viruses (Fig. 4.3B, lanes 2) were analysed, the 480 kDa PIF complexes were detected with P74, PIF1, PIF2, PIF3 and P95 antibodies in both viruses. Again the 480 kDa complexes were not recognized by PIF5 or ODV-E25 antibody and migration patterns of PIF5 and ODV-E25 were the same as in del-rep-*pif4*-HA or del-*pif4* viruses. As expected, HA or PIF5 antibodies did not give any specific signals in the sample of del-*pif5* virus. These results confirmed that P74, PIF1, PIF2, PIF3 and P95 form a complex and that PIF5 and ODV-E25 are not part of this PIF complex. Furthermore, these results showed that deletion of *pif5* did not affect PIF complex formation.

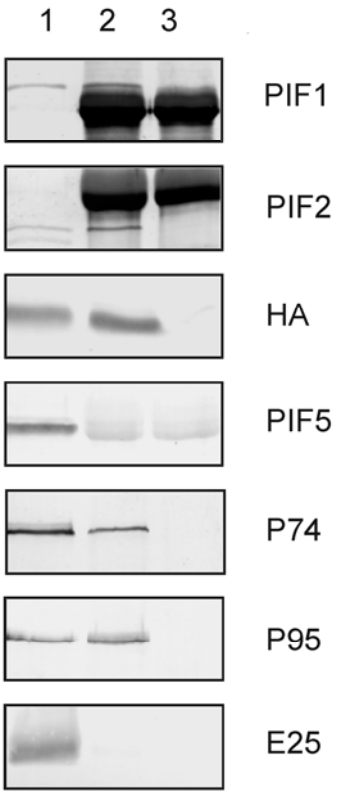

Figure 4.4. Co-immunoprecipitation analysis of the PIF complex. ODV membrane proteins were purified from del-rep-*pif4*-HA virus (CoIP input, lane 1) and CoIP analysis was performed with PIF1 antibody (lane 2) or PIF1 pre-immune serum (lane 3). CoIP input and captured proteins were heated in Laemmli buffer at 95°C for 10 min and separated in SDS-PAGE followed by western blotting with PIF1, PIF2, HA (detecting PIF4-HA), PIF5, P74, P95 and ODV-E25 antibodies.

Co-immunoprecipitation analysis of the PIF complex

The BN-PAGE analysis confirmed the presence of P74, PIF1, PIF2, PIF3, PIF4, and P95 and the absence of PIF5 and ODV-E25 in the complex, as previously revealed by the proteomic analysis. To further verify the components of the PIF complex, another CoIP analysis was performed. ODV membrane proteins were prepared from del-rep-*pif4*-HA virus and were precipitated with PIF1 antibody or PIF1 pre-immune serum. Captured proteins were separated by SDS-PAGE and analysed with P74, PIF1, PIF2, HA (for PIF4-HA), PIF5, P95, and ODV-E25 antibodies. PIF3 antibody was not included because PIF3 has a similar migration rate as the light chain of IgG, which would mask the detection of PIF3 in western blotting (21). P74, PIF1, PIF2, PIF4-HA and P95 were precipitated by the PIF1 antibody (Fig. 4.4 lane 2), but not by the PIF1 pre-immune serum (lane 3). In contrast, PIF5 and ODV-E25 were not precipitated by the PIF1 antibody (lane 2) or pre-immune serum (lane 3). Taken together these results indicate that P74, PIF1, PIF2, PIF3, PIF4 and P95 form a PIF complex and that PIF5 and ODV-E25 are not part of the complex.

PIF1, PIF2, PIF3 and PIF4 form a stable complex and deletion of PIF4 results in stable sub-complexes.

It was previously shown that PIF1, PIF2 and PIF3 form a complex that is stable upon heating in Laemmli buffer at 50°C, but disintegrated at 95°C (117). The apparent molecular mass of this stable complex was estimated to be ≈170 kDa in the denaturing 12% SDS-PAGE, which is larger than the sum of the predicted molecular masses of PIF1, PIF2 and PIF3 (127 kDa). P74, on the other hand, was not a component of this stable complex and probably interacts more loosely with the complex (117). To establish whether the newly identified components PIF4 and P95 are part of the stable complex, ODVs of del-rep-*pif4*-HA or del-*pif4* viruses were purified and heated at 50°C or 95°C. Protein samples were separated in SDS-PAGE and analysed with P74, PIF1, PIF2, PIF3, HA (for PIF4) and P95 antibodies. In the 50°C heated samples of del-rep-*pif4*-HA, the PIF1, PIF2, PIF3, and HA antibodies recognized the same ≈170 kDa protein complex (Fig. 4.5, lane 1), as reported before (117). When the same sample was heated at 95ºC only monomers of PIF1, PIF2, PIF3 and PIF4-HA were detected (lane 2). When the sample of del-*pif4* virus was analysed after heating at 50°C the ≈170 kDa complex was not detected (lane 3). Instead PIF1, PIF2 and PIF3 antibodies recognized smaller complexes (sub-complexes) with molecular masses of ≈150 kDa (lane 3). Again when the same sample was heated at 95°C only monomeric forms of PIF1, PIF2 and PIF3 were detected (lane 4). In del-*pif4* samples HA antibody did not recognize any specific signal (lane 3 and 4) reflecting the deletion of *pif4*. With P74 and P95 antibodies only monomers were detected in all 4 samples, indicating that P95, like P74, is associated loosely with the stable complex.

These results show that the previously identified stable complex contained at least four proteins: PIF1, PIF2, PIF3 and PIF4. Unlike the deletion of *pif1*, *pif2* and *pif3*, the deletion of *pif4* did not completely impair stable complex formation. In the absence of PIF4, the PIF1, PIF2 and PIF3 proteins appear to form one or two stable sub-complexes (best detected by PIF1 Ab in lane 3). It was noticed that PIF1 and PIF2 antibodies recognized the 150 kDa sub-complexes in del-rep-*pif4*-HA viruses in some of the experiments (lane 1) as was also observed in the previous study (117). These sub-complexes detected in del-rep-*pif4*-HA ODVs may be due to partial disassembly of the stable complex under denaturing conditions.

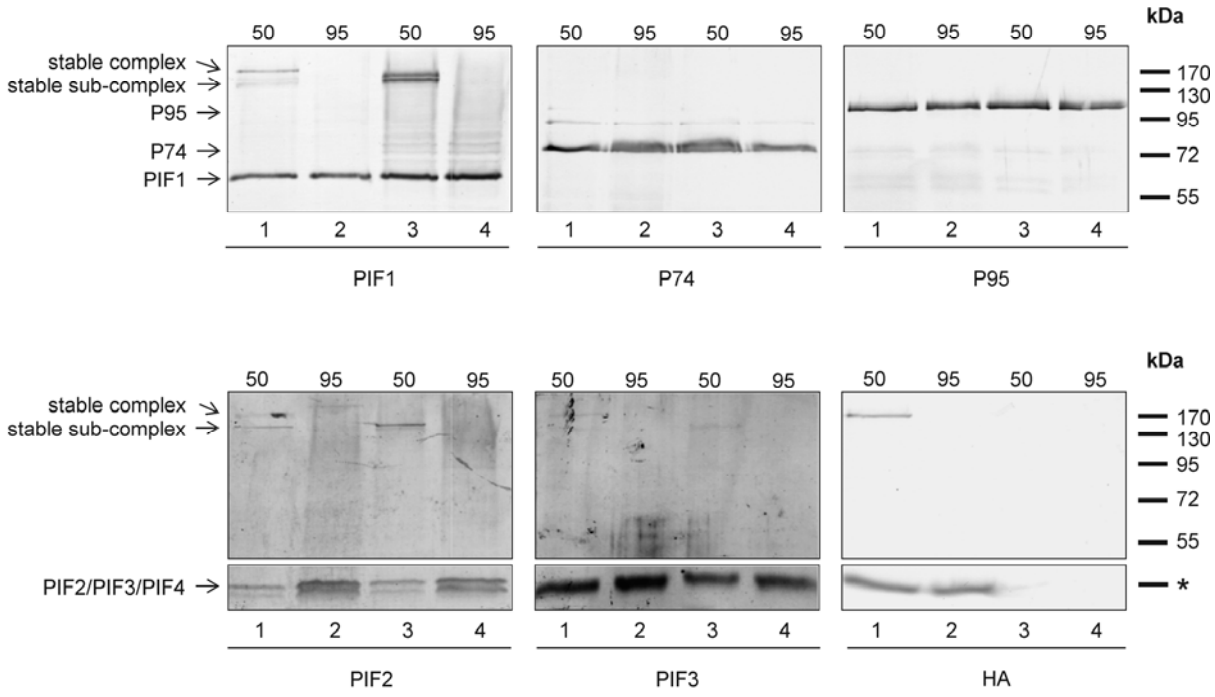

Figure 4.5. SDS-PAGE analysis of the stable complex. ODVs were purified from del-rep-*pif4*-HA or del-*pif4* viruses, heated in Laemmli buffer at 50°C or 95°C and separated in 12% SDS-PAGE followed by western blotting with PIF1, PIF2, PIF3, HA (detecting PIF4-HA), P74, and P95 antibodies. Lanes 1: del-rep-*pif4*-HA ODVs heated at 50°C, lanes 2: del-rep-*pif4*-HA ODVs heated at 95°C, lanes 3: del-*pif4* ODVs heated at 50°C and lanes 4: del-*pif4* ODVs heated at 95°C. Positions of the stable 170 kDa complex, stable sub-complexes and the monomer forms of these proteins are indicated by arrows. Markers are indicated on the right. * indicates that the blot sections shown here are the lower parts of the same original blots.

Discussion

Initiation of baculovirus infection in the midgut occurs under a highly alkaline condition and is mediated by at least 6 viral membrane proteins: P74, PIF1, PIF2, PIF3, PIF4 and PIF5 (42, 45, 58, 77, 107, 120, 143). The recent discovery that ODV-E66 is also important for *per os* infectivity of BmNPV (168) further highlights the complexity of this process. All seven proteins are encoded by baculovirus core genes indicating that the relevant mechanisms are highly conserved and may have arisen early in evolution. Interactions among these PIFs may play essential roles during ODV infection and a complex composed of P74, PIF1, PIF2 and PIF3 was recently identified (117). The current study used three different strategies to analyze the components of the PIF complex. The results showed that the complex contains at least 6 proteins, P74, PIF1, PIF2, PIF3, PIF4 and P95, and suggested three other potential components, AC5, AC68 and AC108. It was also demonstrated that PIF5 is not

present in the complex. Similarly, ODV-E66 was also not found to be a component of the AcMNPV PIF complex (not enriched in the proteomic analysis shown in Fig. 4.2).

The PIF complex seems to have a structural order with a highly stable complex consisting of PIF1, PIF2, PIF3 and PIF4, with which P95 and P74 interact relatively loosely. When the *pif4* gene is deleted PIF1, PIF2 and PIF3 can still form stable sub-complexes, but deletion of *pif1*, *pif2* or *pif3* leads to complete disruption of the complex (117). Therefore the stable complex can be further separated into two formats with PIF1, PIF2 and PIF3 forming a stable core, and PIF4 strongly interacting with this stable core. It has been shown before that formation of the stable complex is not due to covalent disulphide bonds between these PIFs but instead should be the result of large numbers of weak non-covalent bonds (117). Therefore these proteins may have large interacting interfaces potentially giving the complex a compact conformation. Such a conformation might help these four PIFs to embed their functional domains inside the complex to avoid premature action and/or non-specific proteolytic cleavage in the potent digestive environment of the insect midgut (137). These internal functional domains might be exposed upon activation signals such as conformational changes due to receptor binding or specific proteolytic cleavage. Interestingly P74, the less stably interacting component of the PIF complex, was recently found to undergo sequential cleavages mediated by an OB endogenous protease (116) and a host midgut trypin (139). It would be very interesting to investigate whether the P74 cleavage events trigger conformational changes within the complex that activate a programme of events in ODV binding and fusion.

The observation that PIF5 is separate from the PIF complex may suggest that there might be another group of proteins working separately or synergistically with the PIF complex to facilitate ODV infection. Three proteins in the PIF complex, P74, PIF1, and PIF2, were reported to function in ODV binding implying the complex may mediate early steps of ODV entry i.e. binding and fusion. However the presence of three other components with unknown functions, PIF3, PIF4 and P95, leaves the possibility open that the complex may also be involved in events downstream of fusion, i.e. transport of nucleocapsids. In favour of this view, deletion of *pif3* does not affect ODV binding or fusion but oral infectivity was dramatically impaired (107). Further studies toward the functional analysis of the PIF complex, including the identification of host cell ligands for PIFs and determination of potential dynamic conformational changes of the complex during entry, are necessary before the ODV entry mechanism will become understood.

P95 is encoded by a core gene and was found to be a component of the PIF complex in this study. The function of P95 in baculovirus infection is unknown except for the observation that the *Bombyx mori* NPV homologue (BmP95) can stimulate gene expression driven by its own promoter and that of the host actin gene (91). Conserved domain analysis (data not shown) identified a chitin-binding peritrophin A domain in AcP95 (amino acid 227-275). This domain is found in peritrophic matrix proteins of insects and in animal chitinases and is essential for chitin binding (135, 148). Chitin is a component of the peritrophic membrane (PM), a semi-permeable sheath that lines the insect midgut and protects the underlying epithelium from mechanical damage, toxic compounds and pathogens, and aids in regulating nutrient uptake by compartmentalizing digestive processes (62). Once released into the host midgut ODVs need to penetrate the PM before reaching the midgut epithelial cells. It is possible that P95 in the PIF complex plays a role when ODVs are penetrating the PM, maybe through binding to the chitin of the PM to set up the initial contact with midgut cells. In contrast to the identified *pif* genes, *p95* seems also to be an essential gene for *in vitro* infection because insertion/deletion mutants of this gene in BmNPV (*Bm69*) could not be isolated (127). Similarly, construction of a *p95* deletion mutant in AcMNPV was not successful as *p95* deletion bacmid DNA could not develop productive infection in cultured cells (K. Peng, unpublished data). In *Orgyia pseudotsugata* MNPV P95 was found to be a structural component of both BV and ODV (130), but a recent proteomic analysis of AcMNPV BV structural proteins did not identify P95 (153). Further work with mutant *p95* ORFs is necessary to unravel its potential multi-functionality in virus infection *in vivo* and *in vitro*.

Proteomic analysis identified three other proteins, AC5, AC68 and AC108, as PIF1 interacting proteins. As antibodies against these proteins were not available, their presence in the complex could not be analysed by the other assays in this study. AC5 was identified as a structural protein of AcMNPV ODV (16), but not of BV (153). In addition insertion/deletion mutagenesis of *bm134* (homolog of *ac5* in BmNPV) showed that it is not essential for virus replication in cell culture (127). These features appear to be consistent with the supposition that AC5 might have a role in ODV infection. AC68 and AC108 were identified to be associated with AcMNPV ODV in this study for the first time. Like AC5 they were not found in AcMNPV BV (153), which seems to be consistent with their potential functions in ODV infection. The *ac68* gene is conserved among all the sequenced baculovirus genomes. Deletion mutagenesis analysis of *bm56*, the homolog of *ac68,* showed that deletion of this gene did not affect virus production *in vitro* but extends the 50% lethal time in bioassays, in

which BVs were injected into larvae (169). The same study also reported that deletion of *bm56* affects OB morphogenesis leading to production of OB-like structures that did not contain ODVs, and hence, were not infectious to larvae. However, more recently, we found that AC68 does not impact OB formation, but is required for oral infectivity and is also a PIF (Y. Nie, M. Fang, M. A. Erlandson, and D. A. Theilmann, submitted for publication). This discovery supports our observation that AC68 is part of the PIF complex. The *ac108* gene is also highly conserved with homologs present in all Group I and II NPV and all GV genomes except *Plutella xylostella* GV (PlxyGV) (127). It seems to be essential as viruses with an insertion in the BmNPV homolog (*bm91*) could not be isolated (127). Further work is needed to confirm the interactions of these three proteins with the PIF complex and to gain insights in their roles in infection and OB morphogenesis.

It has been envisioned that interactions between the PIF proteins are essential for ODV infection and a previous study identified a PIF complex composed of PIF1, PIF2, PIF3 and P74 (117). The current study revealed more components of the PIF complex and showed that it contains a stable core composed of PIF1-4, with which P74 and P95 interact. This knowledge lays an important foundation for the further investigation of the ODV entry mechanism. Future efforts could be devoted to identification of the host receptors of the PIF complex and the putative sequential conformational changes of the complex during ODV entry. However, these work await the development of a cell line or *in vitro* system that can easily support ODV entry/infection.

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CHAPTER 5

In situ cleavage of the baculovirus occlusion derived virus receptor binding protein P74 in the peroral infectivity complex

Abstract

Proteolytic processing of viral membrane proteins is common among enveloped viruses to facilitate virus entry. The *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) occlusion derived virus (ODV) protein P74 is part of a complex of essential peroral infectivity factors (PIFs). Here we report that P74 is efficiently cleaved into two fragments of about equal size by an OB endogenous alkaline protease during ODV release when AcMNPV occlusion bodies (OB) are derived from larvae. The cleavage is specific for P74 since the other known per oral infectivity factors in the same complex (PIF1, PIF2 and PIF3) were not cleaved under the same conditions. P74 cleavage was not observed in OBs produced in three different insect cell lines suggesting a larval host origin of the responsible protease. P74 in OBs produced in larvae of two different host species was cleaved into fragments with the same apparent molecular mass indicating that the virus incorporates a similar alkaline protease from different hosts. Co-immunoprecipitation analysis revealed that the two P74 subunit fragments remain associated with the recently discovered PIF complex. We propose that under *in vivo* ODV infection conditions P74 undergoes two sequential cleavage events, the first one being performed by an ODV-associated host alkaline protease and the second carried out by trypsin in the host midgut.

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Introduction

For many enveloped viruses proteolytic processing of virus membrane proteins is required to facilitate virus entry. In principle, the proteolytic cleavage converts a proprotein into an active conformation and/or exposes the functional domain, e.g. the fusion domain, to mediate virus binding and/or fusion. Cleavage of these proproteins may occur post-translationally during transportation of the protein through the *trans*-Golgi network. The F protein of many paramyxoviruses, for instance, is cleaved by the host protein furin (141). Alternatively, the protein is cleaved during virus entry either at the surface of recipient cells, such as influenza virus hemaglutinin (HA), which is cleaved by human airway trypsin-like protease (HAT) (12), or in the endosome, as for instance the cleavage of the SARS-Corona virus (SARS-CoV) S protein by cathepsin L (136).

Baculoviruses produce two viral phenotypes that follow different routes of infection (reviewed by Slack & Arif, 2007(137)). The occlusion derived virus (ODV) derived from occlusion bodies (OBs) initiates infection in midgut epithelium cells of host larvae and the budded virus (BV) is involved in systemic spread of the viral infection within the host. Like the F protein of paramyxoviruses, the BV fusion (F) protein of *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) is post-translationally cleaved by furin and this cleavage is essential for the function of F (159).

With baculovirus ODV, the situation is more complex. ODVs are embedded in a large proteinaceous crystal to form OBs. After being ingested by the insect host *per os*, the crystal structure of the OBs dissociates under the alkaline conditions in the midgut (pH 9-11), thereby releasing the ODVs (reviewed by (127)). These ODVs then bind and fuse with the microvilli of midgut epithelium cells to start the first round of infection (137) and this process is known as oral infection. So far six ODV membrane proteins have been found to be essential for oral infectivity and these were named *per os* infectivity factors (PIFs). They are P74 (PIF0), PIF1, PIF2, PIF3, PIF4 and PIF5 (ODV-E56) (42, 45, 58, 77, 107, 120). Three PIFs, P74, PIF1 and PIF2,were shown to function in ODV binding (57, 107), while the function of the other three PIFs is still unknown. Recently, Peng et al. (2010a)(117) reported that at least four of these PIFs, PIF1, 2, 3 and P74 are present in the ODV membrane in the form of a complex. The P74 protein, representative of a class of highly conserved proteins among baculoviruses, was reported to undergo a proteolytic cleavage mediated by insect midgut trypsins releasing a ≈20 kDa fragment from the N-terminus of P74. This cleavage was shown to be essential for P74-mediated *per os* infectivity (139).

For a number of baculoviruses, including AcMNPV, an alkaline protease was found to be associated with larvae-derived OBs (L-OBs) (27, 36, 79, 80, 114, 146, 162). This protease was suggested to function in the degradation of the major matrix protein of OBs (polyhedrin) and/or to assist in the release of ODVs (114, 146). However, the functional significance of the presence of this alkaline protease in OBs is not fully understood yet. Recently, Slack and Arif (2007)(137) envisioned that this OB-associated alkaline protease could play a synergistic role in infection by proteolytic activation of released ODVs, but experimental data supporting this supposition is lacking.

In this study we provide evidence that a potential host-derived alkaline protease associated with AcMNPV L-OB cleaves P74 efficiently and specifically during ODV release under alkaline condition. A sequential proteolytic cleavage model of P74 accommodating both the OB endogenous alkaline protease and midgut host trypsin is proposed.

Materials and methods

Viruses, cells and insects

The AcMNPV E2 strain was used as wild type (wt) virus in this study. The AcMNPV bacmid is derived from the Bac-to-Bac system (Invitrogen). *Spodoptera frugiperda* Sf9 cells (Invitrogen) were propagated in Sf-900II medium (Invitrogen) with 5% FBS. BTI-Tn-5B1-4 cells (Tn-High Five, Invitrogen) were grown in Express Five SFM medium (Invitrogen) and Se-UCR (50) in Grace's Insect Medium (Sigma) supplemented with 5% FBS. All three cell lines were propagated as monolayers at 27ºC. *Spodoptera exigua* (Se) and *Trichoplusia ni* (Tn) larvae were reared on artificial diet at 27ºC, 40% humidity with a 16:8 h (light:dark) photoperiod. In this study, OBs produced from cell culture or larvae are named C-OB or L-OB respectively. Correspondingly, ODVs purified from C-OB or L-OB are named as C-ODV or L-ODV. The origin of OBs and ODVs are also indicated, e.g. Sf9-C-ODV indicates that the ODVs are purified from C-OB produced in Sf9 cells while Se-L-OB indicates that the L-OB is produced in Se larvae.

P74 deletion and repair viruses

An AcMNPV bacmid with a deletion of the *p74* ORF was constructed as previously described (117). For this, a PCR product with 50 bp overhangs homologous to flanking regions of the *p74* gene was generated with primer pair p74-del-F (GCGGCGTCG

TGTCCAACACGACGCCGTTCATGTACATGCAGACCTCCGAGACTGCTCGGATCCA CTAGTAACG) and p74-del-R (GCGTATAGCGAGCTAGTGGCTAACGCTTGCCCCA CCAAAGTAGATTCGTCAAACCTCTAGATGCATGCTCG) with Phusion polymerase (Finnzymes). Primers were designed to replace a fragment from nt 301 to 1638 of the *p74* ORF with the chloramphenicol resistance gene (*cat*). Primer sequences for generating the *cat* gene are underlined. To repair the *p74* deletion bacmid, the coding sequence of the *p74* gene plus its putative promoter region (from position -150 relative to the ATG start codon) were amplified by PCR using the primer pair p74-rep-F (GCGCCATGGGCACAACG AAATGATTATATATTA) and p74-rep-R (GCGGCATGCTTA*TTTGTCATCGTCAT CCTTATAGTC*AAATAACAAATCAATTGTTTTATAAT). N*co*I and *Sph*I restriction sites were introduced by the underlined sequences of the primers and used to clone the sequenced PCR product into a modified pFastBacDual vector that lacks the p10 promoter and contains the entire polyhedrin gene (pFBDΔP10-polh) as described before (117). A Flag tag sequence (italics in the sequence) was introduced by the reverse repair primer to generate a P74 protein with a C-terminal Flag tag. The resulting plasmid was used to construct a '*p74* repair' bacmid using the Bac-to-Bac transposition protocol (Invitrogen). The bacmids were used to transfect Sf9 cells to generate mutant and repair viruses.

Virus production and purification

A wt AcMNPV BV stock was generated as previously described (117). To produce and purify cell culture-derived OBs (C-OBs), Sf9, Tni-High Five or Se-UCR cells were infected with BVs at a MOI of 5 $TCID_{50}$ units per cell and infected cells were collected at 5 days post infection (p.i) by low speed centrifugation. The cell pellet was washed 3 times with deionized H_2O and then resuspended in 0.2% SDS and incubated at 37°C for 2 h or 4°C overnight with gentle rocking. The cell suspension was sonicated and centrifuged at 4,000×g for 20 min. The pellet was washed and resuspended in PBS and centrifuged over a 35-65% (w/w) sucrose gradient at 98,000×g for 60 min at 4°C. The banded OBs were diluted in PBS and collected by centrifugation at 55,000×g for 60 min. The OB pellet was washed 3 times with PBS and stored at 4°C for later use.

OBs derived from larvae (L-OBs) were produced in *S. exigua* or *T. ni* larvae (3rd instar) after oral infection with C-OBs produced in Sf9 cells. To purify L-OB from infected larvae the liquefied or liquefying larvae were collected and grinded in deionized H_2O containing 0.5% SDS and 1% Triton X-100. The suspension was homogenized, sonicated and passed

through two layers of cheesecloth. The L-OB containing material was then centrifuged at 4,000×g for 20 min. The pellet was washed twice with 0.5% SDS/1% Triton X-100 in deionized water, resuspended in PBS and purified through a sucrose gradient in the same way as C-OBs. The resulting L-OB pellet was washed twice with 1M NaCl and once with PBS. Finally, the L-OBs were stored in PBS.

ODV purification under various conditions

Prior to ODV purification, the PBS in the OB suspensions was replaced with ddH_2O (Millipore). For heat inactivation of the endogenous protease, the L-OB suspension was heated at 80°C for 40 min (heated-OB) prior to ODV purification (114). ODVs were either purified at room temperature or at 4°C (low temperature purification). For low temperature purification, OBs were incubated on ice-water (0°C) for 40 min. OBs were then treated with cold (4°C) alkaline DAS solution (0.1 M Na_2CO_3, 166 mM NaCl, and 10 mM EDTA, pH 10.5) for 10 min at 4°C and subsequent steps of purification was also performed at 4°C. In experiments in which the endogenous protease needed to be inhibited, NaOH was added to the ODV releasing solution at a final concentration of 0.1 M, at various time points after the addition of DAS. For time point 0 min, NaOH was mixed with DAS solution and this mixture was used to dissolve OBs. Non-dissolved debris was removed by centrifugation at 1500×g for 3 min. The supernatant was collected and ODVs were pelleted by centrifugation at 20,600×g for 25 min at 4°C.

In experiments where ODVs needed to be purified over a sucrose gradient, ODVs were released from OBs at 4°C and the ODV containing supernatant was layered onto a 25-65% (w/w) continuous sucrose gradient in 10 mM Tris-HCl, pH 7.5 and centrifuged at 98,000×*g* for 90 min at 4°C. The multiple virus bands were collected, diluted in 0.1 × TE (1 mM Tris-HCl pH 7.5, 0.1 mM EDTA), and collected by centrifugation at 55,000×*g* for 60 min at 4°C.

Co-immunoprecipitation

In CoIP analysis, Se-L-OB of the Ac-Rep-P74-Flag virus was used in order to detect both P74-N and P74-C-Flag subunits. For each experiment L-ODV was liberated from 2×10^9 L-OB and the L-ODV was purified through a sucrose gradient as described above. The ODV pellet was resuspended in 600 µl IP buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.2) containing 0.5% Triton-X100, sonicated briefly and incubated at 4ºC for 3 h with gentle

rotation. Meanwhile 15 µl of PIF1 antiserum or pre-immune serum (117) was incubated with 20 µl bed-volume of protein G agarose (Pierce) in 500 µl of IP buffer at 4ºC for 3 h. Subsequent steps of CoIP were performed as previously described (117). A portion of the supernatant was reserved as input sample. The captured proteins and input sample were treated with Laemmli buffer (125 mM Tris-HCl, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8) at 95ºC for 10 min. Proteins were then separated in10% SDS-PAGE.

Polyclonal antibody generation for P74

A 5' segment of 1299 bp of the *p74* ORF was selected for making an expression construct for P74 polyclonal antibody (P74-PAb) generation. The DNA fragment was PCR amplified with the primers P74-Ab-F (GCGGGATCCATGGCGGTTTT AACAGCC) and P74-Ab-R (GCGAAGCTTTTAAGCGATTCGAGTTAACGCT). *Bam*HI and *Hin*dIII restriction sites (underscored) were introduced by the forward and reverse primers, respectively. The amplified DNA was ligated into the pJet1.2 vector (Fermentas) for sequencing analysis and then cloned into the pET28a vector (Novagen) between the *Bam*HI and *Hin*dIII sites for protein expression. The pET28a-P74 construct codes for an N-terminal fusion of a 6×His tag with the AcMNPV P74 fragment. *Escherichia coli* BL21 cells were transformed with pET28a-P74 and expression was induced with isopropyl-ß-D-thiogalactopyranoside (IPTG, 1mM) for 4 h at 37°C. After washing with PBS for three times, the bacteria pellet was resuspended in lysis buffer (0.1% SDS, 1% Triton-X100, PBS, pH 7.4), incubated at 37ºC with gentle rotation for 30 min and then sonicated. Protein purification from the bacterial inclusion bodies was performed as previously described (117). The purified protein was analyzed with SDS-PAGE, Coomassie brilliant blue staining and western blotting with anti-His and P74 monoclonal antibodies (P74 MAb) to determine the specificity, purity and concentration. Purified protein was sent to Eurogentec (Seraing, Belgium) to generate polyclonal antibodies in rabbits.

SDS-PAGE, antibodies and western blot analysis

ODV samples were heated in Laemmli buffer at 95ºC for 10 min and separated in 10% SDS-PAGE. For non-reducing SDS-PAGE, samples were treated with Laemmli buffer lacking 2-mercaptoethanol. The non-reduced samples were also heated at 95ºC for 10 min. Anti-His monoclonal antibody from mouse and anti-Flag polyclonal antibodies from rabbit

were purchased from Sigma. The P74 MAb (45), anti-PIF1, anti-PIF2 and anti-PIF3 antibodies were previously described (117). Western blot analysis was performed as previously described (117, 118). Dilutions of the primary antibodies were as following: His MAb (1:2,000 dilutions), Flag PAb (2ug/ml), PIF1 PAb (1:2,000 dilution), PIF2 PAb (1:2,000 dilution), PIF3 PAb (1:1,000 dilution), P74 MAb (1:50 dilution) or P74 PAb (1:2,000 dilutions). Membranes were then probed with appropriate alkaline phosphatase-conjugated secondary antibodies (Sigma). The signal was developed with NBT/BCIP (Roche).

Computational analysis

Conserved domain analysis of the P74 amino acid sequence was performed with the NCBI conserved domain server: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. Transmembrane domain prediction of P74 was performed with the TMHMM 2.0 server as described before (118). Disulfide bonds were predicted by the DiANNA server: http://clavius.bc.edu/~clotelab/DiANNA/ (46).

Results

Cleavage of P74 during ODV release from L-OB

When purified ODV proteins from OBs derived from *S. exigua* larvae (Se-L-OB) (purified at 20°C) were separated in SDS-PAGE and analyzed with P74 MAb, a 35 kDa fragment instead of 72 kDa full-length P74 protein was detected (Fig. 5.1A, lane 1). Since the P74 MAb is specific to the N terminus of P74 recognizing an epitope located between S_{68} and R_{195} (138), the observed 35 kDa fragment may represent an N terminal fragment of P74 (P74-N) suggesting proteolytic cleavage of P74. The same ODV protein samples were also analyzed with antibodies against PIF1, PIF2 and PIF3, which are the other three known components of the PIF complex. Under the same purification conditions these other three PIFs were not cleaved (Fig. 5.1 B-D, lane 1). Cleavage of P74 could be prevented by heating the L-OB at 80°C for 40 min (Fig. 5.1A, lane 3). Heating had no effect on the other three PIFs (Fig. 5.1 B-D, lane 3). This indicates that an OB endogenous alkaline protease, which can be inactivated by heating, is responsible for P74 cleavage.

In previous reports the temperature optimum of the endogenous alkaline protease of L-OB was reported to be 30 to 40°C (114, 149). Therefore L-ODVs were purified under low temperature condition with the intention to avoid P74 cleavage. L-OBs were incubated at 0°C

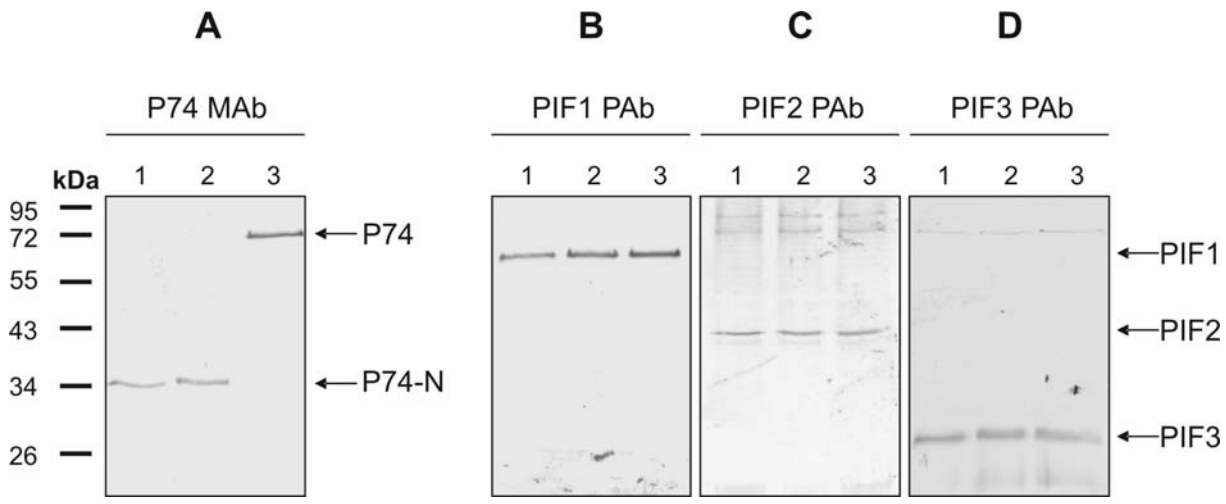

Figure 5.1. P74 is cleaved efficiently and specifically during L-ODV purification. OBs produced from *S. exigua* larvae were used (Se-L-OB). L-ODVs were purified under three different conditions. For the samples in lane 1, L-OB was dissolved in DAS at room temperature. For the samples in lanes 2, L-OB was pre-cooled in ice-water for 40 min and dissolved with cold DAS (4°C). For the sample in lanes 3, L-OB was heated at 80°C for 40 min and then dissolved in DAS solution. For all three treatments L-ODVs were purified at 4°C after DAS treatment. ODV samples were separated in 10% SDS-PAGE and analyzed with P74 Mab (A), or PIF1, PIF2 and PIF3 antibodies (B,C and D). Positions of full-length P74, P74-N, PIF1, PIF2 and PIF3 are indicated by arrows. P74-N represents the N terminal P74 fragment after cleavage.

for 40 min and exposed to cold DAS solution (4°C) and ODVs were purified at 4°C. However, as shown in Fig. 5.1A lane 2, even under the low temperature condition P74 was cleaved completely. Again the other three PIFs were not affected (Fig. 5.1 B-D lane 2). These results indicated that the P74 cleavage is not only specific but also very efficient.

No P74 cleavage with OBs produced in cell lines

To address the question whether P74 cleavage also occurs in OBs produced in cell cultures (C-OBs), AcMNPV OBs were produced in Sf9, Tni-High Five and Se-UCR cell lines. ODVs were purified from Se-L-OBs and the different C-OBs with (Fig. 5.2, lanes 2, 4, 6 and 8) or without (Fig. 5.2, lanes 1, 3, 5 and 7) heat treatment. P74 in these ODVs was analyzed with P74 MAb. In C-ODVs from three different cell lines full length P74 was detected whether or not OBs were treated at 80°C prior to ODV purification (Fig. 5.2, lanes 3-8). Cleavage of P74 did not happen in C-OBs even when the OBs were dissolved at 37°C and ODVs were purified at room temperature (data not shown). In contrast, P74 on L-ODV was cleaved completely (Fig. 5.2, lanes 1) in non-heated Se-L-OB. As before, heating of Se-L-OB prior to ODV purification prevented P74 cleavage and full-length P74 was detected (Fig. 5.2

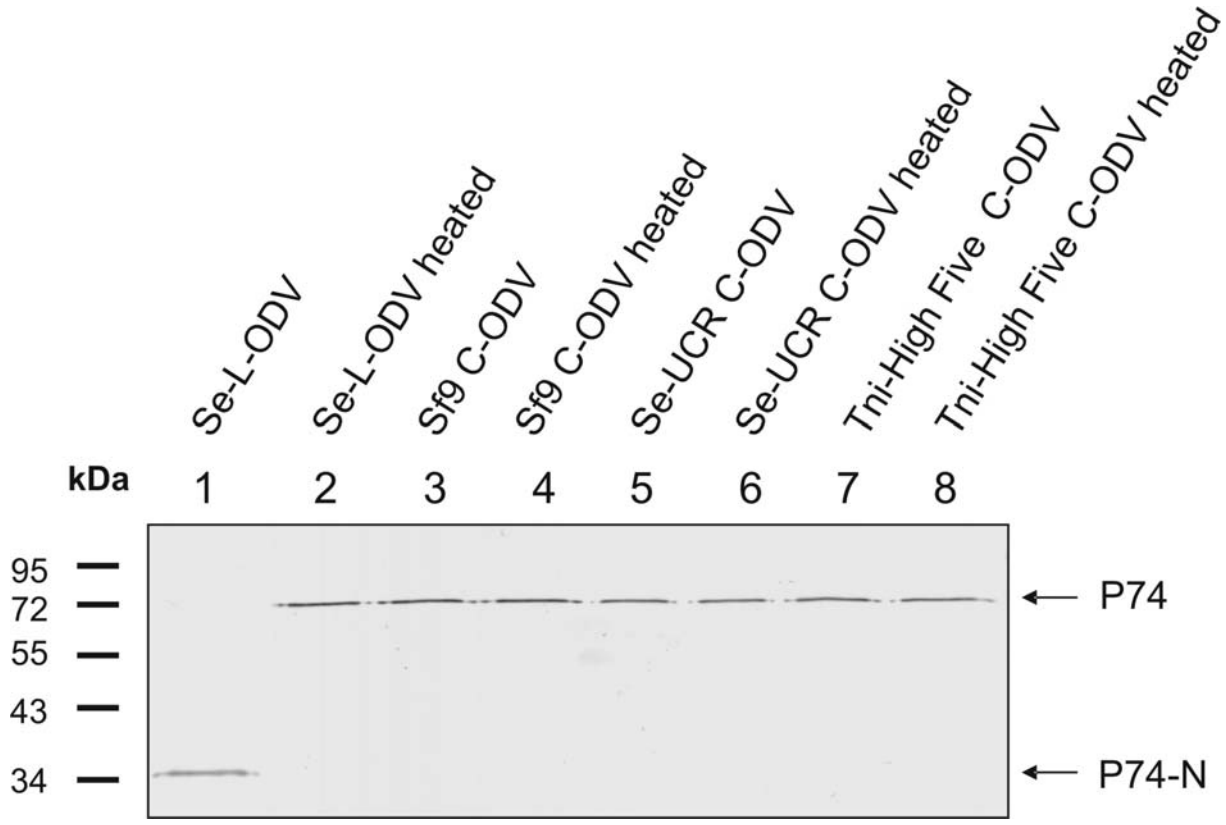

Figure 5.2. P74 cleavage does not happen in C-OBs produced in three cell lines. OBs produced in *S. exigua* larvae (Se-L-OB) and C-OBs produced in Sf9, Tni-High Five or Se-UCR cells were used in this experiment. OBs were directly used for ODV purification or first heated at 80°C for 40 min before dissolving in DAS at 20°C. Purified ODVs were analyzed in 10% SDS-PAGE and western blot analysis was performed with P74 MAb. The full-length P74 and P74-N fragment are indicated by arrows.

lane 2). These results suggest that the protease responsible for P74 cleavage is derived from the larval host and not of viral origin.

P74 cleavage is conserved in AcMNPV L-OBs from two different host larvae

Since Se-L-OBs appear to contain a host protease that cleaves P74, the next question is whether L-OBs derived from different hosts would incorporate similar proteases to cleave P74 in the same way. To address this question, AcMNPV OBs were also produced in *T. ni* larvae. Like Se-L-OB, OBs produced from *T.ni* larvae were named Tni-L-OB. ODV were purified from heated or non-heated Se-L-OB, Tni-L-OB and Sf9-C-OB and analysed with P74 MAb on immunoblots. P74 in non-heated Se- or Tni-L-OB was cleaved completely (Fig. 5.3, lanes 1 and 5) and in each case the P74-N fragment migrated with a molecular mass of approximately 35 kDa. Heat treatment blocked P74 cleavage in both Se- and Tni-L-OB and

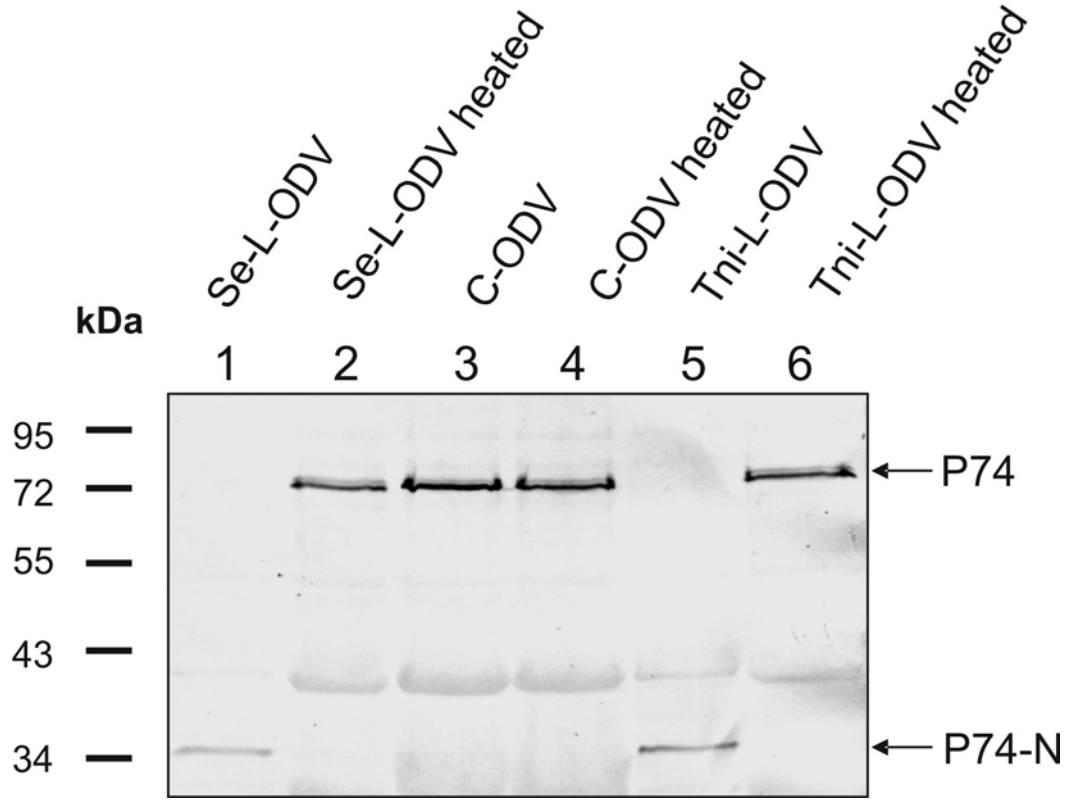

Figure 5.3. P74 cleavage is conserved in L-OBs produced in two different hosts.
L-OBs were purified from infected *S. exigua* and *T. ni* larvae. Sf9-C-OBs were included as a control. OBs were either treated with DAS to purify ODVs directly or were heated at 80°C for 40 min before dissolving in DAS. Purified ODVs were separated in 10% SDS-PAGE. Western analysis was performed with P74 MAb. Arrows indicate the positions of full-length P74 and cleaved P74-N fragment.

full-length P74 was detected (Fig. 5.3, lanes 2 and 6). In contrast, P74 on C-ODV was not cleaved and was present as a full-length protein no matter whether the Sf9-C-OB was heated or not (Fig. 5.3, lane 3 and 4). The fact that P74 in L-OBs derived from different hosts was cleaved into similar, if not identical, protein fragments suggests that similar proteases from different hosts are incorporated into AcMNPV OBs. Alternatively the cleaved region of P74 is sensitive to and can be cleaved by different proteases.

Cleavage of P74 is highly efficient

To further estimate the efficiency of P74 cleavage the endogenous alkaline protease was inactivated with NaOH during ODV release from OB as previously reported (114). To this aim L-OBs were exposed to DAS solution supplemented with NaOH to a final concentration of 0.1 M at different times after exposure to DAS. The purified ODVs were then analyzed with P74 MAb. As shown in Fig. 5.4, if NaOH was added to the DAS solution

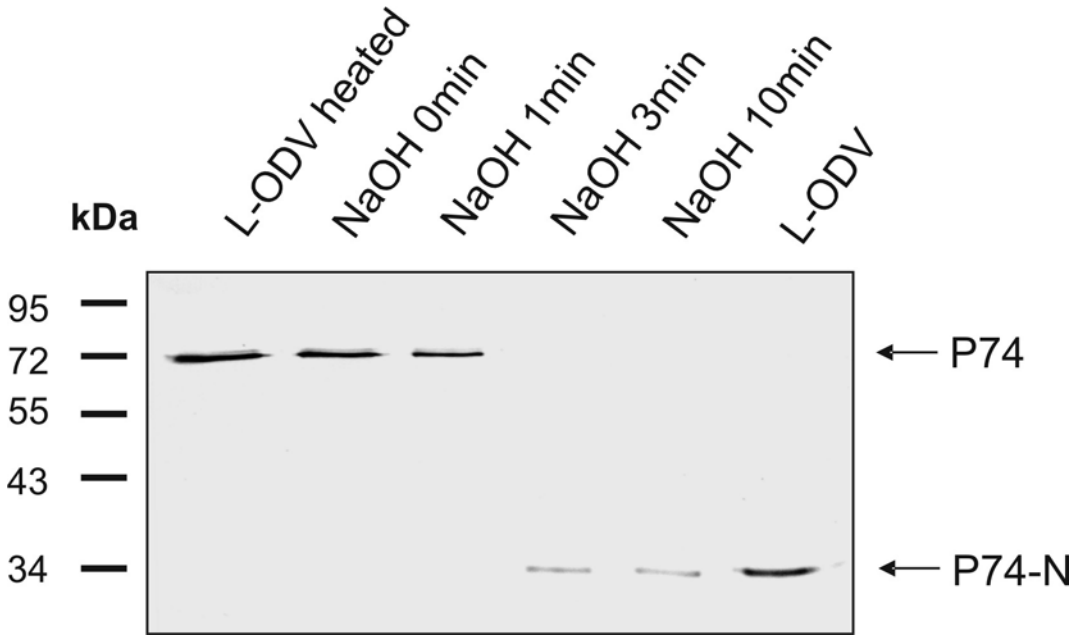

Figure 5.4. P74 cleavage is highly efficient. Se-L-OB was used in this experiment. Heated OBs were incubated incubated at 80°C for 40 min before ODV purification. L-OBs were dissolved in DAS and NaOH was added to the OB dissolving solution to a final concentration of 0.1 M at the indicated time after adding DAS. For the sample "NaOH 0min", NaOH was mixed with DAS and the mixed solution was used to dissolve L-OBs. Purified ODVs were separated in 10% SDS-PAGE and western analysis was performed with P74 MAb. Full-length P74 and the cleaved P74-N fragment are indicated by arrows.

at 0 or 1 min, only full-length P74 was detected (Fig. 5.4, lane 2 and 3) indicating inactivation of the protease. However, if NaOH was added after L-OBs were exposed to DAS solution for 3 or 10 min, only the P74-N cleaved fragment was detected (Fig. 5.4, lane 4 and 5). When L-ODVs were purified without prior heat treatment and without NaOH treatment, P74 was also cleaved completely (Fig. 5.4, lane 6). Like before, when L-OB was heated prior to ODV purification full-length P74 was detected (Fig. 5.4, lane 1). These results showed that the alkaline protease can be inactivated by NaOH and P74 cleavage is accomplished within 3 min after L-OBs were exposed to alkaline conditions (DAS). It should be noted that in NaOH treated samples (Fig. 5.4, lanes 4 and 5) the signal intensity of the P74-N fragment was lower than that of the untreated sample (Fig. 5.4, lane 6) despite the same amount of OBs used. The pH value of the DAS solution mixed with 0.1 M NaOH is around 12.8 while the pH value of DAS solution alone is around 10.5. It is possible that the higher pH in the NaOH treated solutions weakened the association between the P74-N fragment and the ODV, and therefore, less P74-N fragment was co-purified with ODV. Protein-protein interactions are often sensitive to pH as has been previously reported, e.g. (69, 82, 121, 144).

P74 is cleaved into two fragments

Without detecting the P74-C fragment it is difficult to judge whether P74 is cleaved into two subunits or whether the C terminal fragment will undergo further processing. A polyclonal antibody of P74 was generated in this study with the intention to cover more epitopes of P74. However, in practice the PAb only efficiently recognized the P74-N fragment like the P74 MAb and potential P74-C fragment(s) were not identified (data not shown). To address this issue, a bacmid with a deletion of the *p74* ORF was constructed and from this a P74-repair bacmid was made encoding a P74 with a C terminal Flag tag (Fig. 5.5A). The recombinant virus Ac-Rep-P74-Flag was as infectious as wt AcMNPV while the

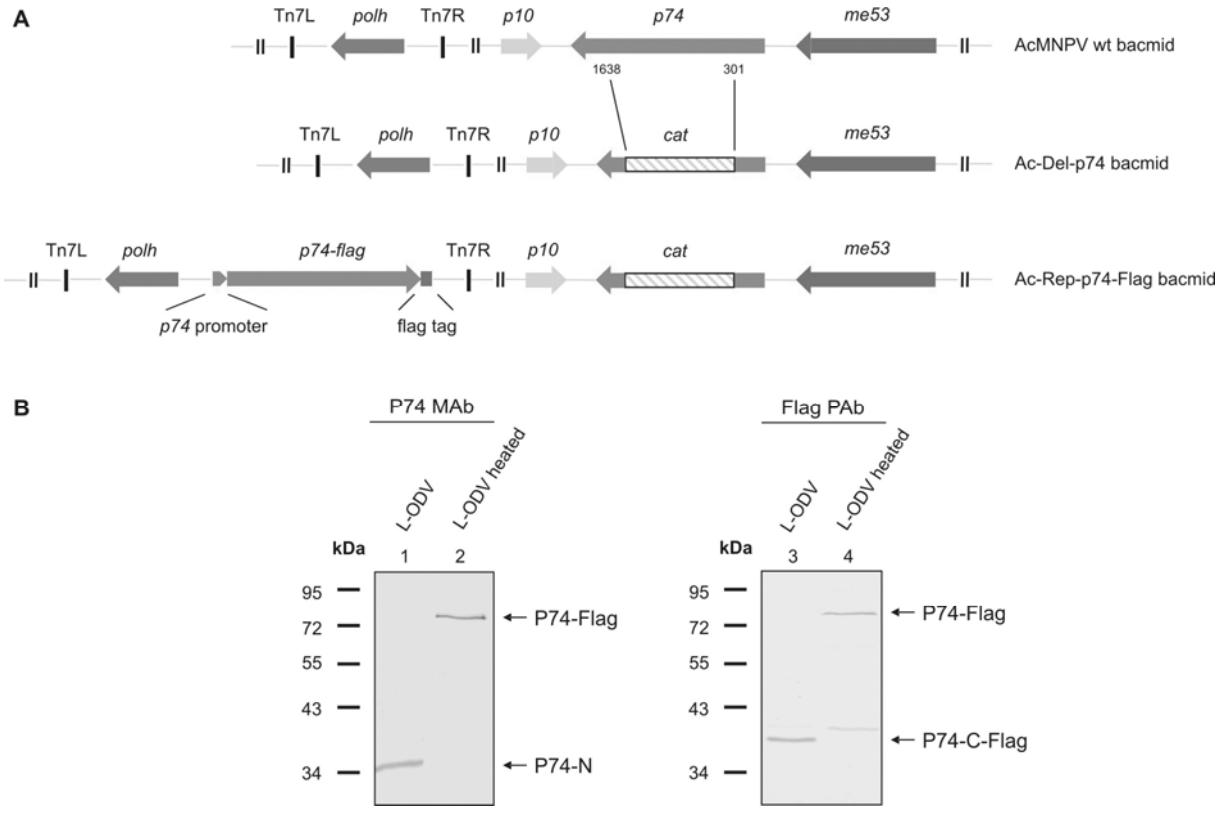

Figure 5.5. P74 is cleaved into two subunits.
(A) Schematic of the *p74* recombinant bacmids constructed in this study. The orientations of *p74* and the adjacent *p10* and *me53* genes in the bacmids are indicated. All bacmids contain the *polh* ORF under its own promoter. Part of the *p74 ORF*, ranging from nt 301 till 1638, was replaced with the chloramphenicol acetyl transferase gene by homologous recombination to generate the Ac-del-p74 bacmid. In the repaired bacmid, the *p74* ORF with its putative promoter region and a Flag tag sequence fused to the 3'end were included. wt: wild type; Del: deletion; Rep: repair.
(B) Detection of the P74-N and P74-C-Flag subunits. Ac-Rep-P74-Flag L-OBs were produced and purified from *S. exigua* larvae. Heating was performed at 80°C for 40 min for heated OBs. ODVs were purified from non-heated or heated OBs and were separated in 10% SDS-PAGE. Western analysis was performed with P74 MAb or Flag PAb. Positions of full-length P74-Flag and the P74-N and P74-C-Flag fragments are indicated.

p74 deleted virus was not orally infectious (data not shown). The Ac-Rep-P74-Flag virus was then used for analyzing the cleaved P74-C fragment. L-OBs of Ac-Rep-P74-Flag virus were produced in *S. exigua* larvae and purified. L-ODVs were then prepared from heated or non-heated L-OBs and ODV samples were separated by SDS-PAGE and analyzed with P74 MAb and Flag PAb to detect P74-N and P74-C subunits respectively. With the MAb, a 35 kDa P74-N fragment was detected for the non-heated L-OB (Fig. 5.5B, lane 1) while a 75 kDa P74-Flag protein was detected with the heated L-OB (Fig. 5.5B, lane 2). When the same samples were probed with Flag PAb a 40 kDa P74-C fragment (P74-C-Flag) and a 75 kDa P74-Flag protein were detected in the non-heated and heated samples, respectively (Fig. 5.5B lane 3 to 4). The molecular mass of P74-N and P74-C-Flag added up approximately to the molecular mass of full-length P74-Flag of about 75 kDa (the predicted size is 74.8 kDa). These results strongly suggest that P74-Flag was cleaved into two fragments: P74-N and P74-C-Flag.

Both P74-N and P74-C-Flag fragments are associated with the PIF complex

It was noticed that the P74-N subunit could be co-purified with ODV, indicating that the P74-N subunit remains associated with the ODV after P74 cleavage. Our previous study has showed that P74 may associate with a stable complex of PIF1, PIF2 and PIF3 present on the ODV surface (117). To investigate whether in L-ODVs the cleaved P74 subunits still associate with this complex, a CoIP analysis with PIF1 antibody and pre-immune serum as a control was performed with L-ODV membrane proteins. Ac-Rep-P74-Flag L-ODV was used to be able to detect both P74-N and P74-C-Flag fragments. P74 PAb and Flag PAb were used to detect P74-N and P74-C-Flag fragments, respectively. As shown in Fig. 5.6A, P74-Flag was cleaved into P74-N and P74-C-Flag subunits (lanes 1 and 3) and both fragments were precipitated by the PIF1 antibody (lanes 2 and 4). In contrast, the two P74 fragments were not precipitated by pre-immune serum (Fig. 5.6B lanes 2 and 4) although they were present in the input sample (Fig. 5.6B, lanes 1 and 3). Heated Ac-Rep-P74-Flag L-ODV was included to show the position of full-length P74-Flag (Fig. 5.6A, B lane 5). Similarly, PIF1 and PIF2 were both precipitated by the PIF1 antibody (Fig. 5.6A, lanes 6-9), but not by the pre-immune antiserum (Fig. 5.6B, lanes 6-9). These results showed that both P74-N and P74-C-Flag fragments remain associated with the PIF complex after the cleavage.

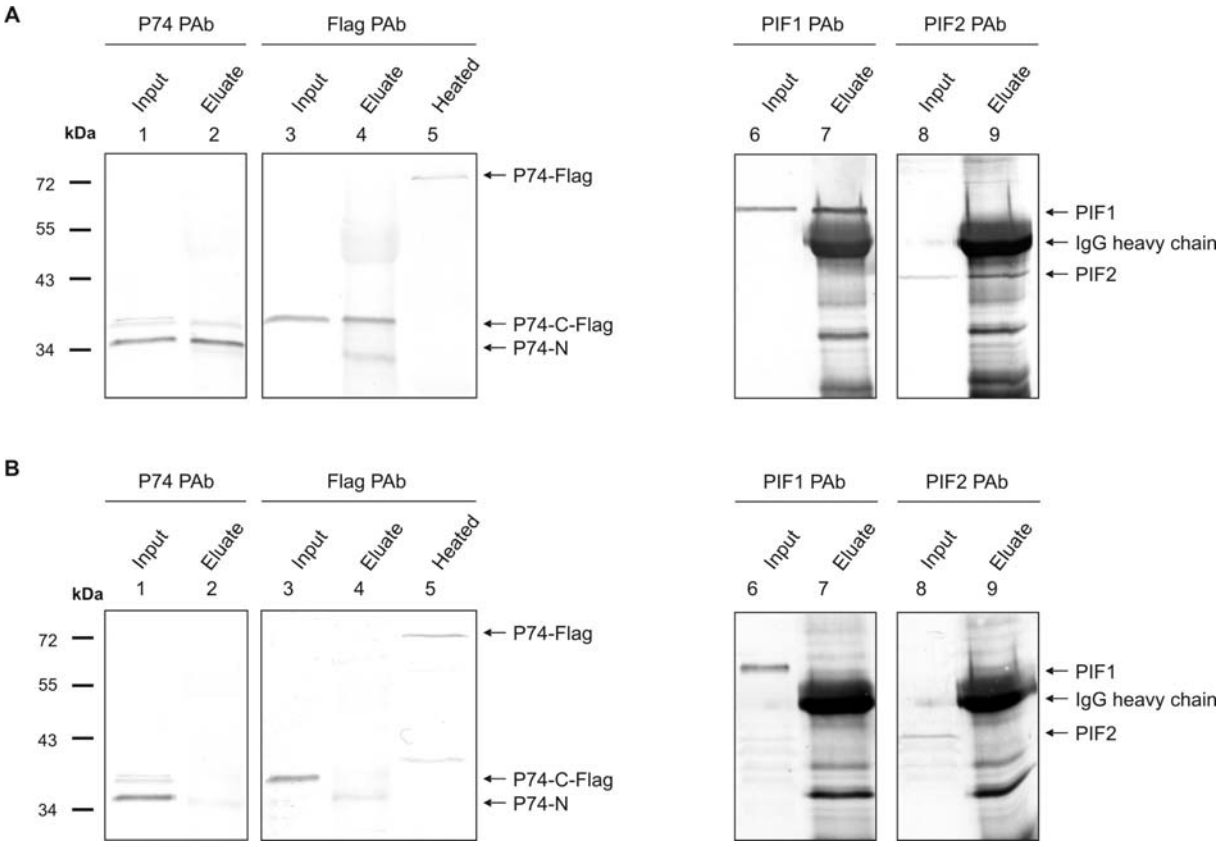

Figure 5.6. The cleaved P74-N and P74-C-Flag subunits associate with the PIF complex after cleavage. Ac-Rep-P74-Flag L-ODVs were released from Se-L-OB and purified through a sucrose gradient. ODV membrane proteins were extracted for CoIP analysis. CoIP was performed with PIF1 antiserum and with pre-immune serum as negative control. CoIP input and eluate were separated in 10% SDS-PAGE followed by western analysis with P74 PAb, Flag PAb, PIF1 or PIF2 antibodies. To show the position of full length P74-Flag, L-ODV was purified from heated L-OBs and detected with Flag PAb. Positions of full-length P74-Flag, P74-N, P74-C-Flag, PIF1, PIF2 and IgG heavy chain are indicated by arrows. (A) CoIP results with PIF1 antibody. (B) CoIP results with PIF1 pre-immune serum.

The P74-N and P74-C-Flag fragments do not interact in a covalent way

For many viral membrane proteins the cleaved subunits interact with each other either in a covalent (141, 145, 159) or a non-covalent way (48). The fact that both P74-N and P74-C-Flag subunits were found in association with the PIF complex suggests that these two subunits may also interact with each other. To investigate whether the P74-N and P74-C-Flag subunits interact in a covalent way, non-reducing SDS-PAGE analysis was performed. L-ODVs of Ac-Rep-P74-Flag virus were purified from heated and non-heated L-OBs and treated with protein sample buffers with or without the reducing agent 2-mercaptoethanol. Samples were separated by SDS-PAGE followed by western analysis with P74 MAb and Flag

PAb. In the non-reduced sample the P74-N fragment migrated as a 32 kDa protein (Fig. 5.7A, lane 1), while in the reduced sample the fragment had a molecular mass of 35 kDa (Fig. 5.7A, lane 2). The P74-C-Flag subunit migrated with a molecular mass of 40 kDa under both reducing and non-reducing conditions (Fig. 5.7B, lanes 1 and 2). The position of full-length P74-Flag was shown in heated ODV sample (Fig. 5.7A, B, lane 3). These results suggested that the two P74 fragments are not associated covalently through disulphide bonds.

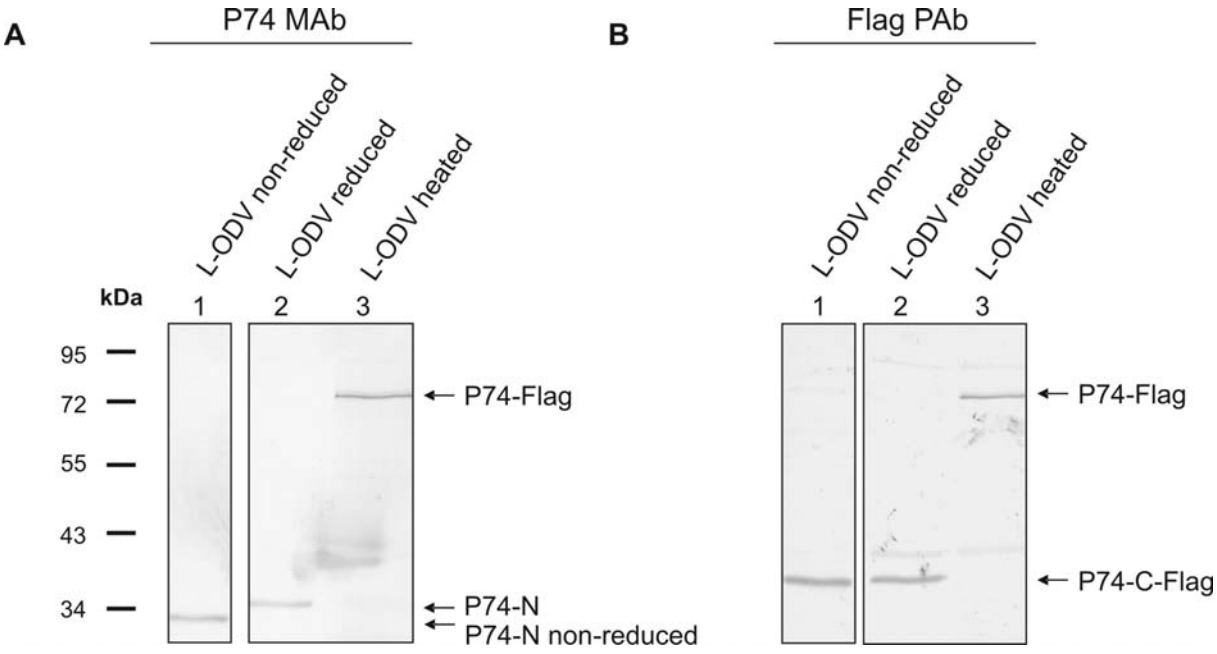

Figure 5.7. P74-N and P74-C-Flag subunits are not associated through disulphide bonding. L-ODVs of Ac-Rep-P74-Flag virus were purified from heated or non-heated L-OBs. The non-heated L-ODV sample was treated with reducing or non-reducing Laemmli buffer and the heated L-ODVs were treated with reduced Laemmli buffer. Proteins were separated in 10% SDS-PAGE. Western analysis was performed with P74 MAb and Flag PAb. Arrows point at full-length P74-Flag, P74-N, P74-N non-reduced and P74-C-Flag.

Discussion

Host-derived alkaline proteases have been found in a number of baculovirus L-OBs, but their functional significance remains enigmatic, except for some suggested roles in solubilization of the OB matrix or the release of ODV from polyhedra (114, 146). Recently, Slack and Arif (2007)(137) speculated that such proteases could play a synergistic role by ensuring activation of released ODVs for virus infection. Here we show that P74, a conserved ODV protein with midgut epithelium binding properties (57) and a component of the PIF

complex, is cleaved by the OB alkaline protease during release of ODVs from OBs and probably prior to infection. This proteolytic cleavage could be part of a mechanism for activation of P74 to function in ODV binding to midgut microvilli and/or further downstream steps in the entry process.

P74 cleavage occurred in a highly efficient way as evidenced by the fact that cleavage was accomplished within 3 min after L-OBs were exposed to the alkaline solution. Furthermore P74 was cleaved completely even if L-ODVs were purified at 4°C. It was reported before that ODVs are released from OBs within 12 min post entry into the insect midgut (1, 137). Therefore, it is possible that P74 is already cleaved before ODV is fully released into the midgut lumen and hence before it contacts its host receptor. If this is true the cleaved P74 should represent a functional form. To test this possibility L-ODVs from Tni-L-OB were purified in the same way as described in the previously reported functional analysis of AcMNPV P74 (57) and we found that P74 was present only in the cleaved form in the ODV (data not shown). Whether it is the cleaved form of P74 that is functioning in ODV binding needs further investigation.

P74 is encoded by a core gene of baculoviruses and it also has homologues in several other large invertebrate, nuclear-replicating DNA viruses such as nudiviruses (156), salivary gland hypertrophy viruses (49), and whispoviruses (155), as well as polydnavirus particles (8). Therefore, the function and/or mode of action of P74 in virus entry might be conserved in all these viruses. Analysis of sequence data shows that the P74 protein contains two conserved domains, each belonging to a specific superfamily: the Baculo_p74_N superfamily and the Baculo_p74 superfamily (Fig. 5.8A). Judging by the size of the P74-N subunit (35 kDa), the cleavage is likely to occur in the region between these two conserved domains. It was previously shown that in ODVs purified from C-OBs full-length P74 associates with complex of PIF1, PIF2 and PIF3 (26). CoIP analysis showed that after cleavage both P74-N and P74-C subunits remain associated with the PIF complex. These results suggest that cleavage of P74 is not prerequisite for its association with the other three PIFs in the complex. Non-reducing SDS-PAGE showed that the two subunits are not associated covalently, but they may interact with each other in a non-covalent way like the S1 and S2 fragments of the murine coronavirus S protein or HIV GP120 and GP41 (48, 111). Whether the two subunits interact with the PIF complex separately or whether they interact with each other in a non-covalent way and then associate with the PIF complex is unknown yet. It is tempting to

speculate, that these two domains form a conserved functional conformation after proteolytic cleavage to facilitate oral infection.

Cleavage of P74 was not observed in OBs produced in cell lines derived from three different insects. Similar results were reported in previous studies (114, 162). This suggests that the protease in the L-OB is not encoded by the virus, but is derived from the host. The

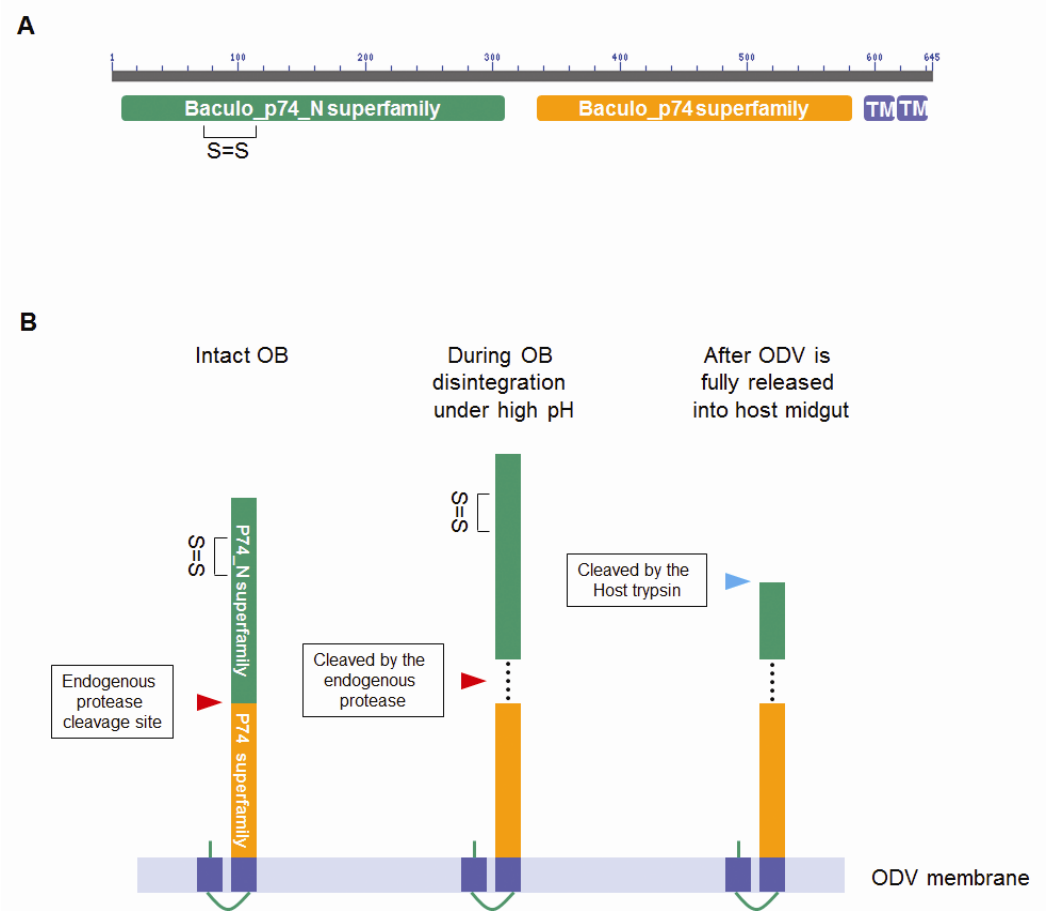

Figure 5.8. Proposed model for sequential cleavage of P74 in L-ODV based on conserved domain prediction and experimental data. (A). Conserved domains in P74. The AcMNPV P74 amino acid sequence was analyzed with the NCBI Conserved domains server. Baculo_p74_N (green block) and Baculo_p74 superfamilies (yellow block) were identified as indicated. The homology with the Baculo_p74_N superfamily starts with aa 5 and lasts till the 309 aa of AcMNPV P74. The Baculo_p74 superfamily domain starts at amino acid (aa) 333 and lasts till aa 583. The only disulphide bond (between cysteine 72 and cysteine 109) predicted with high probability by the DiANNA server is indicated. Score for this prediction was 0.99261 within a range of 0 to 1. Two conserved C terminal transmembrane domains predicted by the TMHMM 2.0 server are also indicated and named as TM (blue block). (B). Model of P74 sequential cleavage. The two conserved P74 domains and the potential intra-molecular disulphide bond in the P74-N subunit are indicated. The first cleavage of P74 is proposed to happen between the two conserved domains and during OB disintegration under alkaline conditions. A potential mode of interaction between the two fragments is indicated by dashed lines. After the first cleavage by the endogenous protease, the P74-N subunit is predicted to undergo a second proteolytic cleavage by a host trypsin present in the insect midgut.

fact that P74 was cleaved in the same way in AcMNPV L-OBs produced in different hosts indicates that the virus is able to select similar proteases from different hosts to cleave P74. This conservation further suggests a functional significance for the association of the protease with the OBs and the subsequent P74 cleavage event. If the protease is indeed derived from the host, how the protease is recognized and accommodated into the OB structure and whether certain viral proteins are involved in these processes would be highly interesting questions for further research.

Where this alkaline protease is located in the AcMNPV OB structure is also unclear. The protease is unlikely to be present on the surface of L-OBs as in this study the OBs were routinely washed with 0.5% SDS and 1 M NaCl, while P74 cleavage was preserved. Spiking C-OBs with larval homogenate before OB/ODV purification also did not lead to P74 cleavage (data not shown), excluding the possibility that cleavage by the protease is due to surface contamination during OB purification. It is possible that the alkaline protease is associated with the polyhedrin matrix. However, this localization may not give the enzyme quick and proper access to P74 upon ODV release. For *S. littoralis* NPV an alkaline protease was found to localize on the ODV membrane (114). Considering the high efficiency of P74 cleavage it is reasonable to assume that the protease, or at least some of the protease, is also located on the ODV membrane in AcMNPV.

The biological significance of the alkaline protease is not clear since in a previous study AcMNPV OBs (C-OB) produced *in vitro* are quantitatively as infectious to cabbage looper larvae as those produced *in vivo* (L-OB) (150). However, a later study showed that mortality occurred significantly more rapidly following infection with L-OB compared to C-OB (11). The endogenous P74 cleavage, which probably happens before ODV is fully released and contacts its putative host receptor, could be one of the reasons to explain this faster mortality. Cleavage of P74 into two associating fragments as demonstrated by CoIP, may facilitate the protein to switch to an active conformation and/or expose certain functional domains. The P74 on C-ODV may be proteolytically cleaved by host alkaline protease(s) present in the midgut after the ODV is fully released. The delay of C-ODV P74 activation may delay the entry process and hence the time of death. The incorporation of alkaline proteases into the ODVs may therefore have evolutionary advantage by speeding up the infection process.

Proteolytic cleavage is a strategy employed by many viruses to activate their membrane proteins. Examples include SARS-CoV S protein (5), paramyxovirus F protein

(141) and influenza virus HA (145). In these viruses cleavage occurs during virus entry on the cell surface or in endosomes inside the cell or post-translationally during protein translocation. The situation is different in the case of baculovirus P74 cleavage. It seems that the virus is able to specifically capture a host-encoded alkaline protease and incorporate it into the virus structure, which will become activated only under alkaline conditions, e.g. in the midgut of infected host insect. The P74 cleavage event reported in this study is likely to happen during the release of ODVs from OBs and results in two associating fragments: P74-N and P74-C. After this cleavage, the P74-N fragment probably undergoes a second proteolytic cleavage by a host midgut trypsin (139), which has been shown to be essential for infectivity (at least for C-OBs). The proposed two-step sequential proteolytic cleavage may represent a novel virus membrane protein activation mechanism (Fig. 5.8 B), which in contrast to vertebrate virus membrane protein activation mechanisms occurs under alkaline conditions. Incorporation of an endogenous alkaline protease into the virus was also reported for other insect viruses such as *Choristoneura biennis* entomopoxvirus (9) and iridescent virus type 6 (44). This common feature suggests that incorporating a protease into the virus structure has an evolutionary advantage for some invertebrate insect viruses.

The present study reports the efficient and specific cleavage of P74 by an endogenous alkaline protease during the release of ODVs from L-OBs and sheds light on the potential significance of this protease in the process of ODV entry. To further investigate the biological significance of this processing event, the cleavage site in P74 for the alkaline protease will be identified in order to generate mutants defective in this P74 cleavage. Computational analysis of P74 proteins of closely-related baculoviruses have not (yet) revealed a common motif for potential alkaline protease cleavage, which complicates the genetic approach somewhat. It would also be interesting to purify and identify the endogenous alkaline protease to address the question how it is selected and incorporated by the virus.

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CHAPTER 6

General discussion

Baculovirus occlusion-derived virus (ODV) is an enveloped virus with a complex virion structure (137). ODV formation takes place in the nucleus of infected cells and the virus gains its envelope from the inner nuclear membrane (18). ODV contains more than 10 different envelope proteins. A group of these is known as *per os* infectivity factors or PIF proteins and they are essential to the ODV oral infectivity. These PIFs mediate ODV entry into insect midgut epithelial cells under alkaline conditions, which is different from the current models of virus entry occurring under either low or neutral pH conditions (97). The genes encoding PIFs are highly conserved among baculoviruses and some other invertebrate, nuclear replicating DNA viruses (151, 154) indicating that the entry mechanism mediated by these proteins might have arisen early in evolution. The special features associated with ODV entry and the complex virion structure makes ODV a unique model to study virion entry and assembly. In this thesis initial efforts were made to gain further insights in the mechanisms of ODV entry and assembly.

ODV assembly

ODV assembly is a sequentially organized process, including viral genome packaging, nucleocapsid assembly, tegument formation, ODV envelopment and embedment in the occlusion body (OB) structure (137). These events involve a plethora of interactions not only between viral proteins but also between viral and host proteins. To begin to investigate these complicated processes, the yeast two-hybrid system was chosen, which is the most frequently used high-throughput system to study protein-protein interaction (PPI) (112). Using this system the interactions among the known structural proteins of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) ODV were studied (Chapter 2). Interactions were screened for 39 proteins including 10 membrane proteins, the latter in truncated forms i.e. without transmembrane domains. Twenty-two interactions including 13 binary interactions and 9 self-associations were identified, of which 17 interactions were identified for the first time. Combined with previously known PPI, a network of PPI among ODV structural proteins and with some host proteins was proposed, which provided new insights on the ODV assembly mechanism.

It should be noted that the identified interactions among membrane proteins in the yeast two hybrid (Y2H) screen should be interpreted more cautiously. This is because the chosen Y2H system, which detects PPI in the nucleus, is not specifically suitable for studying PPI of membrane proteins (47). Indeed in the early stages of this work when full-length membrane proteins were expressed in the Y2H system, no interactions were identified. To overcome this limitation truncated ODV membrane proteins were expressed that did not contain transmembrane domains. A number of interactions were identified among these truncated proteins. However, a concern constantly existed in interpreting these interactions, which is whether these truncated proteins were still able to form their physiological interactions as during ODV assembly. Therefore, the identified interactions among membrane proteins should be carefully verified with alternative methods.

In chapter 2 HearNPV was chosen as a model to study the interactome of baculovirus ODV proteins because of its importance in the bio-control of the cotton boll worm *Helicoverpa armigera*, a major pest of cotton production in China (164). Since HearNPV is a representative of Group II NPV, several ODV components that are specific to Group II NPV or even unique to HearNPV were included in this study. They are HA44, HA100, HA107 and HA122 (33). Identification of interactions of these specific components would shed light on potentially unique mechanisms of Group II ODV assembly. HA44 was found to form homo-oligomers and this was verified by a pull-down assay in this study (Fig. 2.2). The significance of the self-association of HA44 to ODV assembly and viral infectivity should be analyzed with a *ha44* mutant virus in which self-association of HA44 is impaired. HA100 was found to interact with VP80 and IE1 in this study. VP80 of AcMNPV was shown to be important for progeny NC formation and egress of NC from virogenic stroma towards the nuclear periphery (95, 96). Whether VP80 of HearNPV has similar roles and whether the interaction of HA100-VP80 is relevant to these mechanisms are interesting questions for future work. A recent study of a *ha100* deletion virus showed that HA100 and its associated interactions are not crucial for ODV assembly. However, the oral infectivity of the *ha100* deletion virus was significantly reduced (93). Further work therefore should focus on the potential significance of the interactions of HA100 in oral infectivity. On the other hand a number of interactions were identified among proteins encoded by baculovirus core genes (151). These include binary interactions of HA9-EC43, E56-38K, E56-PIF3, LEF3-helicase, LEF3-alkaline nuclease (AN), GP41-38K, 38K-PIF3, 38K-PIF2, E66-PIF3, E66-PIF2 and PIF3-PIF2 and

self-associations of IE1, LEF3, GP41, 38K, and PIF3. These interactions are likely to be conserved among baculoviruses and may be essential for ODV assembly and/or infectivity.

ODV contains a distinct structure known as the tegument, which is not present in budded virus (BV) (137). The tegument structure packages ODV nucleocapsids (NC) and is then enveloped by the ODV membrane. After occlusion in the OB the tegument becomes more condensed around the NCs (78). But why does ODV contain the tegument structure? A possible explanation is that the tegument structure drives the NCs into the route of ODV formation. In the early stage of virus replication NCs are mainly recruited for BV formation to infect adjacent cells. As the virus replication continues NCs start to be trapped inside the cell nucleus to form ODVs, which are eventually occluded into OB (18). The switch between BV and ODV production has been a mystery for a long time and still is. Tegumentation might have something to do with the switch. Probably in the late stage of viral replication, after the structural proteins of the tegument (GP41, EC43 and P95 (137)) have been synthesized, these proteins start to interact with NCs to build up the tegument structure which will then proceed to envelopment inside the nucleus leading to the formation of ODV. In contrast when tegument proteins are not available NCs will follow the pathway of BV production. Further work is necessary to verify this hypothesis. Another significance of the tegument structure might be that the tegument proteins have some roles during ODV infection. After ODV fusion with the microvilli of insect midgut epithelium cells, these tegument proteins will be released into the cytoplasm of epithelium cells. These proteins may then trigger certain signal transduction pathways to facilitate subsequent steps of virus infection, e.g. NC transportation along the microvilli. These theories could be verified with knock out viruses, in which genes for tegument components are deleted to analyze their roles in ODV formation and infection.

One major component of the tegument is GP41, which is a core gene conserved among all the sequenced baculovirus genomes. In this thesis GP41 was found to form homo-oligomers (Chapter 2) and, more interestingly, oligomerization seems to be dependent on disulfide formation among GP41 monomers (K Peng et al., unpublished data). It was further found that the cysteines in GP41 are highly conserved among GP41 homologues indicating that the disulfide bond that determines GP41 oligomerization is conserved among baculoviruses. Oligomerization of GP41 is likely to be important for tegument formation. The disulfide bond of GP41 oligomer seems to be relevant to the condition of ODV infection. After the ODV membrane fuses with the host cell membrane the tegument structure is exposed to the reducing conditions inside the cell cytoplasm (137). This reducing

environment will facilitate break down of the disulfide bonds between GP41 oligomers and probably disassembly of tegument structure leading to exposure of the NC and eventually the viral genome. Making mutations of the cysteines in GP41 and analyzing the effects on ODV formation could verify this hypothesis. GP41 was also found to interact with 38K, a highly conserved component of NCs (165). There should be some interactions between NC proteins and tegument proteins for the tegument to wrap the NC structure. Whether the interaction of GP41-38K is one of the linking interactions between NC and tegument would be an interesting question for further work. It should also be interesting to address the question, whether this interaction contributes to the switch from BV to ODV production.

It has become evident that many viruses incorporate host proteins into their viral structure (3, 19). A recent proteomic analysis of *Autographa californica* MNPV (AcMNPV) BV revealed that 11 cellular proteins are associated with the virus structure (153). Importantly 7 out of these 11 cellular proteins (actin, 14-3-3 zeta protein, Casein kinase II, ADP-ribosylation Factor 1, Small GTP-binding, protein Rab5, Small GTP-binding, protein Rab11 and Ubiquitin Annexin B) are also found within other enveloped virus particles (153), indicating viruses may incorporate a certain group of host proteins to facility either assembly or viral infection. At the time the study in Chapter 2 was performed the host proteins present in HearNPV ODVs had not been identified (and still are not) and therefore the interactions between ODV viral proteins and host proteins were not investigated. Although the roles of these virion-incorporated host proteins in virus infection are not well known, it can be envisioned that they would contribute to viral infectivity. This supposition is supported by the finding in Chapter 5 that ODV incorporates a certain host protease to cleave P74 in a specific way during the process of ODV release, which might offer an advantage to ODV infectivity. Further work can be performed to identify which host proteins are associated with ODVs, how these proteins are incorporated into the ODV structure, and their potential roles in ODV assembly and infection.

ODV assembly is a highly complicated process involving a series of protein-DNA and protein-protein interactions. Protein interactions identified in this study laid foundations for subsequent work to gain deeper insight in the ODV assembly mechanism. Further work can be combined or guided by computational analysis. Preliminary computational analysis has already revealed several conserved domains in some of the proteins covered in this study. For example HA66 and CG30 were found to contain highly conserved coiled-coil domains. Coiled-coil domains are known for mediating protein-protein interactions (124) therefore the

self-association of HA66 and CG30 is likely to be mediated by this domain. Further work could include mutations of key amino acids in this domain to investigate the significance of these interactions in ODV assembly and infectivity. Another interesting direction to follow is to use advanced microscopy analysis (live-cell imaging and super-resolution microscopy) to investigate the kinetics of the assembly process. These efforts would certainly improve our understanding of ODV assembly under nearly physiological conditions to an unprecedented level.

The ODV entry complex

Baculovirus ODV entry is likely to be an evolutionarily conserved mechanism, taking place under highly alkaline conditions. The fact that three PIFs were reported to function in ODV binding (57, 107) triggered the hypothesis that these PIFs may form a dynamic complex to facilitate ODV entry. Work in this thesis (Chapter 3 and 4) showed that the PIF complex contains at least 6 proteins: P74, PIF1, PIF2, PIF3, PIF4 and P95. In addition, proteomic analysis indicated that three other viral proteins, AC5, AC68 and AC108, might also be components of the PIF complex (see Fig. 6.1). Although the association of the latter three proteins with the PIF complex needs further verification, it was recently found that AC68 plays an important role in ODV infection (Dr. D. Theilmann, personal communication). This is in support of its association with the PIF complex. The multi-molecule PIF complex may be

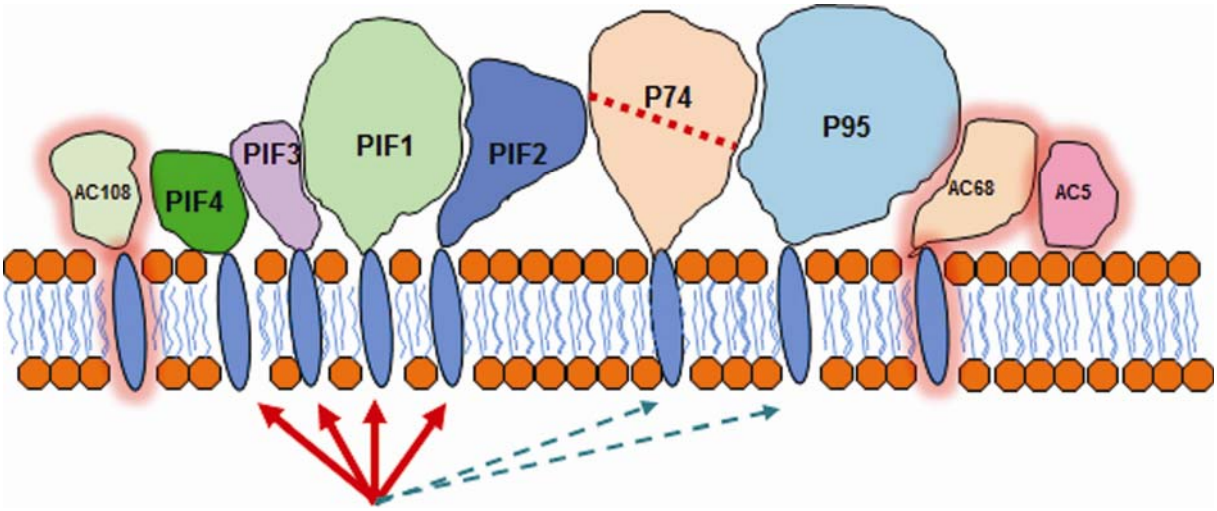

Figure 6.1. Schematic of PIF complex and P74 cleavage. Currently known components or candidates of the PIF complex are shown in the model. Blue ovals represent the simplified version of transmembrane domains of these proteins. Red arrows indicate interactions that are highly stable, while dashed green arrows indicate relatively weak interactions. The red dashed line in P74 represents the cleavage event mediated by the OB endogenous protease during the ODV releasing process. Note that the association of AC5, AC68 and AC108 with the complex needs to be verified with alternative methods.

a reflection of the complexity of the ODV entry mechanism. At the same time, the oral infectivity process is very specific. Swapping *pif1-3* genes between HearNPV and AcMNPV homologs aborts oral infectivity (142). This could be because ODV-receptor interactions do not occur any more or that the PIF complex can no longer be formed in the swapping experiments.

ODVs need to establish infection very efficiently. This is because its target cell, the midgut epithelium cell, is prone to apoptosis and epithelial cells are sloughed away continuously (62). The presence of the PIF complex might be the result of evolutionary selection of an efficient and effective machinery to facilitate ODV infection. Three components of the PIF complex, P74, PIF1 and PIF2, were found to function in ODV binding (57, 107). Why does ODV have three, if not even more, binding proteins? Virus binding, in some cases, undergoes two stages with the first one involving host attachment factors and the second by receptors (97). The initial virus binding to attachment factors serves to concentrate the virus particles on the cell surface. Upon the initial binding, the virus will interact with specific host receptors and this interaction will actively promote entry (one example is the HSV entry (21)). It is possible that some of the binding PIFs interact with host attachment factors to concentrate ODV on microvilli, while other binding PIFs interact with host receptors to mediate efficient entry. Another feature of virus binding is that although the interaction between virus binding protein and receptor is specific, the interactions are often of low affinity (97). However, multiple receptor binding sites on a virus particle can often guarantee nearly irreversible binding (97). Probably ODV employs several proteins in binding to set up binding more efficiently and to guarantee successful infection. This is especially relevant for ODV since infection of its target cell is a race against time. Finally, multisite binding has been found to cluster receptor proteins, which in turn may activate signaling pathways to facilitate subsequent steps during virus entry (31), e.g. translocation of the NCs along the microvilli.

The finding of a highly stable complex composed by PIF1, PIF2, PIF3 and PIF4, which can withstand strong denaturing conditions (Fig. 4.4) is surprising. Why does the virus need such a stable complex? The reason might derive from the circumstance of ODV infection of the insect midgut. The insect midgut contains a wide range of digestive proteases (137). Once exposed into this environment the ODV particles and their surface membrane proteins would be obvious targets for these proteases. If unspecific proteolytic cleavage occurs on these membrane proteins before they mediate ODV entry, they may lose their

functionality and ODV infection will be abortive. Therefore, the virus needs to find a way to protect its membrane proteins from unspecific cleavage. One possible solution is to let the essential proteins form tight complexes with large interacting interfaces. The compact conformation of this complex may shield essential domains of these proteins from the harsh environment. It is even possible that a major role of some components of the PIF complex is to function as shield proteins to protect the essential PIF core proteins from the harsh midgut environment. Obviously these theories should be tested by further studies.

All six components of the PIF complex (P74, PIF1-4 and P95) are encoded by baculovirus core genes. Hence, it is reasonable to assume that within the family *Baculoviridae* PIF complex formation and the entry mechanisms are also highly conserved. These PIFs also have homologs in several other insect viruses (8, 49, 154), but whether those PIF homologs function in similar ways should be investigated. One example is found in bracoviruses (Family *Polydnaviridae*). Bracovirus particles are produced in wasp ovaries and are injected into the body cavity of lepidopteran host together with wasp eggs, allowing successful development of the parasite by inducing host immune suppression and developmental arrest (4). Polydnaviruses can enter cells of host larva and express carried genes, but they do not replicate in the cells as they only release genetic cargo from the host into those cells and contain no viral genome (8). These host genes are involved in blocking the host defense response allowing the parasite egg to develop. The wasp genome carries the polydnavirus genes including those encoding for the virion proteins. One target of polydnaviruses is the haemocyte, which undergo significant changes in response to entry of the virus (4). P74, PIF1-4 and P95 have homologs in these polydnaviruses and the homologous proteins are present in the virus structure (8). However, unlike baculovirus P74 and PIF1-4, which are generally believed to function only in ODV infection of midgut epithelium cells under an alkaline condition, these polydnavirus PIF homologs seem to play a role in infecting haemocytes of larvae. Further work is necessary to address the question whether these polydnavirus PIF homologs mediate virus entry in a similar or a different way.

Despite their essential roles in ODV infection, PIFs are present in ODV with very low abundance (45, 77, 127). A study revealed that for *Spodoptera frugiperda* NPV, a mixture of OBs containing the complete genome and OBs lacking *pif1* and *pif2* genes has higher insecticidal potency than pure OBs with full genomes (22). This suggests that ODVs can set up infection with even less PIFs. Keeping the abundance of entry proteins low is not unique for baculovirus ODV. HIV membrane contains around 10-15 trimers of envelope proteins that

are essential for entry (19). Why do these viruses keep these important proteins at low abundance instead of incorporating more of them to increase binding and entry efficiency? Probably the presence of more of these proteins will actually cause adverse effects during virus maturation e.g. immature fusion with cellular membranes.

Research in this thesis triggered a number of interesting questions for further study. Are the interactions among PIFs triggered by high pH condition or already formed during ODV assembly? What is the stoichiometry of the PIF complex? What are the host receptors of PIFs and do these PIFs undergo conformational changes after binding with their host ligands? Does the PIF complex trigger signal transduction pathways in the host to facilitate virus entry and subsequent replication? To address some of these questions a cell line or a platform that could support ODV binding and fusion or even infection seems to be indispensable. An alternative strategy could be the use of brush border membrane vesicles (BBMV) from the microvilli of insect midgut epithelium cells (134), which have been frequently used in studies of *Bacillus thuringiensis* toxins and are expected to support ODV binding and fusion.

Proteolytic activation

Proteolytic processing or activation of viral membrane proteins is commonly employed by enveloped viruses to confer functional conformation to the proteins and/or to expose functional domains (5, 12, 19, 126). It has been reported that P74 is cleaved by a host midgut trypsin after the ODVs are released into the midgut environment and that cleavage is important for ODV infectivity (139). In this thesis another proteolytic cleavage event was identified, which is only associated with OBs derived from larvae. The results presented here (Chapter 5) suggest that during the procedure of ODV assembly and embedding into OBs a so far undefined host alkaline protease is incorporated into the ODV structure and that the protease cleaves P74 into two fragments during OB disassembly at high pH.

This cleavage event has several distinct features. First, it is performed by a host protease, which is an integral component of OB structure. It is common for a virus to employ host proteases to modify/activate the viral proteins during infection, but incorporating a host protease into the virus structure to perform cleavage is unusual. Second, the virus seems to be able to specifically incorporate a certain kind of protease into its structure as in OBs produced from different hosts P74 is cleaved in the same way. Third, cleavage happens during the process of OB disassembly probably before ODVs are fully exposed into the midgut

environment. This is different from a number of current models of proteolytic cleavage, which happens when virus is bound to a cell surface, during virus fusion or post-translationally after viral proteins have been synthesized (5, 12, 141).

How the protease is incorporated into the OB structure is unknown. Since ODVs are assembled in the nucleus, the question is how this protease ends up in the nucleus. It is possible that certain virus protein(s) are able to recognize and interact with the alkaline protease and translocate it to the sub-cellular location where ODVs are assembled and embedded into OBs (137). It is even possible that the substrate of the protease itself, e.g. P74, interacts with the protease and incorporates it into the virus structure. Apparently, during OB formation and before the OB is exposed to alkaline conditions, the protease remains inactive as long as the condition is not alkaline. When OBs are exposed to the alkaline environment in the host midgut and start to disassemble, the high pH condition activates the protease, which then cleaves P74 efficiently. To study the mechanism of incorporation of the protease into OBs it is necessary to determine which protease it is. This may be achieved by a mass spectrometry analysis of the components of OB derived from host larvae.

P74 cleavage happens highly efficiently as reflected by the fact that P74 was cleaved within 3 min after OBs were exposed to the alkaline condition. It was reported before that ODVs are released from OBs within 12 min post entry into the insect midgut (1, 137). After being fully released ODVs need to penetrate the peritrophic membrane to reach midgut epithelium cells. Therefore, it is possible that it will take ODV more than 12 min to contact its target cells (this speculation is also supported by our EM analysis of ODV entry, which is not shown in this thesis). In this case it is reasonable to speculate that P74 is cleaved before contacting its host receptor. If this is the case, the cleaved P74 should represent a functional form for ODV binding. In a previous study (57), in which P74 was found to function in ODV binding, ODVs were purified from larvae derived OBs (L-OBs) under conditions in which P74 is likely to be cleaved. This would suggest that it is the cleaved form of P74 instead of full-length P74 that functions in binding. To test this possibility ODVs were purified in the same way as described in the previous study (57) and we found that P74 was present only in the cleaved form (data not shown). This suggests that the previous functional analysis was based on the cleaved P74 and that it is the cleaved form of P74 that is functioning in ODV binding.

What would be the significance of this endogenous cleavage event? It was found before that L-OB cause mortality significantly faster than OBs derived from insect cell lines

(C-OB) (11). The presence of the endogenous cleavage in the L-OBs, but not in the C-OBs, might contribute to the higher killing speed of L-OBs. The specific cleavage of P74 may convert this ODV binding protein into a functional conformation and/or expose certain functional domains. Since P74 cleavage is likely to happen before receptor binding, cleavage might ensure that cleaved P74 binds efficiently with its cellular ligands. If this is true the question arises why C-ODV is still infectious? In this case P74 might be cleaved by a host protease only after C-ODVs are fully released into the midgut. This cleavage, however, would happen later than the cleavage event in L-ODV and this time difference might contribute to the lower killing speed of C-ODV.

Of course this hypothesis needs to be supported by experimental evidence. An obvious experiment to verify this is to identify the cleavage site, make a relevant mutant virus and compare the infectivity of the mutant virus with the wild type virus. We aimed to identify the cleavage site in P74 with N terminal protein sequencing, however, due to the very low abundance of P74 in ODV, not enough material could be isolated. Sequence alignment analysis showed that the region, where P74 is likely to be cleaved, is not well conserved among the P74 homologs. This made it difficult to predict candidate sites for mutational analysis. It seems inevitable that a series of mutations need to be constructed in future work to identify the cleavage site and to investigate its potential significance during virus infection. Another interesting question is whether the P74 cleavage event is conserved among baculoviruses. Despite the low level of conservation of the region that undergoes cleavage among P74 homologs, it was found that HearNPV P74 undergoes a similar cleavage event (Dr Hu Zhihong, personal communication). HearNPV is a group II baculovirus while AcMNPV belongs to Group I. Therefore it is possible that this P74 cleavage event is conserved among both Group I and Group II alphabaculoviruses. Future work can reveal whether the P74 cleavage mechanism is conserved in all baculoviruses.

Concluding remarks

The baculovirus ODV assembly and entry mechanisms are highly complex and involve a series of events in spatial and temporal order. Knowledge gained in this thesis is not enough to understand the whole process but offers fundamental insights to the principles of ODV entry and assembly. The concepts developed and technological approaches used here invite for further investigation of these mechanisms.

Based on the knowledge gained in this thesis a preliminary model of ODV entry is proposed. When the virus infects a host larva, during the process of OB formation, a host alkaline protease is incorporated into the OB structure. Upon oral uptake and after OB is transported into the larval midgut, OB disassembles under the midgut alkaline condition and the alkaline protease is activated. The activated protease cleaves P74 into two fragments, which remain associated with the PIF complex. ODV is then fully released from the OB matrix into the midgut environment and a host midgut trypsin further cleaves the N terminal fragment of P74. These sequential cleavage events confer P74 a functional conformation and/or help P74 to expose certain functional domains such as receptor binding domain. The conformational change of P74 might trigger a series of conformational changes to the other components of the PIF complex leading to activation of their functions.

The current hypothesis is that the three PIFs with binding properties, P74, PIF1 and PIF2, mediate ODV binding in a receptor and co-receptor manner, triggering certain signal pathways within the host cells and expose fusion domain(s) between the ODV envelope and the membrane of microvilli of midgut epithelium cells. A direct membrane fusion takes place and nucleocapsids travel along the microvilli into the cytoplasm to proceed with the first round of virus infection. To what extent the PIFs are involved in further downstream processes, such as transport, remains to be investigated.

Much more work is necessary to verify and extend the proposed model and to fill in the gaps of understanding of this mechanism. Subsequent work devoted to identification of relevant host receptors, viral fusion proteins, determination of PIF stoichiometry in the complex and signal pathways activated by these PIFs will prove to be challenging but undoubtedly enable us to appreciate the highly conserved and complicated ODV assembly and entry mechanisms.

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List of Abbreviations

Virus

AcMNPV	*Autographa californica* MNPV
BmNPV	*Bombyx mori* NPV
CfMNPV	*Choristoneura fumiferana* MNPV
CuniNPV	*Culex nigripalpus* nucleopolyhedrovirus
HearNPV	*Helicoverpa armigera* nucleopolyhedrovirus
HSV	Herpes simplex virus
SARS-CoV	SARS-Corona virus
SeMNPV	*Spodoptera exigua* MNPV
SGHV	Salivary gland hypertrophy viruses
SpltMNPV	*Spodoptera litura* MNPV
WSSV	White spot syndrome virus

General

Ab	Antibody
ABC	Ammonium bicarbonate
AN	Alkaline nuclease
ARIF-1	Actin rearrangement-inducing factor
Arp2/3	Actin-related protein 2/3
BBMV	Brush border membrane vesicles
BN-PAGE	Blue native-PAGE analysis
BSA	Bovine serum albumin
BV	Budded virus
C-OB	OB derived from cell culture
C-ODV	ODV derived from C-OB
CoIP	Co-immunoprecipitation
DBP	DNA binding protein
DNA pol	DNA polymerase
ER	Endoplasmic reticulum
F	Group II NPV envelope fusion protein
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
GV	Granulovirus
HA	Influenza virus hemagglutinin
HAD	Haloacid dehalogenase
HAT	Human airway trypsin-like protease
HCF1	Host cell-specific factor 1
HS	Heparan sulphate
HVEM	Herpes virus entry mediator
IE1	Immediately early factor 1, transcriptional factor
INM	Inner nuclear membrane
IPTG	Isopropyl-ß-D-thiogalactopyranoside
LC-MS	liquid chromatography mass spectrometry
L-OB	OB derived from larva
L-ODV	ODV derived from L-OB
MAb	Monoclonal antibody
MNPV	Multiple Nucleopolyhedrovirus
MOI	Multiplicity of infection
MS	Mass spectrometry
NC	Nucleocapsid
NPV	Nucleopolyhedrovirus

nt	Nucleotide
OB	Occlusion body
ODV	Occlusion derived virus
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PIF	*per os* infectivity factor
PK1	Protein kinase-1
PKIP	Protein kinase-interacting protein
PM	Peritrophic membrane
PML	Promyelocytic leukemia protein
POLH	Polyhedrin
PPI	Protein-protein interaction
SM	Sorting motif
SNPV	Single Nucleopolyhedrovirus
TCID50	Tissue culture infective dose of 50%
TFA	Trifluoroacetic acid
TM	Transmembrane domain
Y2H	Yeast-two hybrid
WASP	Wiskott-Aldrich syndrome protein

Summary

Entry into host cells is the first step of virus replication. In this thesis the baculovirus occlusion-derived virus (ODV) was chosen as a model to study virus entry in invertebrates for two main reasons. First, ODV entry is mediated by a group of proteins that are not only conserved in all members of the family *Baculoviridae* but also in other large invertebrate nuclear-replicating DNA viruses, which suggests that the entry mechanism has arisen early in evolution. Second, ODV entry is believed to happen under the alkaline conditions of insect midguts, while the current entry models are documented for viruses entering under either neutral or acidic pH conditions. Taken together ODV entry into invertebrate midgut epithelial cells may represent an evolutionary conserved and uncommon entry mechanism. The ODV is an enveloped virus particle that contains more than 10 different membrane proteins. A subset of these membrane proteins plays essential roles in establishing oral infectivity and is named as *per os* infectivity factors (PIFs). These PIFs mediate ODV binding and probably also direct membrane fusion with the larval midgut epithelial cells. Among the PIFs that are essential for oral infection three have been shown to function in ODV binding and it was speculated that these PIFs work in concert during ODV entry.

The ODV structure can be divided into three layers: envelope, tegument and nucleocapsids. After binding and fusion of the ODV with the host cell membrane the tegument and the nucleocapsid are released into the cytoplasm, where the viral genome is uncoated. Understanding the ODV structure is therefore important to understand both ODV entry and post-fusion events. The ODV structure as a whole is an assembly of a large number of different structural proteins that protect the viral genome. In **Chapter 2** the ODV assembly mechanism was investigated from the perspective of protein-protein interactions. *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV), a group II alphabaculovirus in the family *Baculoviridae* was the model virus in this study. Thirty-nine selected genes of ODV structural proteins were cloned and expressed in the yeast two-hybrid (Y2H) system to study their interactions. This Y2H screen identified twenty-two interactions, of which thirteen were binary interactions [HA9-EC43, E56-38K, E56-PIF3, LEF3-helicase, LEF3-alkaline nuclease (AN), GP41-38K, GP41-HA90, 38K-PIF3, 38K-PIF2, VP80-HA100, E66-PIF3, E66-PIF2 and PIF3-PIF2] and nine were self-associations (IE1, HA44, LEF3, HA66, GP41, CG30, 38K, PIF3 and P24). The binary interaction of LEF3-helicase, LEF3-AN and the self-associations of IE1, LEF3 and 38K, were also identified in *Autographa californica* (Ac) MNPV, the type

member of group I alphabaculoviruses. As HA44 and HA100 were two newly identified ODV proteins only present in group II viruses, their interactions were verified with pull-down and co-immunoprecipitation (CoIP) assays, respectively. A number of interactions were identified among highly conserved proteins such as HA9, EC43, E56, 38K, PIF3, LEF3, helicase, GP41, PIF2, E66, and IE1. The protein-protein interactions between conserved proteins are likely to be preserved across baculoviruses and may form structural subunits for ODV assembly. On the other hand the interactions identified for group II NPV-specific ODV proteins, such as HA44 and HA100, may represent special assembly and downstream mechanisms specific to this group of viruses. The identified interactions lay the foundations for future work to define the ODV assembly mechanism and structure in more detail.

Chapter 2 aimed at a complete screen of interactions among ODV proteins. However, due to the limitations of the Y2H system, interactions among ODV membrane proteins could not be investigated with the same fidelity. Interactions among these membrane proteins are however very important, not only to understand ODV envelopment, but also for elucidating ODV entry mechanisms. The focus of **Chapter 3** was to investigate the potential interactions among PIF1, PIF2, PIF3 and P74 (PIF0), the 4 by then known PIFs, using AcMNPV as model virus. By using protein crosslinking and differential-denaturing SDS-PAGE, it was found that PIF1, PIF2, and PIF3 form a highly stable complex on the surface of ODV particles. Each of these three PIFs is essential for the formation of this complex as deletion of any of the three genes impaired complex formation completely. CoIP analysis independently confirmed the interactions between the three PIF proteins and revealed in addition that P74 is also associated with this complex. However, deletion of the *p74* gene did not affect formation of the PIF1-PIF2-PIF3 complex. Therefore, it is possible that PIF1, PIF2 and PIF3 form a stable core complex with which P74 (PIF0) interacts. It was further shown by immunogold electron microscopy that the complex has a possible uniform distribution over the surface of the ODV. Considering the high level of conservation of these 4 PIFs we proposed that PIF1, PIF2, PIF3, and P74 form an evolutionarily conserved complex on the ODV surface, which plays essential roles in the initial stages of baculovirus oral infection.

More recent studies by other researchers identified two more proteins that are essential for oral infection (PIF4 and PIF5). The very recent discovery of PIF6 indicates that there might be even more PIFs, which may be discovered in the future. The increasing number of PIFs suggests that the ODV entry mechanism is more complicated than previously expected. Without knowing the components of the complete PIF complex it is not possible to

understand the ODV entry mechanism. The aim of **Chapter 4** was to analyze the components of the PIF complex under a more native condition. CoIP coupled with mass spectrometry analysis showed that, besides PIF1-3 and P74, PIF4, P95, AC5, AC68 and AC108 are also components of the PIF complex. In contrast PIF5 is not associated with the complex. The presence of PIF4 and P95 in the complex was further verified with Blue-native PAGE (BN-PAGE) and a second CoIP analysis. BN-PAGE analysis also showed that deletion of the *pif4* gene impaired complex formation while deletion of *pif5* did not affect PIF complex formation. This is consistent with the finding that PIF4 but not PIF5 is part of the complex. Furthermore, PIF4 was found to form a stable complex with PIF1, PIF2 and PIF3, while in the absence of PIF4 the three PIFs remain in a stable sub-complex.

It seems that the currently identified PIF complex has three structural orders, ***i)*** PIF1, PIF2 and PIF3 forming a stable sub-core, ***ii)*** PIF4 strongly interacting with the sub-core to form a stable core, and ***iii)*** P74 and P95 more loosely interacting with this stable core. The putative association with the complex of the other three proteins, AC5, AC68 and AC108, as revealed by mass spectrometry analysis needs further verification. Recently, reports have shown that AC68 (PIF6) and AC108 play important roles in oral infectivity. Our results indicate that these proteins may act in the entry process through interacting with other PIFs.

Besides protein-protein interactions between proteins of viral entry complexes, proteolytic cleavage/activation is also an often observed important requirement for virus entry. Proteolytic cleavage may trigger virus membrane proteins, especially binding and fusion proteins, to adopt functional conformations that facilitate virus infection. In **Chapter 5** it was shown that P74 is cleaved via a novel proteolytic mechanism. P74 is a highly conserved ODV protein known to be involved in binding to midgut cells. The P74 protein was found to be efficiently cleaved into two fragments by an alkaline protease present in the viral occlusion body (OB). The proteinaceous matrix structure of OB surrounds the ODVs in the environment. The protease becomes active upon ODV release from the OBs under the alkaline conditions of the larval midgut. The cleavage is specific for P74 since the other known PIFs in the complex were not cleaved under the same conditions. Only when AcMNPV OBs are derived from larvae (L-OB in contrast to C-OB which is derived from cultured cells) the virus contains this protease suggesting that the protease is derived from the host. The cleavage happens very efficiently as P74 is completely cleaved after OBs are exposed to alkaline conditions for only three minutes. This would suggest that under natural situations P74 is already cleaved before contacting its host receptor. Incorporating the host protease into OBs

seems to be a selective and conserved mechanism as P74 in OBs derived from two different host larvae was cleaved into fragments with the same apparent molecular masses. It was further revealed that after the cleavage the two P74 subunit fragments remain associated with the PIF complex. It was shown before that after ODVs are released into the larval midgut P74 is cleaved by a midgut trypsin and that this cleavage event is important for ODV infection. Since in the natural infection events larvae are infected by L-OBs (containing endogenous protease activity) we propose that during *in vivo* ODV infection P74 undergoes two sequential cleavage events, with the first one being performed by the OB endogenous host alkaline protease and the second one by a host trypsin present in the midgut. The first cleavage event, which is associated with L-OB, might contribute to the faster mortality rate of L-OBs compared to cell derived OBs.

In **Chapter 6** the contribution of knowledge gained in this thesis to the understanding of the ODV assembly and entry mechanisms is discussed. A model of the ODV entry complex and the potential sequential events during ODV entry are proposed. The current hypothesis is that the sequential cleavage events of P74 trigger conformational changes to the ODV entry complex, which activate a program of serial events to mediate ODV entry into its invertebrate host. Several new questions are raised together with suggestions for future work to understand this unique virus entry mechanism of large invertebrate DNA viruses.

Samenvatting

Het binnendringen van gastheercellen is de eerste stap van de virale infectie. In dit proefschrift zijn baculovirussen (Familie *Baculoviridae*) gekozen als model om te bestuderen hoe virussen darmcellen van ongewervelde dieren binnendringen. Baculovirussen infecteren insecten en dringen hun gastheer binnen via de middendarm bij opname van voedsel waarin zich polyeders bevinden. Dit zijn grote kristallen, gemaakt van een viraal eiwit, waarin de staafvormige virusdeeltjes ("occlusion derived virus", ODV's) zijn ingesloten. De polyeders vallen uiteen in het alkalische milieu in het lumen van de middendarm en de vrijgekomen ODV's migreren naar en binden onder deze omstandigheden aan de microvilli van darmepitheelcellen. Dit is in tegenstelling tot wat er bekend is van het binnentreden van membraanvirussen in het maagdarmkanaal van gewervelde dieren, dat onder neutrale of zelfs zure omstandigheden plaatsvindt. Na binding fuseren de membranen van virusdeeltjes en microvilli, waardoor de nucleocapsiden in de epitheelcellen terechtkomen en infectie kunnen bewerkstelligen. Bij deze orale infectie is een aantal virale eiwitten betrokken, de perorale infectiefactoren (PIF), die essentieel zijn voor succesvolle infectie door ODV's. Deze PIF-eiwitten komen voor bij alle baculovirussen, maar daarnaast ook bij andere virussen die ongewervelden infecteren, een groot circulair DNA-genoom hebben en zich in de celkern vermenigvuldigen. Dit doet vermoeden dat het mechanisme van binnendringing van deze virussen evolutionair geconserveerd is. De PIF-eiwitten bevinden zich in of zijn geassocieerd met de ODV-membraan en zijn onder meer betrokken bij de binding van ODV's aan de celmembraan en de daaropvolgende fusie tussen virus- en celmembraan. Van drie PIF-eiwitten (PIF1, PIF2 en P74/PIF0) is daadwerkelijk aangetoond dat ze een functie hebben bij binding van ODV's aan microvilli. Wat het mechanisme van de orale infectie betreft wordt verondersteld dat de verschillende PIF-eiwitten samenwerken om binding en fusie tot stand te brengen en mogelijk ook translocatie van het virus de cel in.

ODV's zijn complexe deeltjes opgebouwd uit één of meer nucleocapsiden, een tegument (tussenzone) en een membraan of envelop. In de envelop zijn tenminste tien verschillende eiwitten aanwezig, waaronder de PIF's. Na binding en fusie van ODV's met de celmembraan komen de nucleocapsiden met tegument in het cytoplasma terecht, waar het genoom wordt vrijgemaakt voor verdere infectie van de cel en vermeerdering van het virus. Kennis van de samenstelling van ODV's en van de onderlinge interacties van de componenten is essentieel voor het begrijpen van de processen, die nodig zijn voor het

binnendringen in de cel, d.w.z. binding, fusie en translocatie. Een analyse van de ODV-componenten van het baculovirus *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV), een groep II alphabaculovirus, is beschreven in **Hoofdstuk 2.** Negenendertig genen die coderen voor ODV-eiwitten werden gekloneerd en tot expressie gebracht in het "yeast-two-hybrid-systeem" (Y2H) om hun onderlinge interacties te bestuderen. Hierbij werden in totaal tweeëntwintig interacties vastgesteld, waarvan twaalf binaire interacties [HA9-EC43, E56-38K, E56-PIF3, LEF3-helicase, LEF3-alkaline nuclease (AN), GP41-38K, GP41-HA90, 38K-PIF3, 38K-PIF2, VP80-HA100, E66-PIF3, E66-PIF2 en PIF3-PIF2] en negen zelf-associaties (IE1, HA44, LEF3, HA66, GP41, CG30, 38K, PIF3 en P24). De binaire interacties van LEF3-helicase, LEF3-AN en de zelf-associaties van IE1, LEF3 en 38K zijn ook gevonden voor *Autographa californica* MNPV (AcMNPV), een groep I alphabaculovirus. Twee nieuwe eiwitten, HA44 en HA100, werden alleen gevonden in groep II alphabaculovirussen en hun zelf-associatie dan wel interacties met andere eiwitten werd bevestigd d.m.v. "pull-down" en co-immunoprecipitatie (CoIP) experimenten. Een aantal van de gevonden interacties werd aangetoond tussen eiwitten die in hoge mate geconserveerd zijn bij baculovirussen, zoals HA9, EC43, E56, 38K, PIF3, LEF3, helicase, GP41, PIF2, E66 en IE1. Het is dan ook waarschijnlijk dat de onderlinge interacties tussen deze eiwitten geconserveerd zijn in de baculovirusfamilie. Mogelijk vormen deze geconserveerde eiwitten structurele sub-eenheden, die nodig zijn voor de assemblage van ODV's. Verder kan het bestaan van interacties tussen ODV-eiwitten die specifiek zijn voor groep II (HA44 en HA100) erop duiden dat het mechanisme van ODV-assemblage en de daaropvolgende processen wat kunnen verschillen tussen groep I en groep II baculovirussen. De in dit hoofdstuk beschreven interacties tussen ODV-eiwitten leggen een goede basis voor verdere en meer gedetailleerde analyse van de ODV-assemblage.

Het doel van het in hoofdstuk 2 beschreven onderzoek was om een compleet beeld te krijgen van de interacties tussen ODV-eiwitten. Het gebruikte Y2H-systeem is prima geschikt voor oplosbare eiwitten, maar heeft een beperkte betrouwbaarheid voor membraaneiwitten zoals de PIF's. Membraaneiwitten en hun onderlinge interacties spelen waarschijnlijk een belangrijke rol bij de entree van ODV's in de cel. Daarom is in **Hoofdstuk 3** in eerste instantie gekeken naar de onderlinge interacties tussen PIF1, PIF2, PIF3 en P74, de tot dan toe bekende PIF-eiwitten van AcMNPV. Door ODV-membraaneiwitten chemisch te koppelen en door verschillende eiwit-denaturerende condities toe te passen voorafgaand aan SDS-PAGE werd ontdekt dat PIF1, PIF2 en PIF3 in de ODV-membraan voorkomen als een zeer stabiel

eiwitcomplex. Na afzonderlijke deletie van het gen voor elk van deze PIF's werd het complex niet meer gevonden, wat aangeeft dat elk van de genoemde PIF's essentieel is voor de vorming van het complex. Verdere analyse met CoIP bevestigde de interacties tussen PIF1, PIF2 en PIF3, maar liet tevens zien dat ook P74 (PIF0) geassocieerd is met het complex. Echter, deletie van het *p74*-gen had geen invloed op de vorming van het PIF1-3-complex. Blijkbaar vormen deze drie PIF's een stabiel "kerncomplex", waar P74 minder hecht mee is geassocieerd. Aangezien de vier genoemde PIFs geconserveerd zijn in baculovirussen en andere invertebraten-infecterende DNA-virussen, is het aannemelijk dat PIF1, PIF2, PIF3 en P74 een evolutionair geconserveerd complex vormen aan het ODV-oppervlak en dat dit complex een essentiële rol speelt in de beginfase van de orale infectie.

Tijdens dit onderzoek werden door andere onderzoekers nieuwe PIF's (PIF4 en PIF5) geïdentificeerd, eiwitten die eveneens essentieel bleken te zijn voor orale infectie. Het toenemende aantal PIF's dat wordt gevonden in de ODV-membraan, doet vermoeden dat het mechanisme van binnendringing in de gastheer een zeer complex proces is. Om dit proces verder te kunnen begrijpen is het van belang alle eiwitcomponenten van de ODV-membraan en hun onderlinge samenhang te kennen en daarom is in **Hoofdstuk 4** een analyse uitgevoerd van de componenten van het PIF-complex onder niet-denaturerende omstandigheden. Het complex werd geïsoleerd met behulp van CoIP en de samenstelling van het complex werd geanalyseerd met behulp van massaspectrometrie. Naast PIF1-3 en P74 bleken ook de eiwitten PIF4, P95, AC5, AC68 en AC108, maar niet PIF5, aanwezig te zijn in het PIF-complex. De associatie van PIF4 en P95 met het complex werd in onafhankelijk experimenten bevestigd door gebruik te maken van Blue-native PAGE (BN-PAGE) en CoIP technologie. Na deletie van het *pif4*-gen werd het complex niet meer gevormd, terwijl zoals verwacht deletie van *pif5* geen effect had op de complexvorming. De resultaten lieten zien dat PIF4, en niet PIF5, samen met PIF1, 2 en 3 een stabiel macromoleculair complex vormt en dat bij afwezigheid van PIF4 de drie overgebleven PIF's (1, 2 en 3) een kleiner, stabiel sub-complex vormen.

Op basis van deze bevindingen kunnen met betrekking tot het PIF-complex tenminste drie structurele organisatieniveaus worden onderscheiden: ***i)*** een stabiel kerncomplex gevormd door PIF1, PIF2 en PIF3; ***ii)*** een stabiel complex gevormd door het kerncomplex samen met PIF4 en ***iii)*** een groter complex bestaande uit dit stabiele complex met daaraan in een losser verband geassocieerd P74 en P95. Onlangs is gerapporteerd dat ook AC68 (nu PIF6 genoemd) en AC108 een rol spelen bij de orale infectie. De uitkomst van de

massaspectrometrische analyse van het complex doet vermoeden dat beide eiwitten, alsmede AC5, mogelijk onderdeel uitmaken van het PIF-complex. Het is van groot belang voor het begrijpen van het infectiemechanisme dat de exacte samenstelling van het complex en de onderlinge interacties tussen de componenten verder wordt onderzocht.

Voor andere virussen, waarbij een macromoleculair complex verantwoordelijk is voor binding en fusie, is vastgesteld dat proteolytische splitsing van één of meer componenten in het complex noodzakelijk is voor het binnendringen van het virus in de cel. Proteolyse kan ervoor zorgen dat de ruimtelijke structuur van eiwitten zodanig verandert, dat deze hun functie in binding en/of fusie kunnen uitvoeren. In **Hoofdstuk 5** is beschreven hoe het PIF-eiwit P74 proteolytisch wordt gesplitst via een voorheen onbekend mechanisme. Zoals eerder vermeld is P74 een zeer geconserveerd ODV-eiwit, betrokken bij binding van ODV's aan de cellen van de middendarm. Uit experimenten blijkt dat het P74-eiwit in twee fragmenten wordt gesplitst door een alkalisch protease, dat aanwezig is in de polyeders waarin de ODV's zijn ingebed. Dit protease wordt geactiveerd in het alkalische milieu van de middendarm tijdens het uiteenvallen van de polyeders en het vrijkomen van de ODV's. Dit protease is specifiek voor P74; de andere PIF-eiwitten worden onder deze omstandigheden niet gesplitst. De splitsing vindt alleen plaats indien de polyeders zijn geïsoleerd uit larven en niet wanneer ze uit geïnfecteerde celkweken komen. Dit suggereert dat het alkalische protease, dat verantwoordelijk is voor de splitsing, afkomstig is van de gastheer. Vergelijkbare splitsing van P74 is vastgesteld met polyeders afkomstig van twee verschillende gastheersoorten, wat duidt op een geconserveerd mechanisme van eiwitsplitsing. Binnen drie minuten na blootstelling van de polyeders aan alkalische omstandigheden is alle P74 gesplitst. De splitsing blijkt dus zeer efficiënt te zijn en dit leidt tot de veronderstelling dat tijdens de infectie alle P74-moleculen gesplitst zijn vóórdat het ODV in contact komt met de vermeende receptor op de darmcellen van de gastheer. Het onderzoek liet verder zien dat de twee P74-fragmenten na splitsing geassocieerd blijven met het PIF-complex. Uit eerder onderzoek is gebleken dat het P74, na vrijkomen uit de polyeders, gesplitst wordt door een trypsine, dat in de middendarm van de gastheer aanwezig is, én dat deze splitsing ook belangrijk is voor een succesvol verloop van de infectie. Blijkbaar ondergaat het P74 twee opeenvolgende proteolytische modificaties, een eerste splitsing door een alkalisch protease dat in de polyeders is opgesloten en geassocieerd is met ODV's en een tweede splitsing door een trypsine in de darm van de gastheer. Dat de eerste splitsing alleen plaatsvindt bij polyeders die

uit larven zijn geïsoleerd zou kunnen verklaren waarom polyeders uit geïnfecteerde larven een hogere mortaliteit veroorzaken in insecten dan polyeders die zijn geïsoleerd uit celkweken.

Het in dit proefschrift beschreven onderzoek heeft aangetoond dat een macromoleculair complex van tenminste zes PIF-eiwitten, die elk essentieel zijn voor orale infectie, aanwezig is in de envelop van de ODV-deeltjes en dat deze PIF's op verschillende wijze met het complex zijn geassocieerd. De waarneming dat één van die eiwitten, P74, in twee stappen wordt gesplitst door enzymen van de gastheer, geeft aanleiding tot de hypothese dat proteolytische modificaties van componenten in het PIF-complex kan leiden tot veranderingen in de vouwing van de eiwitten in het complex, waardoor functionele domeinen ten behoeve van binding en/of fusie worden geactiveerd. In **Hoofdstuk 6** worden de resultaten van het onderzoek integraal bediscussieerd en wordt een model geschetst van de opeenvolgende processen die ODV's doorlopen om zich toegang te verschaffen tot de epitheelcellen in de middendarm van hun evertebrate gastheer om zo een infectie op gang te brengen. Ten slotte worden suggesties gedaan voor verder onderzoek naar de unieke wijze, waarop de baculovirussen, als model voor grote DNA-virussen ongewervelde dieren *per os* infecteren.

摘要

病毒复制的第一步是对宿主细胞的入侵。本论文中我们对杆状病毒的包涵体病毒粒子（occlusion-derived virus, ODV）入侵无脊椎动物的过程进行了研究。选择杆状病毒做为研究对象主要有两个原因。第一个原因是 ODV 的入侵是由一系列高度保守的蛋白介导的。这些蛋白不仅在已测序的所有杆状病毒基因组中高度保守，还在一些其它的大型无脊椎动物核内复制的 DNA 病毒中保守，提示由这些蛋白介导的入侵机理出现于病毒进化的早期。第二个原因是通常的观点认为 ODV 的入侵发生在昆虫中肠的碱性环境下，而目前已知的病毒入侵模式均发生在中性或酸性条件下。综合这两点 ODV 的入侵或许是一种出现于进化早期并具有一定特殊性的机理。对于这一机理的研究将扩展人们对病毒入侵的理解。ODV 是一种囊膜病毒，其囊膜结构中含有 10 多种囊膜蛋白。这些囊膜蛋白中的一部分对于 ODV 的口服感染具有重要作用并因此被命名为口服感染因子（*per os* infectivity factors, PIFs）。这些 PIF 介导了 ODV 与宿主中肠上皮细胞的结合和膜融合。这些 PIF 中的三个被证明在 ODV 的结合中起作用并被推测它们可能形成一种复合体在 ODV 入侵的过程中协同作用。

ODV的结构可以被分为三个层次：囊膜，介层和核衣壳。在ODV与宿主细胞膜发生结合和膜融合后介层和核衣壳被暴露到细胞质中，随后病毒的基因组得到释放。研究ODV的结构对于理解ODV的入侵及融合后的事件均具有重要的意义。ODV是由一系列的结构蛋白组装形成的，这些结构蛋白包裹着病毒的基因组。本论文的**第二章**从蛋白相互作用的角度对ODV组装的机理进行了研究。杆状病毒科中的棉铃虫核型多角体病毒（*Helicoverpa armigera* nucleopolyhedrovirus，HearNPV）是本章中的研究对象。我们将该病毒基因组中的39个结构基因克隆进酵母双杂交（yeast two hybrid, Y2H）系统进行蛋白相互作用的筛选。筛选一共发现了22对蛋白相互作用，其中包括13对异源相互作用（HA9-EC43, E56-38K, E56-PIF3, LEF3-helicase, LEF3-alkaline nuclease (AN), GP41-38K, GP41-HA90, 38K-PIF3, 38K-PIF2, VP80-HA100, E66-PIF3, E66-PIF2 and PIF3-PIF2）和9对自聚集（IE1, HA44, LEF3, HA66, GP41, CG30, 38K, PIF3 and P24）。其中5对相互作用（LEF3-helicase, LEF3-AN, IE1-IE1, LEF3-LEF3 and 38K-38K）已在杆状病毒的模式株苜蓿银纹夜蛾核型多角体病毒（*Autographa californica* multiplenucleopolyhedrovirus AcMNPV）中得到报道。由于HA44和HA100这两个蛋白是组二病毒中新发现的结构蛋白并仅存于该组病毒中我们对这两个蛋白的相互作用分别用pull-down和免疫共沉淀（co-immunoprecipitation, CoIP）的方法进行了验证。本章所发现的一系列蛋白相互作用中的一部分涉及到高度

保守的蛋白，包括HA9, EC43, E56, 38K, PIF3, LEF3, helicase, GP41, PIF2, E66和 IE1。这些蛋白之间的相互作用可能也存在于在其它的一些杆状病毒中并形成ODV组装过程中的一些结构单元。另一方面涉及到组二NPV特异的ODV结构蛋白的相互作用可能只在这组病毒中存在并代表了一些特殊的组装机理。这些新发现的蛋白相互作用为今后深入研究ODV组装的机理奠定了重要的基础。

论文的第二章致力于对ODV结构蛋白间的相互作用进行全面的筛选。然而由于所选的Y2H系统的限制ODV囊膜蛋白之间的相互作用并未得到有效的检测。这些膜蛋白之间的相互作用对于研究ODV囊膜包装和ODV入侵的机理均具有重要的意义。在本论文的**第三章**中我们研究了AcMNPV中4个已知的PIF（PIF1，PIF2，PIF3和P74（PIF0））蛋白之间的相互作用。蛋白交联和变性蛋白胶的实验表明PIF1，PIF2和PIF3在ODV粒子上形成一个非常稳定的复合体。通过对缺失病毒的检测发现缺失这三个蛋白中的任意一成分都将破坏复合体的形成。免疫共沉淀的实验不仅验证了这三个蛋白之间的相互作用同时揭示P74与这三个蛋白形成的复合体结合在一起。免疫金标的实验进一步表明该蛋白复合体在ODV的表面呈均匀的分部。考虑到这四个蛋白的高度保守性我们推测这个蛋白复合体的形成是高度保守的并在杆状病毒口服感染的过程中起重要的作用。

近期的实验发现了另外两个蛋白同样对口服感染具有重要的作用，它们被命名为PIF4和PIF5。最新的文献进一步报道了PIF6，这一发现提示可能还有更多的PIF有待发现。新的PIF蛋白的陆续鉴定提示ODV入侵的机理可能比原先预测地更为复杂。要进一步理解ODV入侵的机理必须鉴定出完整的PIF复合体的成分。论文**第四章**的目的是用更能反应生理条件的方法对PIF复合体的成分进行鉴定。免疫共沉淀和蛋白质谱的分析表明除了先前发现的4个蛋白PIF4, P95, AC5, AC68和AC108也是复合体的成分。相反PIF5不是复合体的成分。PIF4和P95与蛋白复合体的相互作用得到了蓝色非变性胶和免疫共沉淀的验证。其中蓝色非变性胶的实验进一步表明PIF4的缺失会破坏复合体的形成而PIF5的缺失不影响复合体的形成。这与PIF4是复合体成分的发现是一致的。进一步的实验发现PIF4与PIF1，PIF2和PIF3形成一个稳定的复合体，而在PIF4缺失的情况下这三个蛋白会形成另一个稳定的亚复合体。

综合上述结果我们推测PIF复合体可能存在三个结构上的层次，*i.* 由PIF1，PIF2和PIF3构成稳定的复合体核心，*ii.* PIF4和这个复合体核心结合形成更大的稳定复合体，*iii.* P74和P95通过相对弱的相互作用与这个复合体发生结合。该复合体中另外三个成分的存在（AC5，AC68和AC108）还需要进一步的验证。近期已有报道表明

AC68（PIF6）和AC108对于口服感染具有重要的作用，我们的结果提示这两个蛋白可能和其它的PIF协同在ODV入侵的过程中行使功能。

除了病毒入侵复合体蛋白组分间的相互作用外，蛋白酶的剪切激活对于病毒的入侵也常常具有重要的作用。蛋白酶的剪切可以使病毒膜蛋白，尤其是结合和融合蛋白，形成功能性的构象以行使功能。论文的**第五章**中我们发现了针对P74的一个新的蛋白酶剪切机制。P74是一个高度保守的介导ODV与宿主细胞结合的蛋白。这一章中我们发现P74会被病毒包涵体（occlusion body，OB）结构中的碱性蛋白酶剪切成两个片段。当OB在中肠碱性环境中解离释放ODV粒子时该碱性蛋白酶得到激活并对P74进行剪切。在相同条件下其它的PIF并未受到蛋白酶剪切表明剪切是对P74特异的。只有虫体来源的OB（L-OB）才具有该蛋白酶活性而细胞系来源的OB则没有该蛋白酶活性，提示该蛋白酶是来源于宿主的而并非由病毒编码的。研究表明在OB被暴露于碱性环境三分钟以内P74即被完全剪切提示在P74接触宿主受体之前就被剪切了。两种虫体来源的OB对P74具有相同的剪切活性提示包装虫体来源的蛋白酶进入OB结构是一个保守的机理。进一步的实验表明剪切后P74的两个片段仍与PIF复合体结合在一起。前期的报道发现在ODV完全暴露在中肠环境中时P74会被中肠的宿主蛋白酶剪切，而且该剪切过程对于ODV的感染具有重要作用。鉴于在自然环境中病毒的感染是由L-OB介导的，我们推测在口服感染的过程中存在一个P74二级剪切的机理：第一个剪切过程由包装进OB的内源性蛋白酶完成，而在ODV被完全释放进中肠环境后由宿主的中肠蛋白酶完成第二次剪切。而第一个剪切过程可能是L-OB比细胞来源的OB具有更高毒力的原因之一。

在**第六章**中我们讨论了本论文中的结果对于理解ODV组装和入侵机理的意义。我们提出了一个ODV入侵复合体和相应的入侵机理的假说：在ODV感染中肠的过程中P74的二级剪切过程促进了入侵复合体功能性构象的改变并激活了一系列动态过程以介导ODV入侵。针对这一过程我们提出了一些新的问题并对今后的工作提出了一些建议。

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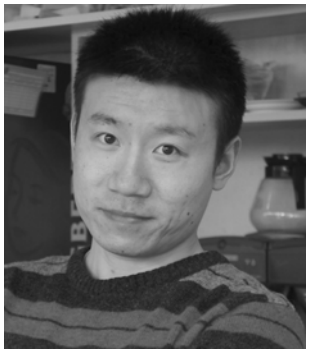
Curriculum Vitae



Ke Peng was born on July 28th, 1980, in Wuhan, China. He studied Microbiology at the Huazhong Agricultural University (HZAU, China) and graduated in July 2002. After graduation he had various professions to explore the "un-biological" society including teaching English in a private education institute. In 2003 he passed the National Graduate Study Entrence examination and was enrolled in an MSc programme in the Wuhan Institute of Virology (WIV) of the Chinese Academy of Sciences under the supervision of Prof. Dr. Zhihong Hu. The MSc programme was later converted into a PhD programme. At his 4th year of study in the WIV, he was offered a 4 year fellowship provided by the joint PhD programme of the Chinese Academy of Sciences (CAS) and the Royal Netherlands Academy of Arts and Sciences (KNAW). He started to work in the Laboratory of Virology at Wageningen University (WUR, The Netherlands) in the research group of Prof. Dr. Just Vlak, Dr ir Jan van Lent and Dr Monique van Oers in January 2008. His research on the mechanisms of baculovirus occlusion derived virus assembly and entry is described in this thesis.

Account

Peng, K., Wu, M., Deng, F., Song, J., Dong, C., Wang, H. & Hu, Z. (2010). Identification of protein-protein interactions of the ODV associated proteins of HearNPV. Journal of General Virology **91**, 659-670.

Peng, K., van Oers, M.M., Hu, Z., van Lent, J.W.M. and Vlak, J.M. (2010). Baculovirus per os infectivity factors form a complex on the surface of occlusion derived virus. Journal of Virology **84**, 9497-9504.

Peng, K., van Lent, J.W.M., Vlak, J.M., Hu, Z. & van Oers, M.M. (2011). In situ cleavage of the baculovirus occlusion derived virus receptor binding protein P74 in the peroral infectivity complex. Journal of Virology **85**, 10710-10718

Peng, K., van Lent, J.W.M., Boeren, S., Fang, M., Theilmann, D.A., Erlandson, M.A., Vlak, J.M. and van Oers, M.M. Characterization of novel components of the baculovirus *per os* infectivity factor (PIF) complex. **Submitted for publication.**

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5 ECTS)

- Baculovirus peroral infectivity of insects: current status, recent advances and future directions (2008)

Writing of Project Proposal (3 ECTS)

- Towards the entry mechanism of baculovirus occlusion-derived virus

Post-Graduate Courses (3 ECTS)

- Transgenesis, gene targeting and *in vivo* imaging; LUMC (2009)
- Electron tomography in life science; LUMC (2009)

Laboratory Training and Working Visits (0.9 ECTS)

- Protein complex production and purification; EMBL, Grenoble (2011)
- Live-cell imaging; Heidelberg University (2011)

Competence Strengthening / Skills Courses (1.8 ECTS)

- PhD Competence assessment; WGS (2009)
- Safe handling of radioactive materials and resources; WGS (2010)
- Effective behaviour; WGS (2011)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.2 ECTS)

- PE&RC Day (2008)
- PE&RC Weekend (2008)

Discussion Groups / Local Seminars and Other Meetings (5.4 ECTS)

- Dutch Annual Virology Symposium; Amsterdam, the Netherlands (2008-2011)
- Program Strategic Scientific Alliances Workshops; KNAW-CAS (2008-2011)

International Symposia, Workshops and Conferences (7.5ECTS)

- Annual Meeting Society for Invertebrate Pathology; oral presentation; Park City, Utah, USA (2009)
- Annual Meeting Society for Invertebrate Pathology; oral presentation; Trabzon, Turkey (2010)
- Gordon Research Conference, Viruses and Cells; poster presentation; Lucca, Barga, Italy (2011)

Lecturing / Supervision of Practicals / Tutorials (2.7 ECTS)

- Molecular virology PGO; 3 days (2009)
- Molecular virology PGO; 3 days (2010)
- Ecological Aspects of Bio-Interactions (EABI); 3 days (2010)

Supervision of 6 MSc Students; 50 days (15 ECTS)

- Functional analysis of White Spot Syndrome Virus ORF115
- Identification of interaction domains of *per os* infectivity factors of baculovirus
- Preparation of brush border-membrane vesicles of insect midgut cells (2 persons)
- Identification of components of *per os* infectivity factor complex of baculovirus
- Functional analysis of P95 of baculovirus

This research was supported by a grant from CAS-KNAW Joint PhD Training Programme (31.849.00024)**.**

Attendance to the Gordon Research Conference in Italy in 2011 was financially supported by a grant of the Rob Goldbach fund.

Cover: Visualization of an AcMNPV ODV binding to microvilli in the insect midgut. Made by Ke Peng and Jan van Lent at the Wageningen Electron Microscopy Center.