The Host Specificities of Baculovirus \textit{per os} Infectivity Factors

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Abstract

Baculoviruses are insect-specific pathogens with a generally narrow host ranges. Successful primary infection is initiated by the proper interaction of at least 8 conserved \textit{per os} infectivity factors (PIFs) with the host's midgut cells, a process that remains largely a mystery. In this study, we investigated the host specificities of the four core components of the PIF complex, P74, PIF1, PIF2 and PIF3 by using Helicoverpa armigera nucleopolyhedrovirus (HearNPV) backbone. The four \textit{pifs} of HearNPV were replaced by their counterparts from a group I Autographa californica multiple nucleopolyhedrovirus (AcMNPV) or a group II Spodoptera litura nucleopolyhedrovirus (SpltNPV). Transfection and infection assays showed that all the recombinant viruses were able to produce infectious budded viruses (BVs) and were lethal to \textit{H. armigera} larvae via intraheamocoeelic injection. However, feeding experiments using very high concentration of occlusion bodies demonstrated that all the recombinant viruses completely lost oral infectivity except SpltNPV \textit{pif3} substituted \textit{pif3-null} HearNPV (vHaBac\textDelta\textit{pif3-SpltNPV-pif3-ph}). Furthermore, bioassay result showed that the median lethal concentration (LC\textsubscript{50}) value of vHaBac\textDelta\textit{pif3-SpltNPV-pif3-ph} was 23-fold higher than that of the control virus vHaBac\textDelta\textit{pif3-HaNPV-pif3-ph}, indicating that SpltNPV \textit{pif3} can only partially substitute the function of HearNPV \textit{pif3}. These results suggested that most of PIFs tested have strict host specificities, which may account, at least in part, for the limited host ranges of baculoviruses.

Introduction

Baculoviruses are insect-specific large DNA viruses, which have important applications in the areas of insect pesticides, protein expression and gene therapy [1–3]. The \textit{Baculoviridae} family is constituted of four genera: \textit{Alpha-}, \textit{Beta-}, \textit{Gamma-} and \textit{Delta-baculovirus}. A typical life cycle of alpha- and betabaculoviruses produces two morphologically distinct virions: the budded virus (BV) and the occlusion-derived virus (ODV) [4]. ODVs enter the epithelial cells of insect...
midgut through direct membrane fusion to initiate primary infection, while BVs are transmitted from cell to cell and cause systemic infection [5, 6].

The successful initiation of infection in the midgut epithelium by the ODVs is largely dependent on a number of virus encoded proteins termed per os infectivity factors (PIFs). So far, eight pif genes have been identified to be conserved in all sequenced baculoviruses, including p74 [7, 8], pif1 [9], pif2 [10, 11], pif3 [12, 13], pif4 [14, 15], pif5 [16, 17], pif6 [18] and pif7 [19]. Absence of any pif gene will result in a profound impairment or complete loss of oral infectivity. All PIFs except PIF7 are conserved in baculoviruses, while PIF7 is conserved only in lepidopteran baculoviruses (alpha- and betabaculoviruses). Interestingly, homologues of a few PIFs are also found in nudiviruses, salivary gland hypertrophy viruses (SGHVs) and the white spot syndrome virus (WSSV), implying an evolutionarily conserved ancient entry mechanism of invertebrate viruses [20].

Among the 8 PIFs, P74, PIF1-3, 5 are all ODV envelope-specific proteins [9, 15, 16, 21, 22], whereas PIF4 and PIF6 were detected in the envelope fractions of both budded virus (BV) and ODV [14]. PIF7 was suggested to be localized in the envelope fractions of ODV by a mass spectrometry study [23]. In Helicoverpa amigera nucleopolyhedrovirus (HearNPV), all the PIFs located in ODV envelope [23]. Recent studies have showed that P74 and PIF1-4 form a protein complex on the surface of ODV. PIF1-3 appear to be the core components, while P74 may be loosely associated with the complex [24, 25]. The PIF complex is likely to play an important role in virus entry into midgut epithelial cells of susceptible insect larvae [24]. Previous studies showed that P74, PIF1 and PIF2 are involved in the binding of ODV onto midgut cells [12, 26]. In contrast, PIF3 may not participate in virus binding process, instead, it is speculated to mediate nucleocapsid translocation along microvilli [12, 27].

Since PIFs are responsible for oral infection, it is reasonable to suggest their involvement in the host range of baculovirus. Unfortunately, studies of the specificity of PIFs are rather scanty. Wu et al. [28] generated a recombinant Autographa californica multiple nucleopolyhedrovirus (AcMNPV) with its p74 gene replaced by that of Spodoptera litura nucleopolyhedrovirus (SpltNPV), but they did not test the oral infectivity of this recombinant virus, which should have been the logical experiment to do. Harrison et al. [16] found that replacement of AcMNPV odv-e56 (pif5) gene with its counterpart from a closely-related Rachiphusia ou multiple nucleopolyhedrovirus (RoMNPV) did not increase virulence against larvae that are more susceptible to RoMNPV than to AcMNPV.

In this study, we tested whether P74, PIF1, PIF2 and PIF3 of HearNPV could be functionally substituted by their homologues from other baculoviruses. HearNPV is a group II alphabaculovirus specific to certain species of Heliothis [29]. The pifs of AcMNPV or SpltNPV were amplified and inserted into the pifs-deleted HearNPV bacmid. AcMNPV is a group I alphabaculovirus with wide host range of 39 lepidopteran species [30], while SpltNPV is a group II alphabaculovirus that infects a single host [31]. All the recombinant viruses produce infectious BVs, which are lethal to H. armigera larvae via intrahaemocoelic injection. However, bioassay results demonstrated that most recombinant viruses lost their oral infectivity completely, except SpltNPV pif3 substituted pif3-null HearNPV (vHaBacΔpif3-Sppif3-ph) which retained only partial oral infectivity. These results revealed the involvement of PIF proteins in host specificities of baculovirus.

Material and Methods

Insect cells, insects and viruses

The Helicoverpa zea ovarian cell line HzAM1 [32] was maintained at 28°C in Grace’s medium (Gibco-BRL) supplemented with 10% fetal bovine serum. H. armigera larvae were reared on an
artificial diet at 27°C. An infectious HearNPV bacmid HaBacHZ8, as well as pifs-deletion bacmids HaBacΔpifs-ph, Hapifs-repaired viruses vHaBacΔpifs-HapifsR-ph and control virus vHaBac-egfp-ph were constructed previously in our laboratory (ph stands for polyhedrin gene) [22, 33]. HearNPV G4 strain, AcMNPV and SpltNPV were maintained as laboratory stocks.

Construction of pifs-pseudotyped HearNPV bacmids

To construct bacmids with substituted pifs, the coding sequence along with the putative promoter region of p74, pif1, pif2 or pif3 were amplified from genomic DNA of AcMNPV and SpltNPV by specific primers listed in Table 1. The PCR products were cloned into pGEM-T easy vector (Promega) for sequencing. Then, these pif genes were inserted into the indicated restriction enzyme sites (Table 1) of the transfer vector pFB-DUAL-ph and further transposed into the respective pif-deletion HearNPV bacmids according to the Bac-to-Bac manual (Invitrogen). The resulting bacmids were identified by PCR analyses and designated as HaBacΔp74-Acp74-ph, HaBacΔpif1-Acpif1-ph, HaBacΔpif2-Acpif2-ph, HaBacΔpif3-Acpif3-ph, HaBacΔp74-Spp74-ph, HaBacΔpif1-Sppif1-ph, HaBacΔpif2-Sppif2-ph and HaBacΔpif3-Sppif3-ph.

Transfection and infection

To produce the recombinant viruses, HzAM1 cells were seeded into tissue culture wells at a density of 5x10^5 cells per well. Transfection was performed with 0.5 μg bacmid DNA using 10 μl lipofectin (Invitrogen). At 6 days post transfection (p.t.), the supernatant containing BVs was harvested by centrifugation to remove cell debris and used to infect a new batch of HzAM1 cells. The titer of each recombinant BV was determined by end point dilution assays (EPDAs).

Electron microscopy (EM)

HzAM1 cells were infected with the individual pif-substituted recombinant HearNPVs or control virus vHaBac-egfp-ph respectively at an MOI of 5 TCID50 units/cell. Infected cells were processed for electron microscopy examination at 72 h p.i. as described previously [22].

Table 1. Primers for amplification of pifs from AcMNPV and SpltNPV genomes.

| Primers   | Sequences                           |
|-----------|-------------------------------------|
| Ac p74 F  | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |
| Ac p74 R  | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Kpn I) |
| Ac pif1 F | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |
| Ac pif1 R | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Kpn I) |
| Ac pif2 F | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |
| Ac pif2 R | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Kpn I) |
| Ac pif3 F | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Xho I) |
| Ac pif3 R | 5’-cggccatgctgctctaaatcatgattcttc-3’ (Kpn I) |
| Splt p74 F| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Xho I) |
| Splt p74 R| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Kpn I) |
| Splt pif1 F| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Xho I) |
| Splt pif1 R| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Kpn I) |
| Splt pif2 F| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |
| Splt pif2 R| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |
| Splt pif3 F| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |
| Splt pif3 R| 5’-cggccatgctgctctaaatcatgattcttc-3’ (Sph I) |

Restriction sites are underlined.

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Western blot analyses of recombinant viruses

Polyclonal antibodies against HearNPV P74, PIF1, PIF2 and PIF3 were generated previously in rabbits [22]. To generate polyclonal antibodies against AcPIFs and SpPIFs, partial coding sequences of the pifs were amplified from AcMNPV or SpltNPV genomes. DNA fragments containing AcP74 (1–1251 nt), AcPif1 (124–1590 nt), AcPif2 (1–1146 nt), AcPif3 (85–612 nt), SpP74 (1–1287 nt), SpPif1 (109–1575 nt), SpPif2 (1–1146 nt), or SpPif3 (73–600 nt) were each cloned into the pET-28a expression vector. These truncated PIF proteins were expressed in E. coli BL21 cells and purified for immunizing rabbit to generate polyclonal antibodies.

HzAM1 cells were infected with vHaBac-egfp-ph, vHaBacΔpifs-Acpifs-ph or vHaBacΔpifs-Sppifs-ph at an MOI of 5. Infected cells were harvested at 96 hours post infection (h p.i.), separated on 12% SDS-PAGE and analyzed by Western blots. ODVs of AcMNPV and SpltNPV were used as positive controls. The PIFs-specific polyclonal antibodies were used as the primary antibodies and an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Gibco-BRL) was used as the secondary antibody. The final signal was detected by using a BCIP/NBT kit (Sino-America).

Immunofluorescence assays (IFA)

HzAM1 cells were infected with vHaBac-egfp-ph, vHaBacΔpifs-Acpifs-ph or vHaBacΔpifs-Sppifs-ph at an MOI of 5. At 72 h p.i., cells were fixed with 4% paraformaldehyde and made permeable with 0.2% Triton X-100. After being blocked with 5% BSA, the cells were incubated with anti-PIFs polyclonal antibodies and then with Alexa Fluor™ 555-conjugated Goat Anti-Rabbit IgG H&L (Abcam) as the secondary antibody. Nuclei were stained with Hoechst stain. The subcellular localization of the PIFs was detected by fluorescence microscopy.

Bioassays

Systemic infection was initiated by intrahaemocoelic injection of BVs into late third-instar H. armigera larvae as described previously [22]. About 10 μl of 10^6 TICD₅₀ units/ml of BVs was injected into the haemocoel of each larva. Grace’s medium was used as a negative control.

Oral infectivity of the recombinant viruses was detected by the droplet method with early third-instar H. armigera larvae described before [22]. The occlusion bodies (OBs) used for bioassay were harvested and purified from diseased larvae. In feeding assay, 10⁸ OBs/ml of each virus were used [34]. To determine median lethal concentration LC₅₀ value, bioassays were conducted by exposing larvae to different virus concentrations: 1×10³, 3×10³, 1×10⁴, 3×10⁴, 1×10⁵, 5×10⁵, 1×10⁶, 3×10⁶, 1×10⁷, 3×10⁷, 1×10⁸, 3×10⁸ and 1×10⁹ OBs ml⁻¹. Probit analysis was used to calculate LC₅₀ values, 95% confidence limits and regression slopes. Data from two replicates were pooled to calculate the final LC₅₀ values, as long as there was no significant difference between the LC₅₀ values and regression slopes of the replicates. LC₅₀ values between each pair of recombinant viruses were compared by the lethal dose ratio method of [35].

In all the above bioassay experiments, larvae were kept separately in 24-well plates and monitored daily until all larvae had either pupated or died as a result of virus infection. At least 48 larvae were used per treatment. All the bioassays were done in duplicates.

Result

Construction and characterization of pifs substituted HearNPVs

In order to investigate the host specificities of baculoviral PIFs, the HearNPV bacmids, each with an individual pif deletion and containing egfp marker gene [22] were used as a backbone to insert the pif counterparts from AcMNPV and SpltNPV (Fig 1A). The recombinant bacmids
HaBacΔpifs-Acpifs-ph and HaBacΔpifs-Sppif-ph were identified and verified by PCR (Fig 1B). To generate recombinant HearNPVs, each constructed bacmid DNA was used to transfec and then infect HzAM1 cells. The successful productions of infectious progenies from all the recombinant bacmids were characterized by the proliferation of green fluorescence (Fig 1C) and appearance of polyhedra (data not shown). Further EM observation showed that all the pif-substituted recombinant viruses formed normal OBs with embedded ODVs, much like the control virus (Fig 2).

Expression of PIFs in infected HzAM1 cells

In cells infected with vHaBacΔp74-Acp74-ph or AcMNPV ODV, a band of the expected size of AcP74 (~70 kDa) reacted with anti-AcP74 antibody (Fig 3A-left). This band was absent in cells infected with vHaBacΔp74-Hap74-ph (Fig 3A-left). By using anti-SpP74 antibody, a band of the size of SpP74 (~72 kDa) was detected in extracts of vHaBacΔp74-Spp74-ph infected cells and SpltNPV ODVs, but not in vHaBacΔp74-Hap74-ph infected cells (Fig 3A-middle). Anti-HaP74

Fig 1. Construction and identification of HearNPV bacmids with substituted pifs and recombinant viruses. (A) Schematic overview of constructed HearNPV bacmids. (B) PCR identification of Acpifs and Sppifs genes in recombinant HearNPV bacmids. (C) HzAM1 cells were transfected with constructed HearNPV bacmids (upper panels) or infected with pif-substituted HearNPV recombinant viruses (below panels). EGFP was used to monitor the transfection and infection by fluorescence microscopy at 72 h p.t. and 72 h p.i.

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Fig 2. EM analysis of recombinant viruses-infected HzAM1 cells. HzAM1 cells were infected with the respective viruses at an MOI of 5. Cells were collected at 72 h p.i. and observed under a Hitachi H-7000 electron microscope operated at 75 kV. Bars, 500 nm.

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antibody detected a band of expected size of HaP74 (~76 kDa) in vHaBacΔp74-infected cells, but not in those of vHaBacΔp74-AcP74-ph and vHaBacΔp74-SpP74-ph infected cells (Fig 3A-right). An additional band of ~40 kDa was detected in the vHaBacΔp74-HaP74-ph infected cells, which may represent a partially cleaved HaP74 (Fig 3A-right) (Huang et al., unpublished data).

Similarly, AcPIF1 (~58 kDa), AcPIF2 (~42 kDa) and AcPIF3 (~22 kDa) as well as SpPIF1 (~58 kDa), SpPIF2 (~42 kDa) and SpPIF3 (~22 kDa) were also expressed in cells infected with
AcPif1-, AcPif2-, AcPif3-, SpPif1-, SpPif2- and SpPif3-substituted HearNPV recombinant viruses, but not in cells infected with vHaBacΔpif1-Hapif1-ph, vHaBacΔpif2-Hapif2-ph or vHaBacΔpif3-Hapif3-ph (Fig 3B–3D, left and middle panels). In contrast, HaPif1 (~58 kDa), HaPif2 (~42 kDa) and HaPif3 (~21 kDa) were detected only in the cells infected with vHaBacΔpif1-Hapif1-ph, vHaBacΔpif2-Hapif2-ph or vHaBacΔpif3-Hapif3-ph, but not in samples of pif-substituted viruses (Fig 3B–3D, right panels). These results confirmed the correct expression of all the four heterologous SpPIFs and AcPIFs in the recombinant viruses-HzAM1 system.

Localization of PIFs in infected HzAM1 cells

IFA was performed to detect the subcellular localization of the heterologous PIFs in infected HzAM1 cells. As shown in Fig 4-left panel, the four PIFs of HearNPV were mainly localized to the nuclear ring zone region of their native host cells. Except for AcP74, SpP74 and SpPif2, whose antibody was not suitable for use in IFA, the rest 5 heterologous PIFs, including AcPif1, AcPif2, AcPif3, SpPif1 and SpPif3 also accumulated in the ring zone region of HzAM1 cells (Fig 4-right panel). Therefore, most of the tested Ac- and SpPIFs were properly transported to the nuclear ring zone region of HzAM1 cells.

All viruses with substituted pifs, except vHaBacΔpif3-SpPif3-ph retained their systemic infectivity but lost their oral infectivity

To test the systemic infectivity of the pif-substituted viruses, supernatants containing BVs of parental controls, pif-substituted and pif-repaired viruses were injected into the haemolymph of late third-instar *H. armigera* larvae with a titer of 10^6 TCID_{50} units/ml. All the recombinant viruses killed the tested larvae, suggesting that the pif-substituted viruses retained their systemic infectivity (data not shown).

**Fig 4. Subcellular localization of PIFs in infected cells.** HzAM1 cells were infected with the parental control virus vHaBac-egfp-ph (left panel) or pif-substituted viruses (right panel). At 72 h p.i., cells were fixed, permeabilized and probed with the corresponding anti-PIF polyclonal antibody/Alexa Fluor™ 555-conjugated goat anti-rabbit antibody and viewed using a fluorescent microscope. Nuclei were stained with Hoechst stain. Bars, 5 μm.

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To assess the oral infectivity of the pif-substituted viruses, preliminary feeding experiments were carried out. OBs (10^8/ml) of the recombinant viruses isolated from infected larvae were fed to the third-instar *H. armigera* larvae by droplet method. The results showed that all the pif-substituted recombinants except vHaBacΔpif3-Sppif3-ph were not infective to *H. armigera* larvae even with such a high virus dose (Table 2).

A bioassay experiment was further performed to determine the LC50 value. As shown in Fig 5 and Table 3, the LC50 values of vHaBacΔpif3-Sppif3-ph was 7.38×10^4 OBs/ml, which is about 23-fold higher than that of the control virus vHaBacΔpif3-Hapi3-ph (LC50 = 3.23×10^7 OBs/ml).

| Virus Test | 1 Dead/Tested | 2 Dead/Tested |
|------------|---------------|---------------|
| HaBac-egfp-ph | 48/48         | 48/48         |
| HaBacp74-Acp74-ph | 0/48         | 1*/48         |
| HaBacpif1-Acpif1-ph | 2*/48         | 0/48         |
| HaBacpif2-Acpif2-ph | 0/48         | 0/48         |
| HaBacpif3-Acpif3-ph | 3*/48         | 2*/48         |
| HaBacp74-Spp74-ph | 0/48         | 1*/48         |
| HaBacpif1-Sppif1-ph | 2*/48         | 0/48         |
| HaBacpif2-Sppif2-ph | 0/48         | 0/48         |
| HaBacpif3-Sppif3-ph | 46+1*/48     | 47/48         |
| Mock       | 0/48         | 0/48         |

*The death was not due to virus infection.*

OB concentration = 1 × 10^8 OBs/ml.

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**Table 2.** Feeding experiments of recombinant viruses in early third instar *H. armigera* larvae.

**Fig 5.** Bioassays. Forty-eight third-instar *H. armigera* larvae were infected with different concentrations of each recombinant virus by the droplet method. The final mortality for each virus concentration was calculated. Each data point represents the mean value from three separate infections; error bars indicate standard deviation.

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Statistical analyses indicated that the LC$_{50}$ values of HaBac$_{Δ}$pif$_3$-Sp$_{pif3-ph}$ was significantly different from that of vHaBac$_{Δ}$pif$_3$-Ha$_{pif3-ph}$ ($P < 0.05$). The data reveal that SpltNPV PIF3 only partially substituted the function of HearNPV PIF3. Altogether, these results suggested that, although the pifs are conserved in baculoviruses, substitution of individual HearNPV pifs with homologues from AcMNPV or SpltNPV did not yield a virus with efficient oral infectivity, indicating the role of PIF proteins in host specificity.

**Discussion**

We studied the host specificity of HearNPV p74, pif1, pif2 and pif3 by substituting with their counterparts from AcMNPV or SpltNPV. Transfection-infection experiments showed that all the recombinant viruses produced BVs infective to cell culture. Intrahaemocoelic injection of BVs into H. armigera larvae confirmed that all the recombinant viruses retained their systemic infectivity. However, bioassay results demonstrated that only vHaBac$_{Δ}$pif$_3$-Sp$_{pif3-ph}$ could infect H. armigera larvae via oral route but with significantly decreased virulence. All the other recombinant viruses with substituted pifs completely lost their oral infectivity.

In fact apart from AcMNPV and SpltNPV, the p74, pif1, pif2 or pif3 gene of two other group II alphabaculovirus, Spodoptera exigue (Se)MNPV and Chrysodeixis chalcites (Chch)NPV, were also used to substitute their counterparts in HearNPV. Similar experiments were carried out, and none of the above Sepifs or Chchpifs rescued the oral infectivity of pifs-deleted HearNPV (data not shown). These data suggest that the host specificity of P74, PIF1, PIF2 and PIF3 may be universal in baculovirus. It is important to mention the insect also plays an important role in host specificity. Only those with receptors in their midgut columnar epithelial cells specific to certain PIFs allow that virus to gain access to these cells. In other words, host specificity is a mutually dependent property of the virus and the host.

As PIF3 of SpltNPV seems to be an exception since it can partially rescue the oral infectivity of HearNPV, we further studied the phylogeny of PIF3. As shown in Fig 6A, SpPIF3 is evolutionarily closer to HaPIF3 compared to the other examined PIF3 proteins, which is in accordance with the result of other core genes trees [36, 37]. Sequence alignment also showed that the amino acid (aa) identity of PIF3 N-terminus between SpltNPV and HearNPV is relatively higher than with other baculoviruses (Fig 6B). This may provide a possible explanation for the result of partial function rescue of HaPIF3 by SpPIF3. Among PIF74, PIF1, PIF2 and PIF3, the latter shows the lowest conservation. Sequence analyses show that the aa identity among different baculoviruses is 37–63% for P74, 31–59% for PIF1, 45–71% for PIF2 and 31–58% for PIF3 (data not shown), indicating that PIF3 is under less stringent selection pressure than that of the other PIFs. Unlike P74, PIF1 and PIF2, which are believed to be involved in the virus binding to a specific receptor on the midgut cells, PIF3 is suggested to mediate necessary interaction between the host and virus downstream of binding process [12]. The different function of PIF3 may also contributed to its less stringent host specificity in comparison to that of P74, PIF1 and PIF2, at least in the case of SpPIF3.

P74, PIF1, PIF2 and PIF3 constitute the core components of the PIF complex [24, 25]. Yeast two hybrid experiments also showed that some PIF proteins interact with other PIFs [38].

| Table 3. LC$_{50}$ and regression slopes of concentrations-mortality of recombinant viruses. |
|-----------------------------------------------|
| Virus | LC$_{50}$ (PIB/ml) | 95% Confidence limit (PIB/ml) | Regression Slope |
|-------|-------------------|-------------------------------|-----------------|
| HaBac$pif3$-Hapif3-ph | 3.23×10$^3$ | (2.11–4.60)×10$^3$ | 1.197 |
| HaBac$pif3$-Sp$pif3-ph$ | 7.38×10$^4$ | (5.32–1.02)×10$^3$ | 1.158 |

*represent the significant difference between the LC$_{50}$ values of these two viruses compared by the lethal dose ration method [35].

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Therefore, the function of an individual PIF may be dependent on proper recognition and interaction with other PIFs. And this may explain why substituting an individual PIF does not mediate oral infection. It will be interesting to test possible rescue of oral infectivity by substituting a whole set of PIFs instead of just individual PIFs. It also remains to be studied whether the PIF4, PIF5 and PIF6 also exhibit similar host specificity.

In summary, by constructing a series of pif$\!\!\!^s$-substituted pseudotyped baculoviruses, we for first time characterized that PIF proteins (P74, PIF1, PIF2 and PIF3) of baculoviruses have strict host specificities. Only SpltNPV PIF3 is able to partially substitute for the function of HearNPV PIF3. The data provided evidence that PIFs are crucial for host range of baculoviruses. Further research will focus on elucidation of the interaction networks among PIFs and the midgut host factors during oral infection. We are presently investigating a system that will allow substituting several PIFs simultaneously to gain further insight into host specificity. These attempts will help disclose the oral infection mechanism and the host specificity of baculoviruses.

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Author Contributions

Conceived and designed the experiments: MW ZH JS. Performed the experiments: JS XW HH XL DH MW. Analyzed the data: JS MW ZH FD HW. Contributed reagents/materials/analysis tools: JS FD HW. Wrote the paper: JS MW ZH BA.

References

1. Inceoglu AB, Kamita SG, Hinton AC, Huang QH, Severson TF, Kang KD, et al. Recombinant baculoviruses for insect control. Pest Manag Sci. 2001; 57(10):981–7. doi: 10.1002/ps.393. WOS:000171566000015. PMID: 11695193
2. Hitchman RB, Possee RD, King LA. Baculovirus expression systems for recombinant protein production in insect cells. Recent patents on biotechnology. 2009; 3(1):46–54. PMID: 19149722.

3. Ghosh S, Parvez MK, Banerjee K, Sarin SK, Hasnain SE. Baculovirus as mammalian cell expression vector for gene therapy: An emerging strategy, Mol Ther. 2002; 6(1):5–11. doi: 10.1006/mtth.2000.0643. WOS:000176578500003. PMID: 12095297

4. Kedde BA, Aponte GW, Volkman LE. The pathway of infection of Autographa californica nuclear polyhedrosis virus in an insect host. Science. 1989; 243(4899):1728–30. PMID: 2648574.

5. Summers MD. Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of Trichoplusia ni. J Ultrastruct Res. 1971; 35(5):606–25. PMID: 5142394.

6. Volkman LE, Summers MD. Autographa californica nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. J Invertebr Pathol. 1977; 30(1):102–3. PMID: 336795.

7. Kuzio J, Jaques R, Faulkner P. Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. Virology. 1989; 173(2):759–63. PMID: 2688302.

8. Yao LG, Zhou WK, Xu H, Zheng Y, Qi YP. The Heliothis armigera single nucleocapsid nucleopolyhedrovirus envelope protein P74 is required for infection of the host midgut. J Virol. 2004; 104(2):111–21. doi: 10.1128/jvi.04.03.005. WOS:000222982300002. PMID: 15246648

9. Kikhuo I, Gutierrez S, Croizer L, Crozier G, Ferber ML. Characterization of pif, a gene required for the per os infectivity of Spodoptera littoralis nucleopolyhedrovirus. J Gen Virol. 2002; 83:3013–22. WOS:000179901600011. PMID: 12466478

10. Pijlman GP, Pruijssers AJP, Viak JM. Identification of pif-2, a third conserved baculovirus gene required for per os infection of insects. J Gen Virol. 2003; 84:2041–9. doi: 10.1099/vir.0.19133-0. WOS:000184411600011. PMID: 12867634

11. Fang MG, Nie YC, Wang Q, Deng F, Wang RR, Wang HZ, et al. Open reading frame 132 of Heliocoverpa armigera nucleopolyhedrovirus encodes a functional per os infectivity factor (PIF-2). J Gen Virol. 2006; 87:2563–9. doi: 10.1099/vir.0.81788-0. WOS:000239939900013. PMID: 16894194

12. Ohkawa T, Washburn JO, Sitapara R, Sid E, Volkman LE. Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of Heliothis virescens larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. J Virol. 2005; 79(24):15258–64. doi: 10.1128/JVI.79.24.15258-15264.2005. WOS:000234276700007. PMID: 16306597

13. Li X, Song J, Jiang T, Liang C, Chen X. The N-terminal hydrophobic sequence of Autographa californica nucleopolyhedrovirus PIF-3 is essential for oral infection. Arch Virol. 2007; 152(10):1851–8. doi: 10.1007/s00705-007-1012-3. WOS:000249813300008. PMID: 17585368

14. Fang MG, Nie YC, Harris S, Erlanson MA, Theilmann DA. Autographa californica multiple nucleopolyhedrovirus core gene ac96 encodes a per Os infectivity factor (PIF-4). J Virol. 2009; 83(23):12569–78. doi: 10.1128/JVI.01409-08. WOS:000271465600055. PMID: 19759145

15. Huang H, Wang M, Deng F, Wang H, Hu Z. ORF85 of HearNPV encodes the per os infectivity factor 4 (PIF4) and is essential for the formation of the PIF complex. Virology. 2012; 427(2):217–23. doi: 10.1016/j.virology.2012.01.022 PMID: 22386821.

16. Harrison RL, Sparks WO, Bonning BC. Autographa californica multiple nucleopolyhedrovirus ODV-E56 envelope protein is required for oral infectivity and can be substituted functionally by Rachiplusia ou multiple nucleopolyhedrovirus ODV-E56. J Gen Virol. 2010; 91:1173–82. doi: 10.1099/vir.0.017160-0. WOS:000277761100009. PMID: 20032203

17. Sparks WO, Harrison RL, Bonning BC. Autographa californica multiple nucleopolyhedrovirus ODV-E56 is a per os infectivity factor, but is not essential for binding and fusion of occlusion-derived virus to the host midgut. Virology. 2011; 409(1):69–76. doi: 10.1016/j.virology.2010.09.027. WOS:000285450900009. PMID: 20970820

18. Nie YC, Fang MG, Erlanson MA, Theilmann DA. Analysis of the Autographa californica multiple nucleopolyhedrovirus overlapping gene pair lef3 and ac68 reveals that AC68 is a Per Os infectivity factor and that LEF3 Is Critical, but Not Essential, for Virus Replication. J Virol. 2012; 86(7):3985–94. doi: 10.1128/JVI.06849-11. WOS:000301371500004. PMID: 22278232

19. Simon O, Palma L, Williams T, Lopez-Ferber M, Caballerio P. Analysis of a naturally-occurring deletion mutant of Spodoptera frugiperda multiple nucleopolyhedrovirus reveals sIF6 as a new per os infectivity factor of lepidopteran-infecting baculoviruses. J Invertebr Pathol. 2012; 109(1):117–26. doi: 10.1016/j.jip.2011.10.010 PMID: 22041202.

20. Wang YJ, Bininda-Emonds ORP, van Oers MM, Viak JM, Jehle JA. The genome of Oryctes rhinoceros nudivirus provides novel insight into the evolution of nuclear arthropod-specific large circular double-stranded DNA viruses. Virus Genes. 2011; 42(3):444–56. doi: 10.1007/s11262-011-0589-5. WOS:000291492600019. PMID: 21380757
21. Faulkner P, Kuzio J, Williams GV, Wilson JA. Analysis of p74, a PDV envelope protein of Autographa californica nucleopolyhedrovirus required for occlusion body infectivity in vivo. J Gen Virol. 1997; 78:3091–100. WOS:A1997YJ34200003. PMID: 9400957

22. Song JJ, Wang RR, Deng F, Wang HL, Hu ZH. Functional studies of per os infectivity factors of Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus. J Gen Virol. 2008; 89:2331–8. doi: 10.1099/vir.0.2008/002352-0. WOS:000252856000030. PMID: 18753243

23. Hou DH, Zhang LK, Deng F, Fang W, Wang RR, Liu XJ, et al. Comparative proteomics reveal fundamental structural and functional differences between the two progeny phenotypes of a baculovirus. J Virol. 2013; 87(2):829–39. doi: 10.1128/Jvi.02329-12. WOS:000312934400013. PMID: 23115289

24. Peng K, van Lent JWM, Boeren S, Fang MG, Theilmann DA, Erlandson MA, et al. Characterization of novel components of the baculovirus per os infectivity factor complex. J Virol. 2012; 86(9):4981–8. doi: 10.1128/Jvi.02329-12. WOS:000302777800023. PMID: 22379094

25. Peng K, van Oers MM, Hu ZH, van Lent JWM, Vlak JM. Baculovirus per os infectivity factors form a complex on the surface of occlusion-derived virus. J Virol. 2010; 84(18):9497–504. doi: 10.1128/Jvi.00812-10. WOS:000281110500050. PMID: 20610731

26. Haas-Stapleton EJ, Washburn JO, Volkman LE. P74 mediates specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to primary cellular targets in the midgut epithelia of Heliothis virescens Larvae. J Virol. 2004; 78(13):6786–91. WOS:000222153800009. PMID: 15194753

27. Slack J, Arf BM. The baculoviruses occlusion-derived virus: Virion structure and function. Adv Virus Res. 2007; 69:99–165. doi: 10.1016/S0065-3527(06)69003-9. WOS:000244034500003. PMID: 17222693

28. Wu WW, Wang JW, Deng RQ, Wang XZ, He XL, Long QX. An efficient method for precise gene substitution in the AcMNPV genome by homologous recombination in E.coli. J Virol Methods. 2003; 113(2):95–101. doi: 10.1016/S0166-0934(03)00225-8. WOS:000186215500004. PMID: 14553895

29. Chen X, Li M, Sun X, Arf BM, Hu Z, Vlak JM. Genomic organization of Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus. Arch Virol. 2000; 145(12):2539–55. PMID: 11205104.

30. Guo T, Wang S, Guo X, Lu C. Productive infection of Autographa californica nucleopolyhedrovirus in silkworm Bombyx mori strain Haoyue due to the absence of a host antiviral factor. Virology. 2005; 341(2):231–7. doi: 10.1016/j.virol.2005.06.045 PMID: 16087208.

31. Pang Y, Yu J, Wang L, Hu X, Bao W, Li G, et al. Sequence analysis of the Spodoptera litura multicapsid nucleopolyhedrovirus genome. Virology. 2001; 287(2):391–404. doi: 10.1006/viro.2001.1056 PMID: 11531416.

32. McIntosh AH, Grasela JJ. Expression of beta-galactosidase and luciferase in insect cell lines infected with a recombinant AcMNPV. In Vitro Cell Dev-An. 1994; 30A(4):275–8. PMID: 8069450.

33. Wang HZ, Deng F, Pijlman GP, Chen XW, Sun XL, Vlak JM, et al. Cloning of biologically active genomes from a Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus isolate by using a bacterial artificial chromosome. Virus Res. 2003; 97(2):57–63. doi: 10.1016/j.virusres.2003.07.001. WOS:000188630400001. PMID: 14602197

34. Song JJ, Wang ML, Huang HC, Luo X, Deng F, Wang HL, et al. Functional studies of per os infectivity factor 3 of Helicoverpa armigera nucleopolyhedrovirus. J Gen Virol. 2012; 93:374–82. doi: 10.1099/vir.0.035865-0. WOS:000300139500001. PMID: 22031529

35. Robertson J. L. & Preisler H. K. (1992). Pesticide Bioassays with Arthropods. Baton Rouge, LA: CRC Press.

36. Zhu Z, Yin FF, Liu XP, Hou DH, Wang J, Zhang L, et al. Genome sequence and analysis of Buzura suppressaria baculovirus: a group II Alphabaculovirus. Plos One. 2014; 9(1). ARTN e86450doi: 10.1371/journal.pone.0086450. WOS:000330339800045.

37. Herniou EA, Jehle JA. Baculovirus phylogeny and evolution. Curr drug targets. 2007; 8(10):1043–50. PMID: 17979664.

38. Peng K, Wu M, Deng F, Song J, Dong C, Wang H, et al. Identification of protein-protein interactions of the occlusion-derived virus-associated proteins of Helicoverpa armigera nucleopolyhedrovirus. J Gen Virol. 2010; 91(Pt 3):659–70. doi: 10.1099/vir.0.017103-0 PMID: 19906939.