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Baculovirus *Per Os* Infectivity Factors Form a Complex on the Surface of Occlusion-Derived Virus▽

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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* multinucleocapsid nucleopolyhedrovirus (AcMNPV). The complex could withstand 2% SDS–5% β-mercaptoethanol with 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. Coimmunoprecipitation 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 the ODV with a scattered distribution. This distribution did not change for PIF1 or PIF2 when the gene for PIF2 or PIF1 protein was 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.

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) (4) and vaccinia virus (23), which have an entry machinery composed of four and eight 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 an occlusion body (OB) that infect midgut epithelial cells (24). After ingestion of OBs by the host, the proteinaceous OB crystal dissolves quickly due to the alkaline conditions (pH 10 to 11) in the midgut, and the ODV particles are released (reviewed in reference 24). 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 (10, 12, 24). 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 reference 22). While the entry mechanisms of BVs have been studied at least to a certain extent (16, 31, 32), 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 10 different envelope proteins (3). Five of these, denoted PIF1, PIF2, PIF3, PIF4, and P74, have been identified to be essential for *per os* infection of insect larvae (6, 7, 14, 18, 20). 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 (7, 10, 18). Until now, three of these proteins, PIF1, PIF2, and P74, have been reported to function in virus binding (10, 18). Deletion of any of these three proteins leads to a loss of oral infectivity, while only a 3-fold reduction in binding is measured, and no significant reduction in fusion efficiency is observed (10, 18). 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 functions of PIF3 and PIF4 are rather enigmatic although there has been speculation that PIF3 functions in nucleocapsid translocation along the microvilli as it seemed to be dispensable for ODV binding and fusion (18, 24).

All five proteins are highly conserved in *Baculoviridae* and are encoded by so-called core genes (3, 6, 11, 29). 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) (9), nudiviruses (30) and white spot syndrome virus (WSSV) (*Nimaviridae*) (J. A. Jehle, personal communication). *pif* genes are also found in polydnaviruses of braconid wasps (2). 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 evolutionarily ancient entry mechanism of viruses into invertebrate hosts.

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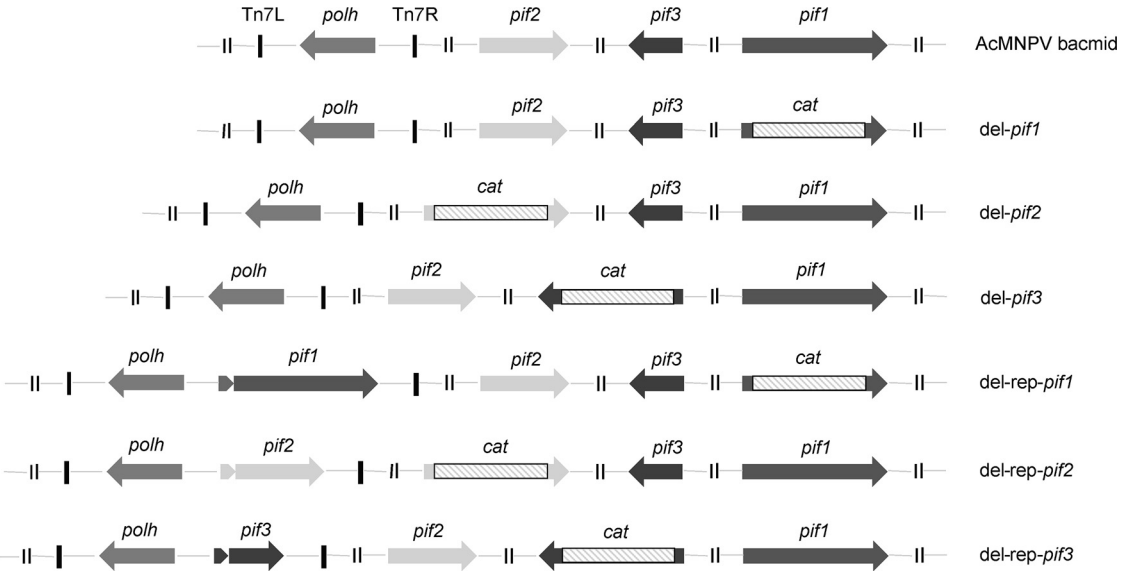

FIG. 1. Schematic of the recombinant bacmids used in this study. The orientation of *pif1*, *pif2*, and *pif3* in the bacmids is indicated. All bacmids contain the polyhedrin ORF under its own promoter. A major part of each of the three genes was replaced with the chloramphenicol resistance gene by homologous recombination. In the repaired bacmids, *pif* ORFs with their putative promoter regions were included.

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, cross-linking, differential temperature SDS-PAGE, and coimmunoprecipitation (CoIP) 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 27°C in Sf-900II medium (Invitrogen) with 5% fetal bovine serum (FBS) in stationary cultures. *Spodoptera exigua* larvae were reared on an artificial diet at 27°C in 40% humidity and with a 16:8-h (light-dark) photoperiod. The *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) E2 strain was used as the wild-type (wt) virus in this study. The AcMNPV bacmid (17) is derived from the Bac-to-Bac system (Invitrogen).

Construction of recombinant viruses. AcMNPV bacmids with a deletion of the *pif1* or *pif2* open reading frame ([ORF] del-*pif1* or del-*pif2*, respectively) were constructed as previously described for the *pif* genes of *S. exigua* MNPV (SeMNPV) (20). To this aim, PCR products with 50-bp overhangs homologous to viral flanking regions of the *pif* genes were generated with the primer pairs pif1-del-F/pif1-del-R or pif2-del-F/pif2-del-R (see Table S1 posted at http://edepot.wur.nl/143042) with Phusion polymerase (Finnzymes). Primers were designed to replace a fragment from nucleotides (nt) 115 to 1489 of the *pif1* ORF and from nt 65 to 1108 of the *pif2* ORF with the chloramphenicol resistance gene (*cat*) (Fig. 1). The *pif3* deletion bacmid was kindly provided by Xinwen Chen of the Wuhan Institute of Virology (15). An AcMNPV *p74* deletion virus was kindly provided by Jeffrey Slack of Great Lakes Forestry Centre Canada (7).

In order to repair the *pif1*, *pif2*, and *pif3* deletion bacmids, the coding sequences of these three genes plus their putative promoter regions (from position −150 relative to the ATG start codon) were amplified by PCR using the 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 (see underlining in Table S1 posted at http://edepot.wur.nl/143042) 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 the EcoRI and PstI restriction sites (pFBDΔP10-polh). The resulting plasmids were used to construct the "repair" bacmids (Fig. 1) using the Bac-to-Bac transposition protocol (Invitrogen).

Proliferation and purification of virus. *S. exigua* fourth instars were infected with OBs of wt AcMNPV. Hemolymph was collected at 2 days postinfection (p.i.) and clarified once at 3,000 × *g* for 10 min at 4°C. The supernatant was filtered through a 0.45-μm-pore-size nonpyrogenic 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 a multiplicity of infection (MOI) of 5 50% tissue culture infective dose ($TCID_{50}$) units per cell, and infected cells were collected at 5 days p.i. The cells were resuspended in 0.1% SDS, incubated at 37°C 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 to 65% continuous sucrose gradient centrifugation. After two washes with deionized water, OBs were stored at 4°C until used. 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 37°C for 10 min. The solution was neutralized with a 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 × *g* for 2 min. The ODVs were collected by centrifugation at 20,600 × *g* for 25 min at 4°C.

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 (see Table S1 posted at http://edepot.wur.nl/143042). 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 His_6 tag. *Escherichia coli* BL21 cells were used to express the proteins after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The bacteria were collected by centrifugation, washed three times with phosphate-buffered saline (PBS), and resuspended in lysis buffer (0.1% SDS, 1% Triton X-100 in PBS [pH 7.4]). They were incubated at 37°C under gentle rocking for 30 min and sonicated. The resulting suspension was centrifuged at 3,000 × *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 95°C 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

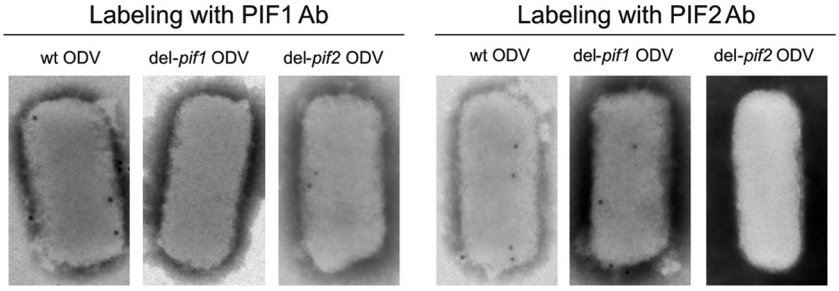

FIG. 2. Immunogold labeling analysis of PIF1 and PIF2 on the ODV surface. ODVs were purified and probed with PIF1 or PIF2 antibodies and then with goat-anti rat IgG conjugated with 10-nm gold particles. Samples were stained with 2% phosphotungstic acid and studied at a magnification of ×15,000. Ab, antibody.

(Aurion). Grids were then incubated for 1.5 h at room temperature with antibodies diluted 300 times in 10% blocking buffer, washed six times for 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. ODVs were observed with a JEOL 1011 transmission electron microscope. Five squares of each grid were arbitrarily selected, the numbers of gold particles on 10 intact ODVs per square were counted, and the average number of gold particles per virion was calculated. Experiments were repeated two times. A Mann-Whitney U test for pairwise comparison (SPSS Inc.) was performed to compare the data obtained with PIF1, PIF2, and PIF3 antibodies to determine the significance of the data.

Cross-linking analysis. To perform cross-linking, 1×10^8 OBs were treated with 2.4 ml of DAS buffer at 37°C for 10 min with gentle rotation. Neutralization with Tris solution was skipped to keep the ODVs under alkaline conditions. Insoluble debris was removed by centrifugation at $800 \times g$ for 2 min. Formaldehyde was added to the supernatant to a final concentration of 0.5%, and cross-linking was allowed for 30 min at 4°C under rotation. ODVs were collected by centrifugation at $20,600 \times g$ for 25 min at 4°C and resuspended in Laemmli buffer. The samples were heated at 65°C (for cross-linking) or 95°C (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 50°C or 95°C. Samples were separated by 12% SDS-PAGE. For nonreducing 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 95°C for 5 min.

Coimmunoprecipitation analysis of ODV membrane proteins. For each CoIP analysis, ODVs were purified from 2.5×10^8 OBs of wt or deletion mutant virus and resuspended in 550 μl of immunoprecipitation (IP) buffer (25 mM Tris, 150 mM NaCl, [pH 7.2]) containing 0.5% Triton X-100 and sonicated briefly. The suspension was incubated at 4°C for 2 h. Meanwhile, 20 μl of antiserum was mixed with a 25-μl bed volume of protein G-agarose (Pierce) in 500 μl of IP buffer and incubated at 4°C for 2 h. The protein G-agarose-antibody complex was collected by centrifugation at $500 \times g$ for 2 min and washed once with 1 ml of IP buffer. The ODV membrane protein suspension was centrifuged at $20,600 \times g$ for 20 min at 4°C, and 500 μl of supernatant was added to the IgG-protein G-agarose and incubated at 4°C overnight. The protein G-agarose was precipitated and washed three times with 1 ml of IP buffer, and captured proteins were eluted in Laemmli buffer by heating at 95°C for 5 min. The remaining 50 μl of the ODV membrane protein suspension was heated in Laemmli buffer and used as the input control. Samples were separated by 12% SDS-PAGE.

Western blot analysis. Western blot analyses were performed with antibodies against PIF1 or PIF2 (1:2,000 dilution), PIF3 (1:1,000 dilution), or P74 (1:50 dilution) using standard detection methods using alkaline phosphatase (19). P74 monoclonal antibody was kindly provided by Gary S. Blissard, the Boyce Thompson Institute at Cornell University (7).

Computational analysis. The transmembrane domains and orientations of PIFs were predicted by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). The cysteine disulfide bonding state prediction was performed with the PredictProtein server (http://www.predictprotein.org/). Cysteines that were predicted to form disulfide bonds with a confidence of >7 (0, low score; 9, high score) are indicated in a schematic representation in Fig. 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 recombinants (Fig. 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 with 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 *S. exigua* MNPV (20, 27), deletion of *pif1* and *pif2* did not significantly affect AcMNPV BV infectivity but led to a total loss of oral infectivity (data not shown). Reinsertion of *pif1* (del-rep-*pif1*, where rep indicates repair virus), *pif2* (del-rep-*pif2*), and *pif3* (del-rep-*pif3*) downstream of their own putative promoter regions 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, immunogold labeling of purified ODVs was performed using antibodies against each of these proteins (Fig. 2 and Table 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 1). With PIF1 antibody, low but significant numbers of gold particles were found on the ODV surfaces of wt (2.9 ± 0.21) and del-*pif2* (2.4 ± 0.26) viruses but not on the 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) and del-*pif1*

TABLE 1. Average numbers of gold particles on ODVs

Antibody type	Avg no. of gold particles (± SD) per ODV virion of:[a]			
	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
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.

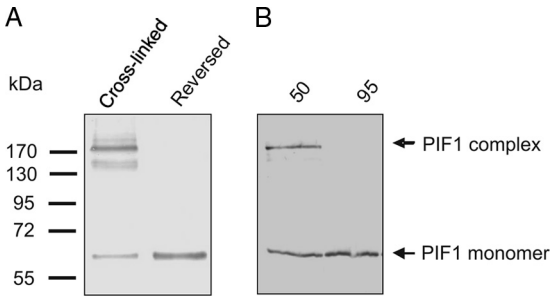

FIG. 3. Detection of the PIF 1-containing complex by Western blot analysis. (A) Cross-linking analysis of wt ODV. ODVs were purified and cross-linked with formaldehyde. Samples were then heated in Laemmli buffer at 65°C or at 95°C for 5 min for the cross-linked and reversed sample, respectively, before analysis by SDS-PAGE. (B) Detection of PIF1 at different temperatures without cross-linking. 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.

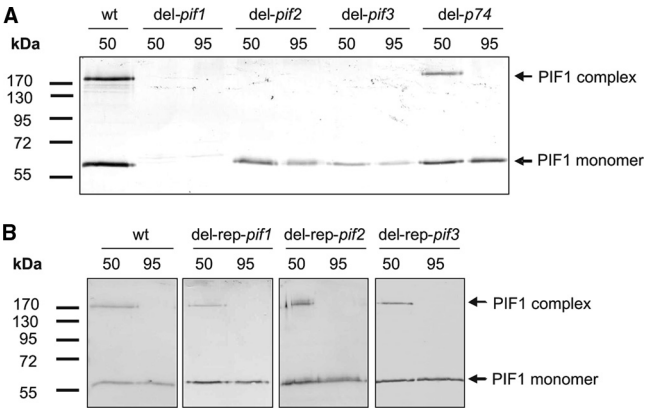

FIG. 4. Detection of the complex by Western blot analysis in deletion and repair viruses. (A) Absence of the complex in the deletion viruses del-*pif1*, del-*pif2*, and del-*pif3* but not in del-*p74*. (B) Detection of the complex in the repair viruses del-rep-*pif1*, del-rep-*pif2*, and del-rep-*pif3*. Wild-type (wt) virus was used as a 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.

(2.3 ± 0.31) virus but not on ODVs of del-*pif2* AcMNPV (0.2 ± 0.12) ($P < 0.05$). The distribution of gold 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 small amount of PIF proteins present in the ODV membrane, which has been reported before (3, 7, 14). 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) compared with labeling of del-*pif3* ODVs (0.4 ± 0.12) (Table 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.

Cross-linking reveals a complex containing PIF1. To investigate whether PIF proteins are present in the ODV membrane in the form of a complex, protein cross-linking with formaldehyde was carried out. The value of cross-linking with formaldehyde is that it is one of the shortest cross-linkers (2.3 to 2.7 Å), and therefore cross-linking between two proteins can occur only when they are in very close proximity, as one might expect for a native protein complex (28). After cross-linking, 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. 3A). In some experiments a fraction of PIF1 migrated at around 150 kDa (Fig. 3A). 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 cross-linking was reversed by heating, PIF1 migrated exclusively as a monomer. The same samples were probed with PIF2, PIF3, and P74 antibodies. For PIF2 and P74, cross-linking 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. In the case of PIF3, the cross-linked sample did not show a significant difference from the reversed sample (see Fig. 1 posted at http://edepot.wur.nl/143042).

The PIF1-containing complex is stable. Subsequently, it was noticed that the higher-molecular-mass complex (>170 kDa) is very stable. When cross-linking 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. 3B). 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 by Western blotting. As expected, no PIF1 was detected in the del-*pif1* ODV (Fig. 4A). For del-*pif2* or del-*pif3* ODV, only monomeric PIF1 was detected in samples treated at both 50°C and 95°C (Fig. 4A). However, for del-*p74* ODV, the complex was observed in the sample treated at 50°C 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 de-

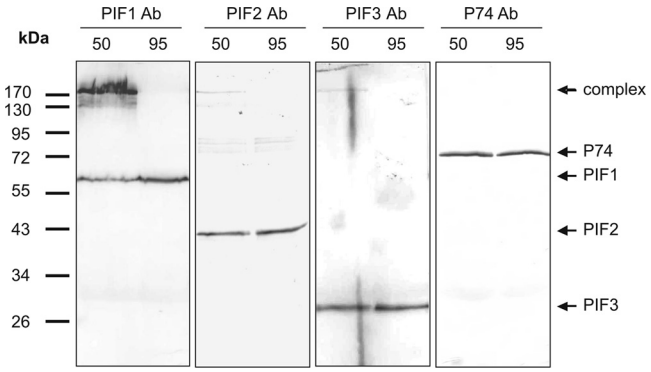

FIG. 5. Analysis of the complex on ODVs with antibodies against PIF1, PIF2, PIF3, and P74 without cross-linking. ODVs were purified and heated in Laemmli buffer at 50°C or 95°C for 5 min and then separated by SDS-PAGE, which was followed by Western blot analysis. Western blotting was performed with PIF1, PIF2, PIF3, and P74 antibodies (Ab). The locations of complex and monomeric forms of each protein are indicated. The predicted molecular masses 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.

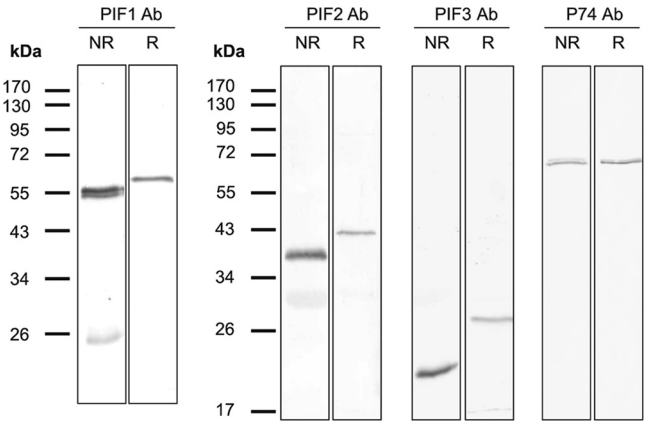

FIG. 6. Analysis of nonreduced and reduced forms of PIF proteins. ODVs were purified in the presence of iodoacetamide in DAS solution and heated in Laemmli buffer with (reduced; R) or without (nonreduced; NR) 5% ß-mercaptoethanol at 95°C for 5 min. Samples were separated by SDS-PAGE and probed with the indicated antibodies.

tected (Fig. 4B). 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. 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 previously (15). Whether this is due to posttranslational modification or to conformation of PIF3 is unknown yet. With P74 antibody, only monomeric P74 was detected in both the sample heated at 50°C and the one at 95°C. For detection with PIF1 antibody, the signal intensity of complex is comparable to that of the PIF1 monomer, but for detection with PIF2 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, parts 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 cross-linking analysis (see Fig. 1 posted at http://edepot.wur.nl/143042). Probably, cross-linking 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. Comparing the amino acid sequences of AcMNPV PIF1, PIF2, and PIF3 with those from other baculoviruses showed that the cysteines in all three proteins are highly conserved. To test whether potential disulfide bridges between these cysteines are responsible for the 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 prevent artificial disulfide bond formation (5), e.g., during ODV purification. Samples were treated with Laemmli buffer with (reduced) or without (nonreduced) 5% ß-mercaptoethanol and probed with PIF1, PIF2, PIF3, and P74 antibodies. As shown in Fig. 6 for PIF1, PIF2, and PIF3, the nonreduced 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 nonreduced samples may be due to formation of intramolecular 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 intramolecular disulfide bonds (Fig. 7). For PIF1 two forms, the doublet bands near the 55-kDa marker (Fig. 6), were detected in the nonreduced sample, which may be due to variation in the intramolecular disulfide bonds. A 25-kDa band was also observed. Whether this was due to breakdown of PIF1 is unknown yet. For P74, nonreduced and reduced protein migrated at the same rates. This is consistent with the fact that P74 does not contain cysteines, with the prediction confidence higher than 7 (Fig. 7). Notably, for PIF1, PIF2, and PIF3, detection in the nonreduced sample yielded a stronger signal than in the reduced sample although the same amount of protein was loaded. This may be due to the fact that the nonreduced proteins may contain conformational epitopes for the polyclonal antibodies generated in this study. Comparatively, detection of P74 with monoclonal antibody did not show a difference of signal intensities between the nonreduced and reduced samples. These results showed that the stable complex of PIF1, PIF2, and PIF3 is not dependent on disulfide bonds.

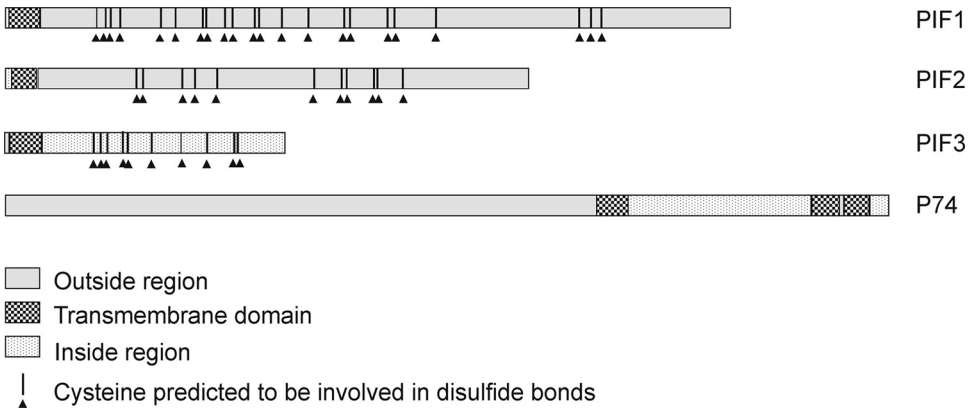

FIG. 7. Schematic structure of PIF1, PIF2, PIF3, and P74. The predicted transmembrane domains and orientations of these proteins are shown in different patterns. The cysteines in these proteins that are predicted to form disulfide bonds with high confidence are indicated.

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, the ODV of the corresponding deletion mutant, i.e., del-PIF1 for PIF1 antibody or del-PIF3 for PIF3 antibody, was used as a negative control. In CoIP of wt ODV membrane proteins with PIF1 antibody, PIF1, PIF2, and P74 were detected in the eluate by Western blotting (Fig. 8). In contrast, for the CoIP eluate of del-PIF1 ODV with PIF1 antibody, 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 in the eluate of CoIP with PIF1 antibody. Possibly the affinity of PIF3 antibody in the CoIP analysis is lower than that of PIF1 antibody, resulting in a 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 molecular mass (25 kDa) similar to that of 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 (6, 14, 20, 25). The fact that deletion of any of these proteins leads to a 3-fold reduction in binding efficiency compared to wt virus (10, 18) 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

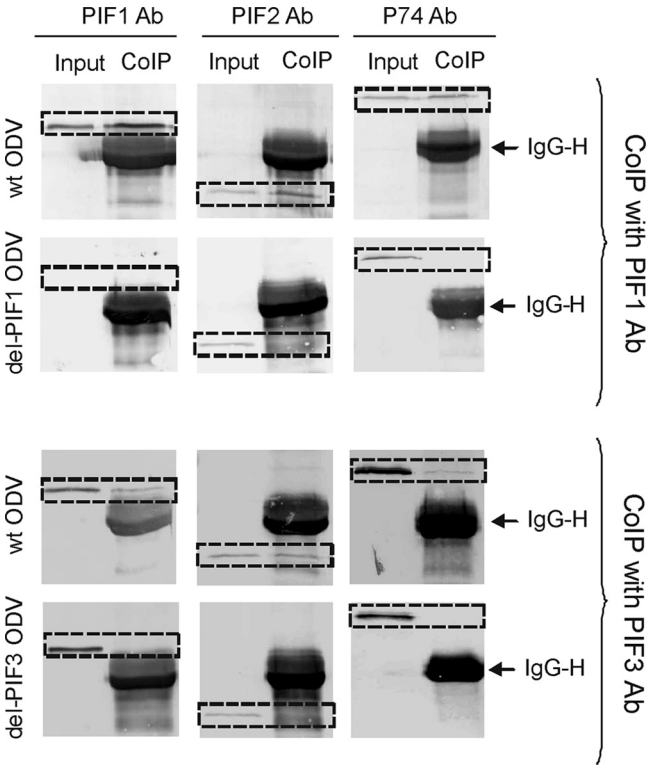

FIG. 8. Coimmunoprecipitation of PIF proteins. wt or corresponding deletion ODVs were purified, and membrane proteins were extracted. The membrane proteins were incubated with protein G-agarose bound to the indicated PIF IgGs. After a washing step, the captured proteins were collected. The input sample and the captured proteins were heated in Laemmli buffer at 95°C for 5 min and separated by SDS-PAGE. Western blot analysis was performed with the indicated antibodies. The positions of the heavy chains of rat/mouse IgG (IgG-H) are indicated. del-PIF1 ODVs were used as negative controls for CoIP with PIF1 antibody, while del-PIF3 ODVs were used as negative controls for CoIP with PIF3 antibody. The frames indicate detection of the PIF proteins.

suggested that P74 may be in a complex with other PIFs on the ODV surface (25). In this study, a PIF1-containing complex was first identified by cross-linking. 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 against PIF1, PIF2, and PIF3, but not P74, can recognize the same stable complex, indicating that the complex is composed of at least 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) (4). 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 (21). 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 3-fold (10, 18). In addition, these proteins do not have an apparent function in fusion as deletion of their genes had no notable effects on fusion efficiency (10, 18). Therefore, the PIF proteins may have additional functions in events further downstream. 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 (18). PIF3 has been suggested to function in the translocation of ODV nucleocapsids along the microvilli (24). 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 (18), 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 (8).

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. 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. 5). The first assumption was that the complex might be determined by disulfide bonding, which is a commonly observed link among stable protein complexes. To investigate this possibility, ODVs were treated under nonreducing conditions and analyzed by Western blotting. The high-molecular-mass complex was not observed in the nonreduced samples; instead, the nonreduced forms of PIF1, PIF2, and PIF3 migrated faster than the reduced forms, indicating the presence of intra- but not intermolecular disulfide bonds (Fig. 6). A 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 noncovalent bonds. These noncovalent bonds may confer on 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 larval 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 (26). 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. 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 on either the ODV surface or on nucleocapsids. Until now, the major part of PIF3 was predicted to be on the inside of the ODV membrane (24), 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 (19). 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 (7).

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 (13). Electron microscopic analysis suggested that both modes might be used for ODV binding and fusion (13). 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 seem 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 (3, 6, 11, 29). 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* to *pif3* and *p74*) have homologues in several other large invertebrate, nuclear-replicating DNA viruses such as nudiviruses (30), SGHVs (9), and polydnaviruses (2). In the last case, it has been hypothesized that the homologues have been derived from an ancient nudivirus (2). The conservation of the 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 the viruses and to gain further insights into the mechanism of baculovirus oral infectivity and the role the PIF proteins.

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