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Characterization of Novel Components of the Baculovirus *Per Os* Infectivity Factor Complex

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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 multimolecular 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 that 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. In order to understand the ODV entry mechanism, the identification of all components of the PIF complex is crucial. Hence, the aim of this study was to identify additional components of the PIF complex. Coimmunoprecipitation (CoIP) combined with proteomic analysis was used to identify the 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. P95 and P74 are more loosely associated with this complex. Three other proteins, AC5, AC68, and AC108 (homologue of SF58), were also found by the proteomic analysis to be associated with the PIF complex. Finally the functional significance of the PIF protein interactions is discussed.

In many cases, cell entry of enveloped viruses 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 virus entry complex/machinery (28). Entry of baculovirus occlusion-derived virus (ODV), an enveloped virion embedded in a viral occlusion body (OB) (30), 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) (8, 9, 11, 15, 19, 23, 31). 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 (8, 10, 19, 31). These PIFs might work in a synergistic way, and it was recently found that P74, PIF1, PIF2, and PIF3 form a complex (21). This complex very likely plays an essential role during the initial steps of ODV entry. Interactions of this complex with PIF4 and/or PIF5 and with other known ODV membrane proteins have 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 (32). All PIFs described so far also have homologs in nudiviruses (35) and in genomes of braconid wasps, which form polydnavirus virion structures (1). P74, PIF1, PIF2, and PIF3 are also conserved in the salivary gland hypertrophy viruses (SGHVs, family *Hytrosaviridae*) and in white spot syndrome virus of shrimp (family *Nimaviridae*) (34). This high level of conservation in a diverse range of large invertebrate DNA viruses suggests that the processes mediated by these proteins and their interactions are probably conserved during evolution.

ODV entry takes place in the alkaline environment of the insect larval midgut (25, 30). This is very different from the well-documented viral entry events under neutral or low pH conditions in vertebrates. Considering the unique alkaline condition during ODV infection and the high level of conservation of the PIFs among large, nuclear-replicating, invertebrate DNA viruses (baculoviruses, nudiviruses, salivary gland hypertrophy viruses, and white spot syndrome virus), 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 a prerequisite to know the components of the putative ODV entry complex composed of PIFs and, possibly, other proteins. In this study, coimmunoprecipitation (CoIP) followed by proteomic mass spectrometric analysis was used to identify components of the PIF complex. Blue native PAGE (BN-PAGE) and another CoIP analysis were 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 (21), 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.

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MATERIALS AND METHODS

Virus and insect cells. The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) E2 strain was used as the wild-type (WT) virus in this study. The AcMNPV bacmid (bMON14272) is derived from the Bac-to-Bac system (Invitrogen). The PIF4 deletion virus (del-*pif4*), PIF4 repair virus (del-rep-*pif4*-HA), in which the deletion was repaired with PIF4 with a C-terminal influenza hemagglutinin (HA) tag, and PIF1 deletion virus (del-*pif1*) used in this study were described previously (8, 21). 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 (7). Briefly, a zeocin resistance gene was amplified using two primers (5′-CAATTGCGGCGCGTGGACAACGTGCCAGACTTTAATTACCACACCAAGCATTCGGATCTCTGCAGCAC-3′ and 5′-TAAAAACAAGACCGCGCCTATCAACAAAATGATAGGCATTAACTTGCCGCTCGAGGTCGACCCCCCTG-3′) and 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 a 3′ flanking primer (5′-GGGTCTGGTTCCGTTGTC-3′) with a second zeocin primer (5′-CTGACCGACGCCGACCAA-3′). The del-*pif5* bacmid was provided with *polh* and green fluorescent protein (GFP) gene sequences by Tn*7*-mediated transposition (17) from plasmid pFAcT-GFP (6).

To tag the C terminus of PIF5 with the influenza HA epitope (CYPYDVPDYASL), *pif5* was amplified from bMON14272 with primers 5′-GCGGCATGCTACACAACAAATGCGCCTT-3′ and 5′-GCGGCGGCCGCTTAGGCGTAGTCGGGCACGTCGTAGGGGTATCGAGGGGCCGTTGTT-3′. The resulting *pif5*-HA PCR product, which encompasses the native *pif5* promoter and open reading frame (ORF), was digested with SphI and NotI and then cloned into the same sites of pFAcT-GFP-Tnie1p(A) (18). The resulting plasmid, pFActac148HA, which contained *pif5*-HA with a downstream *Trichoplusia ni* SNPV (single-capsid nucleopolyhedrovirus) *ie1* poly(A) signal, *polh*, as well as the gene for the GFP marker, was used to introduce *pif5*-HA, *polh*, and the GFP gene into the pif5-del bacmid as described above, finally resulting in the virus del-rep-*pif5*-HA.

SDS-PAGE and Western blot analysis. Protein sample treatment in Laemmli buffer at 50°C or 95°C, SDS-PAGE, and Western blot analysis were performed as previously described (21). 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 as follows: anti-ODV-E25 rabbit antiserum, 1:5,000 (26); anti-ODV-E56 rabbit antiserum, 1:2,000 (2); and anti-P95 rabbit antiserum, 1:4,000 (27). Dilutions of antibodies against PIF1, PIF2, PIF3 (all produced in rat), P74 (mouse monoclonal antiserum), and secondary antibodies conjugated with alkaline phosphatase were as previously described (21). The marker used for SDS-PAGE was Fermentas PageRuler prestained protein ladder number SM0671.

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 (21). 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. Nondissolved 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 X-100, 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% [wt/vol] 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 proteins purified from approximately 6×10^7 OBs. A NativeMark unstained protein standard from Invitrogen (number LC0725) was used as the marker.

Coimmunoprecipitation. Coimmunoprecipitations (CoIPs) of ODV membrane proteins were performed as previously described (21), 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, pH 7.2) containing 0.5% Triton X-100. The ODV membrane protein suspension was centrifuged at 20,600 × *g* for 20 min at 4°C. Simultaneously, 30 μl of PIF1 antiserum was incubated with 40 μl bed volume of protein G agarose in 1,000 μl IP buffer at 4°C for 2 h. The protein G agarose with captured anti-PIF1 IgG was collected and washed as described previously, divided between two Eppendorf tubes, mixed with either the WT or the del-*pif1* sample, and further processed as described previously (21). 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, four sample preparation replicates (four separate CoIPs with the same protein input) were performed.

For CoIP verification of the identified interactions using PIF1 antiserum or PIF1 preimmune serum, del-rep-*pif4*-HA ODVs were purified from 8×10^8 OBs and the ODVs were incubated in 1,000 μl IP buffer–Triton X-100 and processed as described above. Meanwhile, 15 μl of PIF1 antiserum or the PIF1 preimmune 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) were added to the collected IgG-protein G agarose. Further processing and sample preparation in Laemmli buffer were performed as reported previously (21). Samples were analyzed with SDS-PAGE followed by Western blot analysis.

Mass spectrometric analysis. Protein-G agarose beads were washed twice with 200 μl 50 mM ammonium bicarbonate (ABC, pH 7.8) to prepare samples for proteomic analysis after CoIP. The beads were supplemented with 15 μl 50 mM ABC (pH 7.8) containing 50 mM dithiothreitol and incubated at 60°C for 1 h. Afterwards, 18 μl of 50 mM iodoacetamide in 50 mM ABC (pH 8) was added and the sample was incubated at room temperature in the dark for 1 h, followed by the 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 pH 3. The peptides resulting from this digestion were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS-MS) as described previously (14).

The raw data files from the LTQ-Orbitrap were analyzed with MaxQuant software version 1.1.1.36 (4, 5), which also included label-free relative quantitation. The default MaxQuant 1.1.1.36 settings were used, except that asparagine and glutamine deamidations were added as variable modifications and the label-free quantitation and 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 in December 2010. Protein sequences of rat IgG and *Streptococcus* protein G were included. Next to those, a standard contaminants database was used,

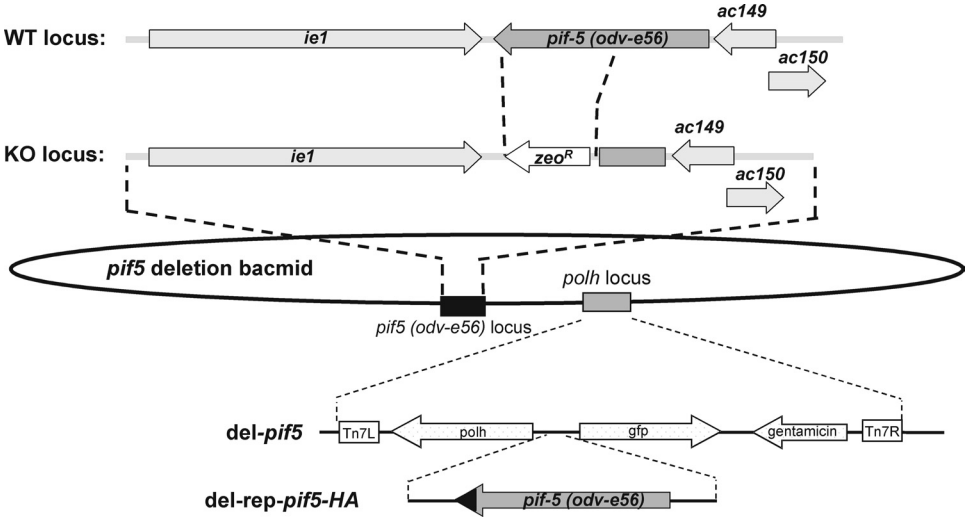

FIG 1 Schematic of the construction of the del-*pif5* and del-rep-*pif5*-HA bacmids. A major part of the *pif5* ORF was replaced by a zeocin resistance gene cassette via homologous recombination in *E. coli* to generate a *pif5* knockout bacmid. The lower part of the figure shows the genes inserted into 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.

which included, e.g., the following entries from the UniProtKB/Swiss-Prot database: 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). Decoy handling was also done with MaxQuant. Bioinformatic 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) (13).

RESULTS

Construction of a *pif5* deletion and repair virus. PIF4 and PIF5 are two recently identified PIFs in AcMNPV and, therefore, their potential interaction with the PIF complex was specifically investigated. Except for the *pif5* knockout and repair viruses, all other viruses used in this study have been described previously (8, 21). The del-*pif5* and del-rep-*pif5*-HA viruses were generated by first deleting the majority of the *pif5* ORF (*ac148*) from the AcMNPV bacmid by homologous recombination in bacteria, thereby replacing it with a cassette expressing the zeocin antibiotic resistance gene (see Materials and Methods and Fig. 1). The *pif5* knockout bacmid was supplemented with *polh* and the GFP marker to generate the del-*pif5* virus or with *polh*, *pif5*-HA, and the GFP marker to generate the *pif5* repair virus, del-rep-*pif5*-HA. The del-*pif5* OBs lacked oral infectivity, while the OBs of the *pif5* repair virus had oral infectivity comparable with that of the wild-type virus (data not shown).

Mass spectrometric analysis of the PIF complex. The ODV membrane fractions of AcMNPV WT and del-*pif1* were subjected to CoIP using PIF1 antiserum and analyzed by LC–MS-MS (Fig. 2). The proteins that were significantly enriched ($P < 0.01$) by the PIF1 antibody in the WT AcMNPV sample are marked in purple in Fig. 2, proteins that were not enriched are marked in blue, and contaminants are marked in yellow. For P74, PIF2, PIF3, and PIF4, enrichment factors of more than 30 (or $\log_{10}$ of 1.5) were observed. Four other viral proteins (AC5, AC68, P95 [AC83], and AC108) were also found to be significantly enriched, 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, 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 amount in the wild-type sample) may be due to contamination of the del-*pif1* sample with the WT virus sample during sample preparation. This contamination could not be detected by less sensitive methods, such as Western blotting (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. The presence of AC5, AC68, and AC108 in the complex was not further verified due to a lack of corresponding antibodies and recombinant viruses.

PIF4 and P95 are components of the PIF complex. BN-PAGE is a technique in which membrane protein complexes are charged with Coomassie brilliant blue and subsequently separated in a native gel. It has been proven to be a powerful technique to analyze membrane protein complexes in their native state and has been successfully used, for example, to identify mitochondrial and chloroplastic membrane protein complexes (24). Therefore, this method was chosen to analyze 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 analyzed by Western blotting with a panel of antibodies against P74, PIF1, PIF2, PIF3, HA (for PIF4 or PIF5), PIF5, P95, and ODV-E25.

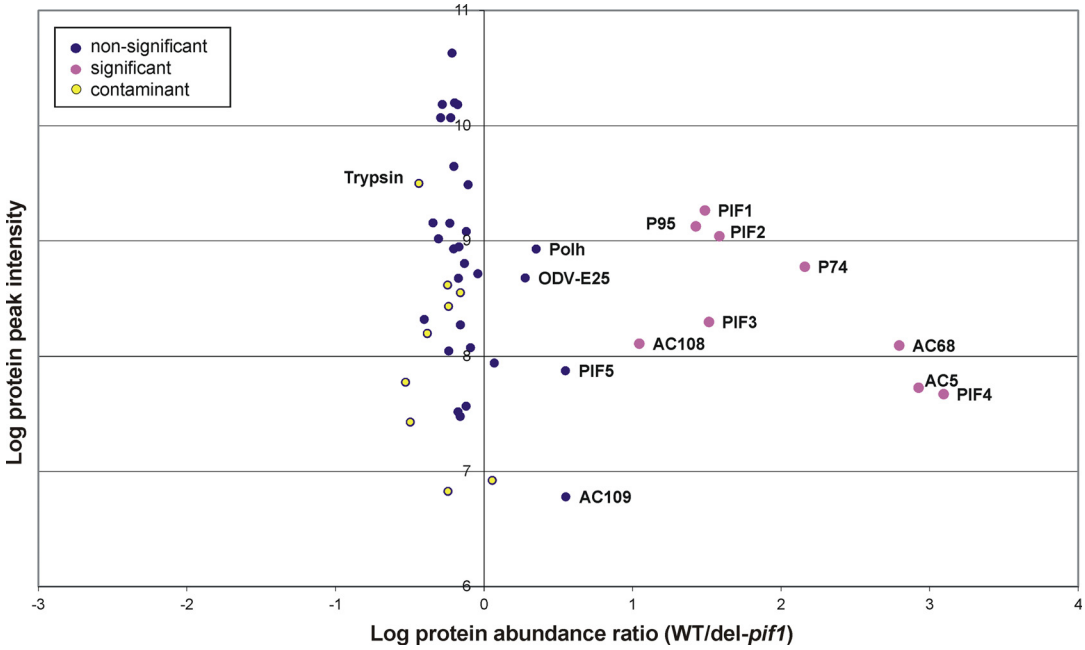

FIG 2 Mass spectrometric proteomic analysis of the PIF complex. ODV membrane proteins were prepared from wild-type or del-*pif1* virus and precipitated by PIF1 antiserum. The precipitation experiments were repeated 4 times from the same starting material, and precipitated proteins were analyzed with LC–MS-MS analysis using MaxQuant software. 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 compared to their levels in 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.

When proteins from the del-rep-*pif4*-HA virus were analyzed, antibodies against P74, PIF1, PIF2, PIF3, HA (detecting PIF4-HA), or P95 recognized one or more protein complexes with molecular masses of approximately 480 kDa (Fig. 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 analyzed (Fig. 3A, lanes 2), the ~480-kDa complexes were no longer

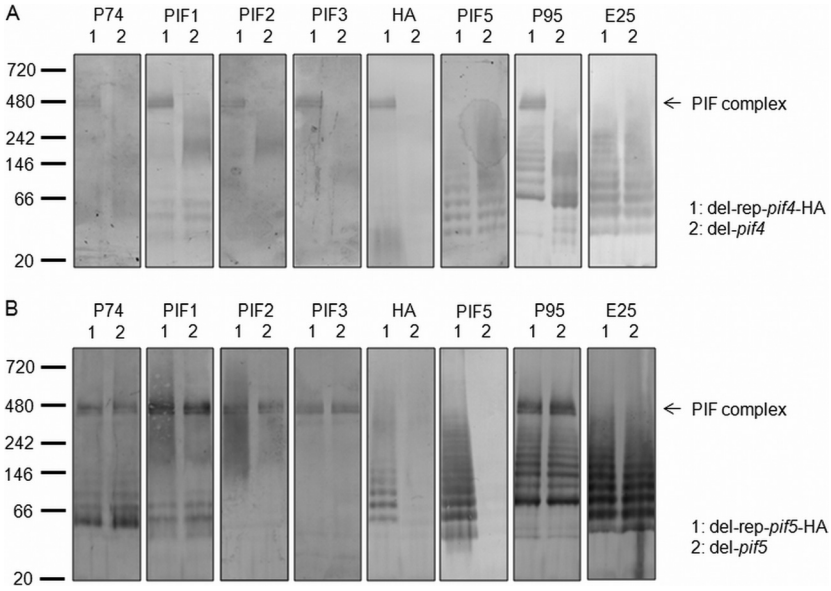

FIG 3 Blue native PAGE analysis of the PIF complex. (A) Analysis of PIF4. ODV membrane proteins were purified from del-rep-*pif4*-HA (lanes 1) or del-*pif4* (lanes 2) viruses and separated in BN-PAGE, followed by Western blot analysis with antibodies against P74, PIF1, PIF2, PIF3, HA (detecting PIF4-HA), PIF5, P95, or ODV-E25 as indicated above the panels. (B) Analysis of PIF5. ODV membrane proteins from del-rep-*pif5*-HA (lanes 1) or del-*pif5* (lanes 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 at the left.

present. 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 multimolecular complexes and that deletion of the *pif4* gene impaired complex formation. In contrast, PIF5 and ODV-E25 are not associated with the PIF complex. It is notable that in the del-*pif4* sample, PIF1 and PIF2 antibodies detected diffuse signals with identical migration rates between the marker proteins of 146 and 242 kDa (Fig. 3A). Whether these diffuse signals represent a subcomplex composed of PIF1 and PIF2 needs to be further verified. 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. 3B, lanes 1) and del-*pif5* viruses (Fig. 3B, lanes 2) were analyzed, 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 the migration patterns of PIF5 and ODV-E25 were the same as for del-rep-*pif4*-HA or del-*pif4* viruses. As expected, HA and 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.

Coimmunoprecipitation 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 these data, another CoIP analysis was performed. ODV membrane proteins were prepared from del-rep-*pif4*-HA virus and were precipitated with PIF1 antibody or PIF1 preimmune serum. Captured proteins were separated by SDS-PAGE and analyzed with P74, PIF1, PIF2, HA (for PIF4-HA), PIF5, P95, and ODV-E25 antibodies. PIF3 antibody was not included because PIF3 has a migration rate similar to that of 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, lanes 2) but not by the PIF1 preimmune serum (lanes 3). In contrast, PIF5 and ODV-E25 were not precipitated by the PIF1 antibody (lanes 2) and preimmune serum (lanes 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 the deletion of PIF4 results in stable subcomplexes. It was previously shown that PIF1, PIF2, and PIF3 form a complex that is stable upon heating in Laemmli buffer at 50°C but disintegrates at 95°C (21). 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 interacted more loosely with the complex (20, 21). 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 analyzed with P74, PIF1, PIF2, PIF3, HA (for PIF4), and P95 antibodies. In the samples of del-rep-*pif4*-HA heated to 50°C, the PIF1, PIF2, PIF3, and HA antibodies recognized the same ≈170-kDa protein complex (Fig. 5, lanes 1) as reported before (21). When the same sample was heated at 95°C, only monomers of PIF1, PIF2, PIF3, and PIF4-HA were detected (Fig. 5, lanes 2). When the sample of del-*pif4* virus was analyzed after heating at 50°C, the ≈170-kDa complex was not detected (Fig. 5, lanes 3). Instead, PIF1, PIF2, and PIF3 antibodies recognized smaller complexes (subcomplexes) with molecular masses of ≈150 kDa (Fig. 5, lanes 3). Again, when the same sample was heated at 95°C, only monomeric forms of PIF1, PIF2, and PIF3 were detected (Fig. 5, lanes 4). In del-*pif4* samples, HA antibody did not recognize any specific signal (Fig. 5, lanes 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. It should be noted that P95 migrated as a 115-kDa protein in the 10% SDS–PAGE (Fig. 5, blot probed with P95 antibody), while the predicted molecular mass is 96 kDa. This difference in molecular mass has been reported before (27) and is probably due to currently unknown posttranslational modifications.

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 subcomplexes (best detected by PIF1 antibody, as shown in lanes 3 of Fig. 5). It was noticed that PIF1 and PIF2 antibodies recognized the 150-kDa subcomplexes in del-rep-*pif4*-HA viruses in some of the experiments (Fig. 5, lanes 1), as was also observed in the previous study (21). These subcomplexes detected in del-rep-*pif4*-HA ODVs may be due to partial disassembly of the stable complex under denaturing conditions.

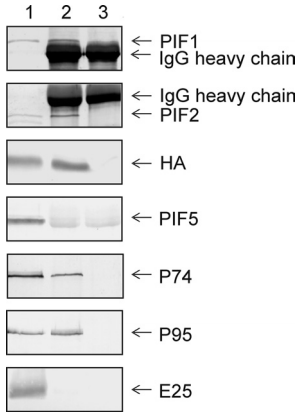

FIG 4 Coimmunoprecipitation analysis of the PIF complex. ODV membrane proteins were purified from the del-rep-*pif4*-HA virus (CoIP input, lane 1), and CoIP analysis was performed with PIF1 antibody (lane 2) or PIF1 preimmune serum (lane 3). CoIP input and captured proteins were heated in Laemmli buffer 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. The positions of precipitated proteins and the heavy chain of IgG are indicated by arrows.

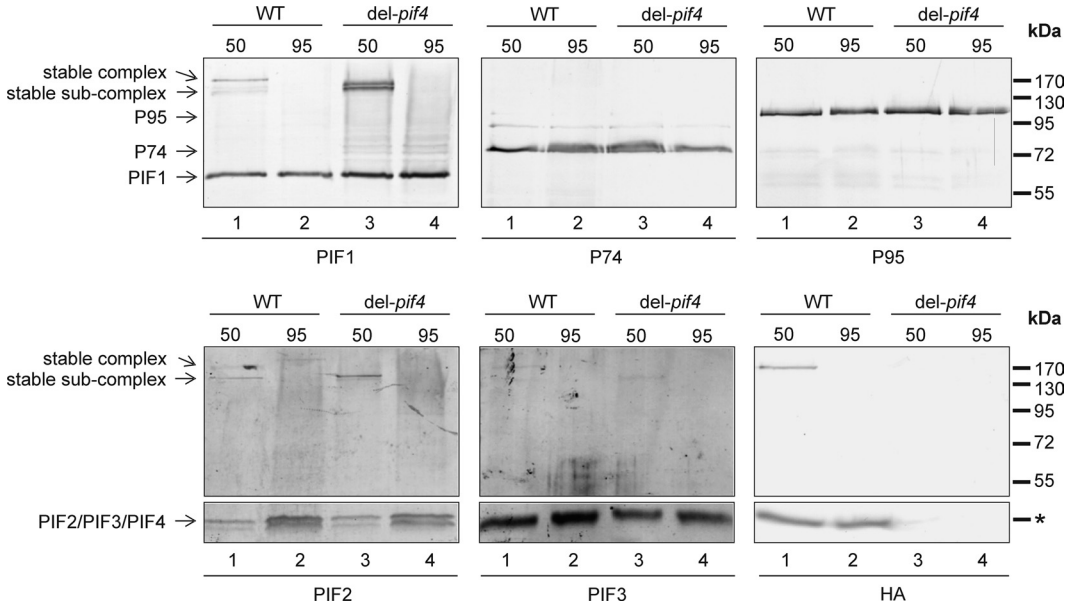

FIG 5 SDS-PAGE analysis of the stable complex. ODVs were purified from del-rep-*pif4*-HA or del-*pif4* viruses, heated in Laemmli buffer at 50°C or 95°C, and separated in 12% SDS-PAGE, followed by Western blotting with PIF1, PIF2, PIF3, HA (detecting PIF4-HA), P74, and P95 antibodies. Lanes 1, del-rep-*pif4*-HA 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; lanes 4, del-*pif4* ODVs heated at 95°C. Positions of the stable 170-kDa complex, the stable subcomplexes, and the monomeric forms of these proteins are indicated by arrows. Markers are indicated at the right. The blot sections indicated by an asterisk are the lower parts of the same original blots shown above them. WT, wild type.

DISCUSSION

The initiation of baculovirus infection in the midgut occurs under alkaline conditions and is mediated by at least 6 viral membrane proteins, P74, PIF1, PIF2, PIF3, PIF4, and PIF5 (ODV-E56) (8–10, 15, 19, 23, 31). The recent discovery that ODV-E66 is important for *per os* infectivity of *Bombyx mori* NPV (BmNPV) (36) further highlights the complexity of this process. All seven proteins are encoded by baculovirus core genes, suggesting 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 (21). 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. 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 subcomplexes, but the deletion of *pif1*, *pif2*, or *pif3* leads to complete disruption of the complex (21). Therefore, the stable complex can be further separated into two formats, with PIF1, PIF2, and PIF3 forming a stable core and PIF4 interacting strongly with this stable core. It has been shown before that the formation of the stable complex is not due to covalent disulfide bonds between these PIFs but, instead, should be the result of large numbers of weak noncovalent bonds (21). 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 nonspecific proteolytic cleavage in the potent digestive environment of the insect midgut (30).

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 that 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 open the possibility that the complex may also be involved in events downstream of fusion, i.e., transport of nucleocapsids. In favor of this view, the deletion of *pif3* does not affect ODV binding or fusion but oral infectivity is dramatically impaired (19). Further studies toward the functional analysis of the PIF complex, including the identification of host cell ligands for PIFs and the 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 BmNPV homologue (BmP95) can stimulate gene expression driven by its own promoter and that of the host actin gene (16). Conserved domain analysis (data not shown) identified a chitin-binding peritrophin A domain in AcP95 (amino acids 227 to 275), suggesting that P95 might have chitin-binding ability. Chitin is a component of the peritrophic membrane (PM), a semipermeable sheath that lines the insect midgut and protects the underlying

epithelium from mechanical damage, toxic compounds, and pathogens (12). The PM also serves as a barrier for ODV infection of the midgut epithelial cells. Chitin in the PM is synthesized by chitin synthase, which is membrane bound on midgut epithelial cells, and polymerizing chitin is secreted at the apical side of the epithelial cells to be incorporated into the PM (38, 39). Therefore, the potential chitin-binding ability of P95 might be involved in mediating interactions between ODVs and PM or between ODVs and epithelial cells, the target cells of ODV infection.

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 (25). Similarly, the 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 budded virus (BV) and ODV (27), but a recent proteomic analysis of AcMNPV BV structural proteins did not identify P95 (33). Further work with mutant *p95* ORFs is necessary to unravel its potential multifunctionality 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 analyzed by the other assays in this study. AC5 was identified as a structural protein of AcMNPV ODV (3) but not of BV (33). In addition, insertion/deletion mutagenesis of *bm134* (homolog of *ac5* in BmNPV) showed that it is not essential for virus replication in cell culture (25). These features appear to be consistent with the supposition that AC5 might have a role in ODV infection. AC68 and AC108 were identified as being associated with AcMNPV ODV in this study for the first time. Like AC5, they were not found in AcMNPV BV (33), 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 the deletion of this gene did not affect virus production *in vitro* but extended the 50% lethal time in bioassays in which BVs were injected into larvae (37). The same study also reported that deletion of *bm56* affected OB morphogenesis, leading to the production of OB-like structures that did not contain ODVs and, hence, were not infectious to larvae (37). However, more recently, we found that AC68 does not affect OB formation but is required for oral infectivity and is also a PIF (18a). 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 NPVs and all granulovirus (GV) genomes except that of *Plutella xylostella* GV (PlxyGV) (25). It seems to be essential, as viruses with an insertion in the BmNPV homolog (*bm91*) could not be isolated (25). Importantly, a recent study reported that SF58, homolog of AC108 in *Spodoptera frugiperda* multiple nucleopolyhedrovirus, is a new PIF (PIF6) (29). This discovery is consistent with our result that AC108 might be part of the PIF complex. Further work is needed to confirm the interactions of these three proteins with the PIF complex and to gain insights into 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 (21). The current study revealed more components of the PIF complex and showed that it contains a stable core composed of PIF1 to -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 works await the development of a cell line or *in vitro* system that can easily support ODV entry/infection.

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