Baculoviruses require an intact ODV entry-complex to resist proteolytic degradation of *per os* infectivity factors by co-occluded proteases from the larval host

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Abstract

Baculoviruses orally infect caterpillars in the form of occlusion-derived viruses (ODVs). The ODV-envelope contains a number of proteins which are essential for oral infectivity, called *per os* infectivity factors (PIFs). Most of these PIFs are involved in the formation of an ODV-entry complex that consists of a stable core, formed by PIF1, PIF2, PIF3 and PIF4, and the more loosely associated PIFs P74 (PIF0) and P95 (PIF8). PIF1, PIF2 and PIF3 are essential for formation of the stable core, whereas deletion of the *pif4* gene results in the formation of a smaller complex. P74 is not needed for formation of the stable core. We show here in larva-derived ODVs of the *Autographa californica* multicapsid nucleopolyhedrovirus that PIF-proteins are degraded by host-derived proteases after deletion of a single *pif*-gene. Constituents of the stable core-complex appeared to be more resistant to proteases as part of the complex than as monomer, as in ODVs of a *p74* deletion mutant only the stable core was found but no PIF monomers. When the stable core lacks PIF4, it lost its proteolytic resistance as the resulting smaller core complex was degraded in a *pif6* deletion mutant. We also identified PIF6 as a loosely associated component of the entry complex that appeared nevertheless important for the proteolytic resistance of the stable core, which was degraded after deletion of *pif6*. We conclude from these results that an intact entry-complex in the ODV-envelope is prerequisite for proteolytic resistance of PIF-proteins under the alkaline conditions of the larval midgut.

INTRODUCTION

Baculoviruses infect the larval stages of insects in the orders Lepidoptera, Hymenoptera and Diptera [1]. The name baculovirus refers to the bacillus-shaped nucleocapsid, in which a large circular, double-stranded DNA genome is packaged. The genome sizes range from 80 to 180 kbp, encoding 90–180 genes [2]. Baculoviruses have a biphasic replication cycle in which two virion-types are produced, first the budded virus particles (BVs) and later in infection occlusion-derived virus particles (ODVs). BVs consist of a single nucleocapsid which is enveloped by a membrane derived from the plasma membrane of the host cell. BVs establish systemic infection within the larvae after a primary infection. ODVs are of two types, single or multiple nucleocapsids in single capsid (SNPVs) and multicapsid nucleopolyhedroviruses (MNPVs), within an envelope derived from the inner nuclear membrane of the host cell. ODVs are occluded in a crystalline protein-matrix of polyhedral, forming occlusion bodies (OBs). When larvae consume OB-contaminated plant material, ODVs establish the primary infection in the insect. The OBs dissolve in the alkaline environment of the insect midgut to release the ODVs. The released ODVs then enter insect midgut epithelial cells by means of the activity of *per os* infectivity factors (PIF-proteins) which are present in the ODV-envelope. So far, nine different PIF-proteins have been identified: P74 or PIF0 (encoded by the *Autographa californica* (Ac) MNPV gene *ac138*), PIF1 (*ac119*), PIF2 (*ac22*), PIF3 (*ac115*), PIF4 (*ac96*), PIF5 (ODV-56/*ac148*), PIF6 (*ac68*), PIF7 (*ac110*) and P95 or PIF8 (*ac83*) [3–11]. Most of these PIFs have been shown to be part of an entry complex in the ODV-envelope that consists of a stable core complex and some more loosely associated components. The stable core complex is formed by PIF1, PIF2, PIF3 and PIF4 and resists treatment with Laemmli buffer containing 2 % SDS and 5 % 2-mercaptoethanol at 50 °C for SDS-PAGE analysis [12]. Of these components, PIF1, PIF2 and PIF3 appeared essential...
for formation of the stable core, while deletion of pif4 resulted in the formation of a smaller complex of only PIF1, PIF2 and PIF3 [13]. P74 and P95 have been shown to associate with the core-complex in a more loose way, as these components can only be detected as part of the entry complex under non-denaturing conditions [13]. P74 appeared not essential to form the stable core complex as this complex can be formed by a p74 deletion mutant [12, 13]. PIF5 has not been found in association with the entry complex [13]. Whether the other identified PIFs are part of the entry complex is currently not known.

Earlier studies reported proteolytic activity from co-occluded alkaline proteases in larva-derived OBs (L-OBs) for various baculovirus-host combinations [14–18]. The biological significance of this proteolytic activity is poorly understood, but it has been suggested that it may be important for optimal release of ODVs from the protein matrix of occlusion bodies, as significant amounts of the matrix protein polyhedrin was still associated with the ODVs after OB dissolution in alkaline buffer when these proteases were inhibited [18]. Although degradation of the matrix protein was most prominent, peptide analysis showed that proteins of the ODVs were also degraded during treatment with alkaline buffer [18]. Furthermore, it has been shown that P74 is cleaved into two fragments by an endogenous co-occluded protease when ODVs were released from OBs that were produced in Spodoptera exigua larvae [19]. P74 has not been found to be cleaved when the ODVs were isolated from cell culture-derived OBs (C-OBs), suggesting that the co-occluded protease was obtained from the larval host [19]. As well, cleavage of P74 was not observed when the L-OBs were heated prior to the release of the ODVs as a consequence of heat-inactivation of these proteases. These data suggest that the presence of co-occluded proteases in larva-derived OBs have significant effects on PIF-proteins as shown with P74.

Here, we study how co-occluded proteases affect PIF-proteins by comparing isolated cell-derived and larva-derived ODVs (C-ODVs and L-ODVs respectively) in SDS-PAGE analysis. By using wild-type and various pif deletion mutants for this analysis, we were able to compare the proteolytic sensitivity of PIF-proteins as part of the stable core-complex with monomeric PIF-proteins. This study revealed that deletion of only a single pif-gene resulted in enhanced proteolytic degradation of the remaining PIFs, regardless of whether the missing PIF is member of the stable core or a loosely associated component of the entry complex. So the entire entry complex, comprised of the stable core complex and loosely associated PIFs, needs to be intact to resist the proteolytic activity of co-occluded proteases from the larval host.

RESULTS

The PIF-core complex is affected by co-occluded proteases from the host

Peng et al. [12] showed that the PIF-core-complex appeared as a 170 kDa band in western blot analysis with PIF1 antiserum, when a protein sample of isolated C-ODVs (released from cell-culture derived OBs) was heated at 50 °C, instead of the 95 °C normally used for complete denaturation. On the other hand, the complex completely dissociates after denaturation at 95 °C. Here, we compared the stability of the core-complex in presence and absence of lepidopteran proteases. Therefore, we analysed the complex of wild-type AcMNPV in C-ODVs and L-ODVs [released from OBs produced by cultured insect cells (C) or S. exigua larvae (L)] after heating at 50, 60, 75 and 95 °C by western blot analysis with PIF1 antiserum (Fig. 1). In C-ODVs, the 170 kDa core-complex and the PIF1 monomers were found after heating at 50, 60 and 75 °C, while only PIF1 monomers were found when the sample was heated at 95 °C (Fig. 1a). These results indicate that the complex is very stable and only dissociates after heating at temperatures above 75 °C. However, with L-ODVs, degradation products of the core complex between 120 and 70 kDa were detected when the sample was heated at 50 °C and monomers were not found, probably because of the proteolytic activity of co-occluded proteases present in L-OBs that are activated during ODV-release in alkaline solution (Fig. 1b). When the protein sample was heated at 60 or 75 °C, the 170 kDa complex was found again, as were the PIF1 monomers. Possibly, the higher incubation temperatures sufficiently inactivated the proteases in the L-ODV protein sample. As the complex of C-ODVs and L-ODVs were more comparable when heated during sample preparation at temperatures above 50 °C, all further analyses were conducted at 60, 75 and 95 °C.

PIF1 is more resistant to proteolytic degradation as part of the core-complex than as monomer

To assess how proteases affect PIF-proteins during OB-dissolution in a pif-deletion mutant that still contains the 170 kDa core-complex in the ODV-envelope, C-ODVs and L-ODVs of a p74 deletion mutant were subjected to western blot analysis with PIF1 antiserum (Fig. 2). In C-ODVs, the complex and PIF1 monomers were found when heated at 60 and 75 °C, and the complex was fully dissociated into monomers when completely denatured at 95 °C (Fig. 2a), just as observed with the wild-type virus. In contrast, in L-ODVs of a p74 deletion mutant, only the core-complex was found when heated at 60 and 75 °C but not the PIF1 monomers (Fig. 2b). Even after complete dissociation of the core-complex at 95 °C, no PIF1 monomers were found. As it was suspected that the co-occluded proteases degraded the PIF1 monomers, the proteases were inactivated by heat-treatment of L-OBs at 80 °C for 40 min prior to ODV release in alkaline buffer. The PIF1 monomers were indeed detected again after heat treatment of L-OBs, further supporting the involvement of proteases in the degradation of PIF1 (Fig. 2c). After deletion of p74, PIF1 is apparently more sensitive in monomeric form to the co-occluded proteases than as part of the stable core complex, which is still formed in this deletion mutant.
PIF4 is important for the proteolytic resistance of the stable core-complex

Peng et al. [13] showed that deletion of pif4 resulted in the formation of a smaller core-complex of approximately 150 kDa, instead of the 170 kDa core-complex of the wild-type virus. To determine the impact of the co-occluded proteases on this smaller complex, C-ODVs and L-ODVs of a pif4 deletion mutant were analysed by western blot analysis with antiserum against PIF1. When C-ODVs of a pif4 deletion mutant were analysed, the approximately 150 kDa complex and PIF1 monomers were detected with expected sizes when heated at 60 °C (Fig. 3a). However, in L-ODVs, neither the smaller sized complex nor the PIF1 monomers were found after heating at 60 °C (Fig. 3, left panel). Only when the proteases associated with L-OBs were heat inactivated prior to L-OB dissolution in DAS-buffer, the ~150 kDa complex and PIF1 monomers were detected (Fig. 3b, right panel). Furthermore, when C-ODVs of a pif4 deletion mutant were analysed after heating at 75 °C, only PIF1 monomers were found and not the ~150 kDa complex. This was also observed in L-ODVs isolated from heat treated OBs. Thus the smaller complex that lacks PIF4 completely dissociates into monomers when heated at 75 °C. However, when the deletion mutant was repaired with a HA-tagged pif4 gene, the 170 kDa complex and PIF1 monomers were again detected in the ODVs after heating at 60 and 75 °C, even without prior heat inactivation of the co-occluded proteases in L-OBs (Fig. 3c). Apparently, the smaller complex is degraded more easily by these proteases than the ~170 kDa complex that still contains PIF4. These observations indicate that PIF4 is an important stabilising factor of the core-complex to resist proteolytic activity of host derived co-occluded proteases.

P74, PIF2 and PIF3 are also degraded by co-occluded proteases in L-ODVs of various pif-deletion mutants

The results above show that PIF1 monomers are prone to proteolytic degradation by co-occluded proteases in L-ODVs after deletion of p74- or pif4. To determine whether PIF1 monomers are also degraded in other pif-deletion mutants, ODVs of pif1-, pif2-, and pif3 deletion mutants were analysed for the presence of PIF1 monomers. As this research question only relates to PIF-protein monomers, all ODV-samples were fully denatured by heating at 95 °C and analysed by western blot analysis with antiserum against PIF1. In C-ODVs, PIF1 monomers were found in all pif deletion mutants except for the pif1 deletion mutant, while PIF1 monomers in L-ODVs were only detected in wild-type and the pif4 repair mutant (Fig. 4a, b). However with ODVs released from heat-treated L-OBs, the PIF1 monomers were found again in all pif deletion mutants, except the pif1 deletion mutant (Fig. 4c). Also some non-specific bands of unknown origin were found of approximately 50 kDa.
which are unlikely PIF1 degradation products as these bands were also detected in the pif1 deletion mutant. The L-ODVs released from L-OBs without prior heat treatment were also analysed with antiserum against P74, PIF2 and PIF3 to assess whether these PIFs are degraded as well (Fig. 5). Just as previously observed with PIF1, these PIFs were only found in L-ODVs of wild-type and pif4 repair viruses and not in the pif deletion mutants, indicating that these PIFs are degraded as well. In ODVs of wild-type and the pif4 repair mutant, PIF2 and PIF3 were detected at the

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expected sizes of 40 and 25 kDa respectively, P74 was detected at 35 kDa rather than 74 kDa due its cleavage by co-occluded host derived proteases as observed previously [19]. These data indicate that not only PIF1, but also P74, PIF2 and PIF3 are proteolytically degraded by host derived proteases in the various pif-deletion mutants.

PIF6 is a loosely associated component of the entry complex

Prior research identified the protein AC68 as a PIF-protein (denoted PIF6) and as a possible interaction partner of PIF1 [8, 13]. Here the envelope proteins of a HA-tagged pif6-repair mutant were analysed under denaturing and non-denaturing conditions to determine whether PIF6 is part of the core-complex or is a relatively loosely associated component of the entry complex similar to P74. Under denaturing conditions, when samples were heated at 60 °C, anti-HA antibodies detected PIF6 only as 15 kDa monomers and not in the complex (Fig. 6a, right panel). However, when the blot was treated with antiserum against PIF1, the ~170 kDa complex and PIF1 monomers were found as expected (Fig. 6a, left panel). PIF6 therefore does not appear to be part of the 170 kDa core-complex. When the ODV-envelope proteins of the HA-tagged pif6 repair mutant were analysed under non-denaturing conditions, a double band was detected with antiserum against PIF1 and antibodies against the HA-tag just below the 480 kDa marker, similar to what was observed with wild-type virus and anti-PIF1 antiserum in Blue native-PAGE gels (Fig. 6b). These results show that PIF6 is also a loosely associated component of the entry complex.

PIF6 is important for the proteolytic resistance of the core-complex against co-occluded proteases

As the PIF6 protein appears to be a loosely associated component of the entry complex, L-ODVs of a pif6 deletion mutant were analysed by SDS-PAGE and western blot analysis with and without prior heat treatment of L-OBs, to determine whether the presence of PIF6 is important for the proteolytic resistance of the core-complex and PIF monomers. When the co-occluded proteases in the L-OBs were inactivated by heat prior to dissolution, the core-complex and PIF1 monomers were found after heating of the isolated ODVs at 60 °C with antiserum against PIF1 (Fig. 7a). After heating at 95 °C, the complex was fully dissociated and only PIF1 monomers were found in the western blot analysis. When analysing L-ODVs without prior heat treatment, the anti-PIF1 antibodies only detected a pattern of degradation products below the 170 kDa marker when heated at 60 °C, and the PIF1 monomers were absent (Fig. 7b). When the complex was completely dissociated by heating at 95 °C, the PIF1 monomers were not detected either. However, when L-ODVs of the pif6-repair mutant were analysed, the core-complex (together with degradation products) and PIF1 monomers were detected at 60 °C, and only PIF1 monomers were detected upon heating at 95 °C (Fig. 7c). These results indicate that PIF6 is important for the proteolytic resistance of the core-complex, despite being a loosely associated component of the entry complex.

DISCUSSION

Various studies have shown that the proteins in ODVs released from L-OBs are affected by co-occluded host-derived proteases [15, 18, 19]. Here we show that the baculovirus ODV-entry complex, consisting of a stable core-complex and at least three different loosely associated PIFs (P74, P95 and now also PIF6), needs to be completely intact to resist proteolytic activity of these proteases during L-OB dissolution under alkaline conditions in vitro. Even in wild-type L-ODVs with an intact entry complex, we observed that the stable core-complex was largely degraded after

![Fig. 4. Western blot analysis of PIF1 monomers in ODVs of various pif deletion mutants, derived from cultured insect cells (C) and infected larvae (L). ODVs were purified from a wild-type (wt) construct, Δp74-, Δpif1-, Δpif2-, Δpif3-, Δpif4- and the pif6 repair mutant (rpif4HA), heated in Laemmli buffer at 95 °C for 5 min and separated by SDS-PAGE for western blot analysis with anti-PIF1 antiserum. The ODVs were released from C-OBs (a), L-OBs (b) or heat-inactivated L-OBs (c). In the analysis with C-ODVs and heat inactivated L-ODVs, some nonspecific bands were also detected in some samples but are from unknown origin. Detection of capsid protein VP39 with anti-VP39 antiserum was used as loading control (lower panel).](image-url)
treatment in Laemmli buffer at 50°C, whereas the core remained largely intact after heating at 60 or 75°C. This observation can be explained by dissociation of the loosely associated components of the entry-complex from the stable core due to the presence of SDS in the Laemmli buffer for SDS-PAGE analysis, leaving the core-complex more exposed to proteases. It also implies that the co-occluded proteases are still present in the protein sample after L-OB dissolution and that these are still active in SDS-containing Laemmli buffer at elevated temperatures. This is in accordance with previous reports about co-occluded proteases in various other baculoviruses, which showed that the proteolytic activity of the proteases was mainly associated with the virus particles [15], and that the proteases are thermostable and compatible with detergents such as SDS and reducing agents [15–17]. As such, when the isolated L-ODVs were incubated in Laemmli buffer at 50°C, the proteases were likely still active and degraded the exposed core-complex and dissociated PIFs. In contrast, when the L-ODVs were heated at 60 or 75°C, the proteases appeared sufficiently inactivated as both the core-complex and PIF monomers were found again, as they were with C-ODVs. Consequently, to assess only the effects of the co-occluded proteases on PIFs during OB-dissolution in DAS-buffer (mimicking the alkaline environment of the larval midgut), we heated the protein samples at 60°C or higher to prevent further degradation during SDS-PAGE sample preparation. Although these experimental conditions are not biologically relevant, they can nevertheless be used to assess the importance of the different components of the entry complex with regard to the proteolytic activity of co-occluded proteases during OB dissolution in the larval midgut. The presented results provide some insight in the properties of the co-occluded proteases in L-OBs, but further characterisation of these proteases is still necessary to unravel the biological consequences and importance of their activities.

Analysis of a p74 deletion mutant initially suggested that PIFs in the core-complex are more resistant to co-occluded proteases than as monomers, as only the stable core-complex and not the PIF1 monomers were found in ODVs

Fig. 5. Western blot analysis of PIF-protein monomers on ODVs of various pif deletion mutants, released from L-OBs. ODVs were purified from L-OBs without prior heat treatment of a wild-type (Wt) construct, Δp74-, Δpif1-, Δpif2-, Δpif3-, Δpif4 mutants and the pif4 repair mutant (rif4HA), heated in Laemmli buffer at 95°C for 5 min and separated by SDS-PAGE for western blot analysis with antiserum against P74 (a), PIF2 (b) and PIF3 (c). Detection of the major capsid protein VP39 with anti-VP39 antiserum was used as loading control (d).
without prior heat treatment of the L-OBs. In contrast, the stable core-complex was found degraded in the pif4 and pif6 deletion mutants, indicating that the core-complex requires these components to maintain proteolytic resistance. It was previously shown that deletion of pif4 resulted in the formation of a smaller core-complex of PIF1, PIF2 and PIF3 [13]. Apparently, PIF4 is not essential for forming the stable core, but it does seem important for the resistance of the core-complex against the proteolytic activity of co-occluded proteases. In contrast to PIF4, PIF6 proved not to be part of the core-complex but was identified as a loosely associated component of the entry complex. Despite this more loose association with the core-complex, PIF6 appeared to be more important for the proteolytic stability of the core-complex than P74. Apparently, the importance of the various PIF-proteins for the proteolytic resistance of the core-complex can differ independently of whether the missing PIF-protein is a core component or a loosely associated component of the complex. To better understand this aspect, further information is needed about the protein-protein interactions in the ODV entry complex and the stoichiometry of the individual components.

The importance of an intact entry complex in the ODV-envelope for the proteolytic resistance of PIF-proteins can also be nicely illustrated by the current observations on P74 monomers. P74 has previously been shown to be cleaved into two fragments of approximately 35 and 37 kDa in L-ODVs [19]. However, current study showed that deletion of any pif-gene affected proper cleavage of P74 and resulted in proteolytic degradation of this PIF-protein instead. Apparently, the protein-protein interactions in the entry complex ensures that P74 is cleaved only at the directed cleavage site and prevent total degradation, but when a PIF-protein is missing due to deletion of a pif-gene, co-occluded proteases are able to degrade P74 along with the other PIF-proteins.

The observations presented in this study indicate that deletion of a pif-gene affects the structure of the entry-complex which consequently leads to degradation of the remaining PIF-proteins by most likely co-occluded alkaline proteases during OB-dissolution. This finding conflicts with previous observations on binding and fusion properties of pif1-, pif2- and pif3 deletion mutants of AcMNPV with midgut epithelial cells in Heliothis virescens larvae [9]. These authors showed that deletion of pif1 or pif2 compromised binding and fusion of the L-ODVs with epithelial cells in the larval midgut, while the pif3 deletion mutant was still functional in terms of binding and fusion. In addition to PIF1 and PIF2, P74 has also been shown to be crucial for binding and
fusion of ODVs to epithelial midgut cells [20]. However, our study indicates that deletion of pif3 results in proteolytic degradation of P74, PIF1 and PIF2 under alkaline conditions, which possibly affects the binding and fusion capacities of ODVs in the larval midgut. Furthermore, PIF3 has been shown to be essential for formation of the stable core complex and was also shown to interact with P74 in AcMNPV [12]. A recent yeast-two-hybrid study suggests that the interaction between PIF3 and P74 is indirect, but this needs to be validated by further experiments [21]. Nevertheless, deletion of pif3 results in the inability to form the entry complex as a consequence of lost protein-protein interactions between the PIFs known to be involved in binding and fusion, and might additionally be degraded by co-occluded proteases. Consequently, it can be hard to draw firm conclusions about the involvement of a single PIF during the early stages of oral infection by making pif deletion mutants as the remaining PIFs in the ODV-envelope are severely affected by loss of protein-protein interactions and presence of host derived co-occluded proteases.

Conclusions
This study demonstrates the importance of an intact ODV entry complex to resist proteolytic activity of host-derived proteases that are co-occluded in L-OBs, as upon deletion of a pif gene the remaining PIFs are more sensitive to proteolytic degradation. This observation could be biologically relevant as in the alkaline environment of the midgut, the co-occluded proteases can be activated as did the alkaline buffer in our in vitro experiments. But even when the co-occluded proteases are not activated in the larval midgut, proteolytic resistance is still important as the alkaline proteases normally present in the midgut lumen can supplement the co-occluded proteases. Thus, proteolytic resistance against proteases can be considered as an important selective pressure, explaining the high level of conservation of PIF-proteins among the different baculoviruses in order to maintain complex formation and preserve oral infectivity.

METHODS

Culture of insect cells and lepidopteran larvae
Sf21 cells were cultured in T25 flasks with Graces medium (Fisher N.V.), supplemented with 10% fetal bovine serum (FBS) and 50 µg ml⁻¹ gentamycin. Sf21 suspension cultures were grown in SF900II medium (Fisher N.V.) supplemented with 5% FBS and 50 µg ml⁻¹ gentamycin. Spodoptera exigua larvae were reared on artificial diet in a 26 °C climate room with 40% humidity and a 16 : 8 h (light : dark) photoperiod.

Bacmids
The AcMNPV bacmid bMON14272 was derived from the Bac-to-Bac system (Invitrogen; Luckow et al., 1993). The construction of the bacmids with the individual pif-gene
deletions (del-p74, del-pf1, del-pf2 and del-pf4) has been described previously [12, 13, 19]. The pf3 deletion bacmid was kindly provided by Dr. Xinwen Chen of the Wuhan Institute of Virology [22]. The construction of the HA-tagged pf4 repair bacmid has also been described previously [3, 13]. The bacmids of the Ac68 (pf6) deletion mutant and the corresponding HA-tagged repair construct has also been described previously [8]. In all deletion and repair bacmids, the polyhedrin gene was restored to enable the resulting virus to form OBs. The polyhedrin (polh) gene was restored by using the Bac-to-Bac transposition protocol with a pFastBacDual vector (Invitrogen) from which the p10 promoter was deleted and the ORF of the polh gene was inserted behind the polyhedrin promoter as previously described [12]. The pf ORFs needed to repair the deletion bacmids were introduced simultaneously with the polh gene and were placed under control of their native promoters.

**Virus proliferation and purification**

SF21-cells were transfected with the bacmids using Express2TR reagent (Express2ion Biotechnologies) to generate first generation BV-stocks. Subsequently, the obtained BV-stocks were amplified in SF21 cells by an additional round of infection at low MOI. Cell-derived OBs (C-OBs) were generated by infecting a 80 ml suspension cultures of SF21-cells at 1.5×10⁶ cells ml⁻¹ with 1 ml of amplified BV-stock (approximate MOI of 1) and incubated for a week. After a week of incubation, the cells were pelleted by centrifugation at 4000 g for 30 min. The cells were suspended in 0.1 % SDS dissolved in demi water and incubated for 2 h at 37 °C with gentle agitation. Then the cells were sonicated for 1 min at 7 Watt. Total cell lysis was verified by light microscopy and the OBs were pelleted by centrifugation at 4000 g for 30 min. Larva-derived OBs (L-OBs) were obtained by injection of 1 µl of the amplified BV-stock into the haemocoel of L4 Spodoptera exigua larvae, using an Humapen Luxura insulin pen (Lilly) (TCID₃₀=5×10⁻²⁻¹×10⁸ per ml). Three to four days post injection, the liquefied larvae were collected, homogenized in sterilized water and filtered through two layers of cheese cloths. The debris in the flow-through was pelleted by centrifugation at 500 g for 10 min and the L-OBs in the supernatant were subsequently harvested by centrifugation at 4000 g for 30 min. The pellet of the first centrifugation step, containing the cell debris, was washed multiple times with water followed by centrifugation to further purify the OBs. Finally, the purified OBs were washed twice in sterilised water. Both C-OBs and L-OBs were further purified by centrifugation over a 30–60 % (w/w) sucrose gradient in a Beckmann SW32 rotor at 90 000 g for 1 h at 4 °C. The band with OBs was withdrawn from the gradient with a Pasteur pipette and subsequently pelleted by centrifugation at 4000 g for 30 min.

**SDS-PAGE and Western blot analysis**

ODVs were released from approximately 3.0×10⁸ OBs by treatment with DAS buffer (0.1M Na₂CO₃, 166 mM NaCl and 10 mM EDTA, pH 10.5) for 10 min at 37 °C. When endogenous alkaline proteases needed to be inactivated, 1-OBs were heated at 80 °C for 40 min prior to dissolution in alkaline DAS-buffer. After verification of OB dissolution by light microscopy, the ODV suspension was neutralised with 1/10 vol of 0.5M Tris-HCl pH 7.5. Non-dissolved debris was removed by centrifugation at 1500 g for 2 min and the ODVs in the supernatant were pelleted by centrifugation at 20 800 g for 25 min at 4 °C. The ODVs were resuspended overnight with gentle rotation at 4 °C in 0.1× TE buffer, supplemented with Complete Protease Inhibitor Cocktail (Roche). Then 4× Laemmli buffer was added to the ODV-suspension which was then heated at 50, 60, 75 or 95 °C for 5 min. The samples were separated in a 12 % SDS-PAGE gel and blotted onto a PVDF membrane (Millipore Immobilon-P). Western blot analyses with antisera against various PIF-proteins were performed as previously described in [12]. In brief, anti-P74 antiserum (1 : 1000 dilution), produced in rabbit, and anti-PIF1 (1 : 2000 dilution), anti-PIF2 (1 : 1000 dilution) and anti-PIF3 antiserum (1 : 1000 dilution), all produced in rats, were used as primary antibodies. To detect the HA-tagged PIF6 in the pf6-repair mutant, anti-HA antibodies were used as primary antibody (1 : 2000; Roche 3F10). Detection of capsid protein VP39 with anti-VP39 antibodies (1 : 1000), produced in mice, was used as loading control. Goat anti-rabbit (1 : 2000 dilution; Dako), goat anti-rat (1 : 2000 dilution; Sigma A8438) and goat anti-mouse antibodies (1 : 2000 dilution; Sigma A5153), conjugated to alkaline phosphatase, were used as secondary antibodies to detect the proteins of interest by conversion of NBT-BCIP substrate (Sigma) in a blue-purple coloured precipitant in AP-buffer (0.1M Tris-HCl, 0.1M NaCl, 5 mM MgCl₂, pH 10.5).

**Blue-native PAGE**

ODVs of 3.0×10⁸ OBs were isolated as described above. The ODV pellet was resuspended in 100 µl extraction buffer (6.25 mM Tris, 37.5 mM NaCl, 0.5 % Triton-X100, pH 7.2) and incubated overnight at 4 °C with gentle rotation to extract the ODV envelope proteins. The envelope fraction was separated from the nucleocapsids by centrifugation at 16 000 g for 30 min at 4 °C. The supernatant containing the envelope fraction, was collected and mixed with 4x BN-PAGE sample buffer (200 mM Bis-Tris, 64 mM HCl, 200 mM NaCl, 40 % glycerol, 0.004 % Ponceau S, pH 7.2). The protein samples were supplemented with 5 % Coomassie G-250 to a final concentration of 0.1 %. Electrophoresis of 4–16 % Bis-Tris gradient gels was performed as described in the manual for the NativePAGE Novex Bis-Tris Gel system (Invitrogen). NativeMark unstained protein ladder (Invitrogen LC0725) was also stained with Coomassie and used as marker. Blotting to a PVDF membrane was performed with NuPAGE Transfer buffer (Invitrogen) according to the manufacturer’s protocol.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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