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Specific Binding of *Autographa californica M* Nucleopolyhedrovirus Occlusion-Derived Virus to Midgut Cells of *Heliothis virescens* Larvae Is Mediated by Products of *pif* Genes *Ac119* and *Ac022* but Not by *Ac115*

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Per os infectivity factors PIF1 (Ac119) and PIF2 (Ac022), like P74, are essential for oral infection of lepidopteran larval hosts of *Autographa californica M* nucleopolyhedrovirus (Ac*M*NPV). Here we show that Ac115 also is a PIF (PIF3) and that, unlike PIF1 and PIF2, it does not mediate specific binding of Ac*M*NPV occlusion-derived virus (ODV) to midgut target cells. We used an improved in vivo fluorescence dequenching assay to compare binding, fusion, and competition among control Ac*M*NPV ODV and the ODVs of Ac*M*NPV PIF1, PIF2, and PIF3 deletion mutants. Our results showed that binding and fusion of PIF1 and PIF2 mutants, but not the PIF3 mutant, were both qualitatively and quantitatively different from those of control ODV. Unlike control and PIF3-deficient ODV, an excess of PIF1- or PIF2-deficient ODV failed to compete effectively with control ODV's binding to specific receptors on midgut epithelial cells. Moreover, the levels of PIF1- and PIF2-deficient ODV binding were depressed threefold compared to control levels. Binding, fusion, and competition by PIF3-deficient ODV, however, were all indistinguishable from those of control ODV. These results implicated PIF1 and PIF2 as ODV envelope attachment proteins that mediate specific binding to primary target cells within the midgut. In contrast, PIF3 mediates another unidentified, but critical, early event during primary infection.

Autographa californica M nucleopolyhedrovirus (Ac*M*NPV) (family *Baculoviridae*, genus *Nucleopolyhedrovirus*) produces two viral phenotypes during productive infection: occlusion-derived virus (ODV) and budded virus (BV). Each viral phenotype has a specific biological function. ODV establishes the first round of infection within its larval lepidopteran host, and BV, produced by the ODV-infected midgut cells, transmits infection to juxtaposed tracheal cells that service them (13, 38–41). Subsequent generations of BV transmit infection throughout the host until ultimately the insect dies and liquefies, releasing millions of viral occlusions (1).

Each viral phenotype performs its function under extremely different environmental conditions and targets different cell types. BV is a generalist; it infects a broad spectrum of cell types and circulates within a slightly acidic (pH 6.4 to 6.8), protein-rich medium called hemolymph (5). BV is the more infectious of the two phenotypes in cell culture, and for Ac*M*NPV, this difference is ~1,700-fold (on a PFU/pg viral protein basis) (37). Accordingly, most of what we understand about baculovirus infection pertains to BV in cell culture.

In contrast to BV, ODV is a specialist. In nature, it only infects columnar (and less frequently undifferentiated) epithelial cells within the larval midgut. Upon being ingested by feeding lepidopteran larvae, the occlusions encounter highly alkaline midgut fluids (pH 8.7 to 11) that induce their rapid dissolution and liberate the ODV. The ODV, bathed in harsh alkaline digestive fluids, must make contact with the microvillar membrane of columnar cells in order to establish infection. Access to columnar cells is limited, however, by the peritrophic membrane, a secreted extracellular matrix that borders the midgut epithelium. The microvillar tips of columnar cells can penetrate through this structure, however, and become exposed to the luminal contents (2). This condition may explain, at least in part, why columnar cells are accessed by ODV through the distal ends of their microvilli. ODV entry, clearly documented by electron microscopy, occurs by fusion of the viral envelope with microvilli (1, 15, 21, 33). How ODV gains functional entry at this location in the absence of apparent viral fusogenic machinery and how nucleocapsids are subsequently transported basally through microvilli packed with a bulky F-actin core and cross filaments (9) are still open questions. Elucidation of the roles of a few highly conserved "per os infectivity factor" genes (the *pif*s), essential for ODV infectivity but completely dispensable for BV infectivity (28), should shed some light on these processes.

The absence of any one of the *pif* gene products leads to a block in infection prior to viral gene expression in midgut cells (14, 17, 23, 28). These data indicate that the PIFs are structural proteins involved in early infection events. Ohkawa identified four *pif* genes in *Bombyx mori* NPV by screening a library of over 150 viral mutants, each generated by the removal of a large fragment of an individual open reading frame (ORF) and subsequent insertion of a *lacZ* reporter cassette in its place (28). Those mutants that retained viability in cell culture were further tested in insect larvae both orally (occlusions) and intrahemocoelically (BV). Of the four *pif* genes identified in

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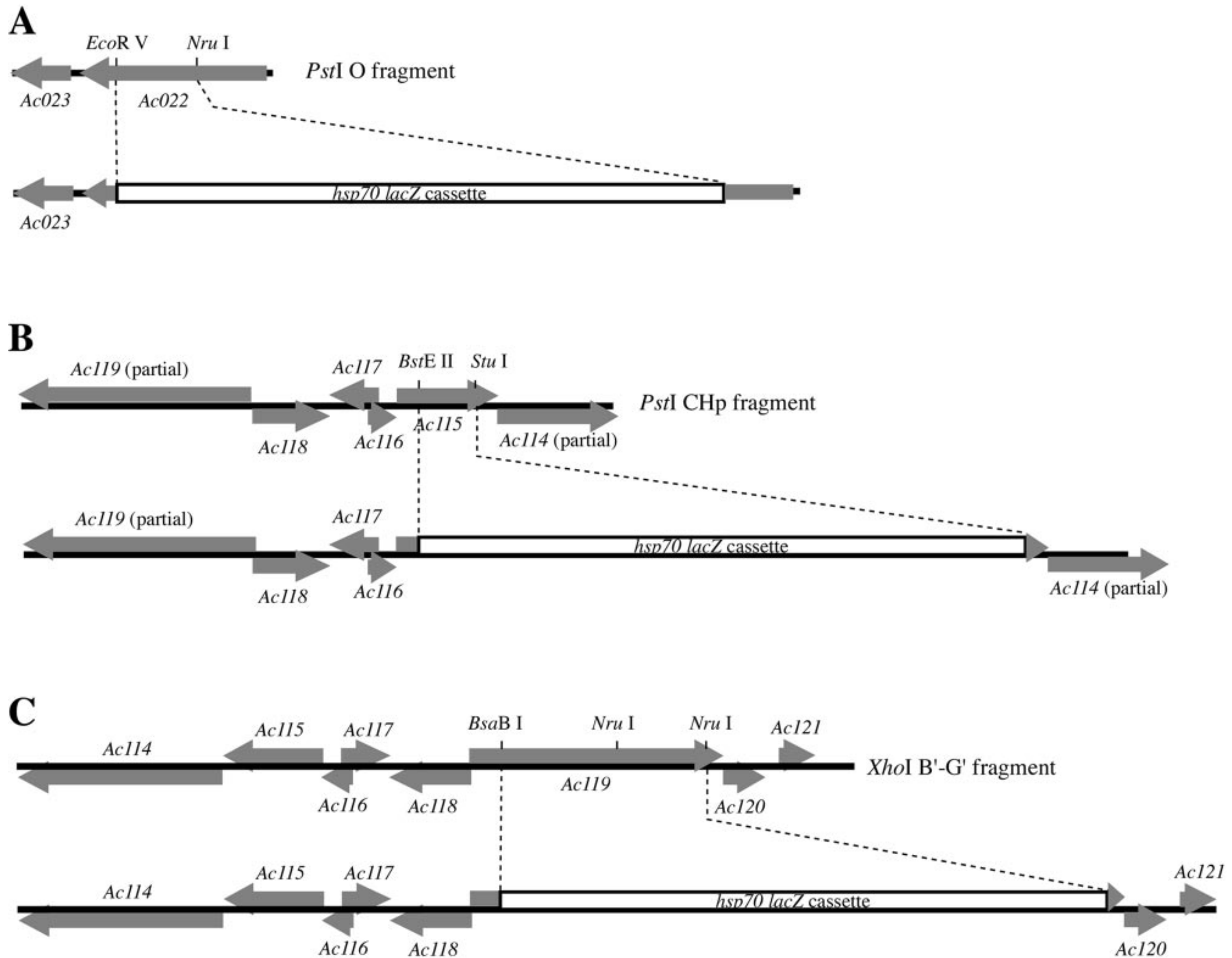

FIG. 1. Construction of plasmids used for making Ac*M*NPV *pif* gene mutants. A cassette containing the β-galactosidase reporter gene driven by the *Drosophila hsp70* promoter was inserted into ORFs *Ac022* encoding PIF2 (A), *Ac115* encoding PIF3 (B), and *Ac119* encoding PIF1 (C) at the locations indicated. Subfragments containing the relevant *pif* gene were cloned and exposed to pairs of restriction enzymes as follows: EcoRV and NruI for *Ac022*, BstEII and StuI for *Ac115*, and BsaBI and NruI for *Ac119*.

this screen, one (*p74*) had been described previously (23) and two (*pif* and *pif2*) have by now been described by others in different systems (22, 29). The fourth *pif* gene, however, *Ac115* (and homologues), is presented here for the first time. We propose to designate *Ac115* (and homologues) *pif3* and to add a "*1*" to *pif* (*pif1*) to normalize the nomenclature.

Thus far, P74 is the only PIF protein that has been assigned a function: specific binding of ODV with midgut columnar cell microvilli (17, 45). Using evidence generated with an improved in vivo fluorescence-dequenching assay, we show that PIF1 and PIF2 also are involved in initial binding of ODV to midgut cells, whereas PIF3 is not. PIF3, therefore, must mediate another critical early event during primary infection.

MATERIALS AND METHODS

Inoculation and maintenance of test larvae. For all experiments, we used fourth instar larvae of *Heliothis virescens*, *Trichoplusia ni*, or *Spodoptera exigua* reared from eggs provided by the USDA Western Cotton Research Laboratory, Phoenix, AZ. All larvae were reared in groups at 28 ± 2°C on a modified wheat germ diet (Stoneville) until the onset of quiescence at the end of the third instar, indicative that larvae were preparing to molt to the fourth instar. For some experiments, large numbers of quiescent third instars were held between 4 and 15°C for a maximum of 12 h until a sufficient number of the same developmental stage were available for testing. Each larva was individually inoculated with occlusions, ODV, or BV in 0.5- to 2-μl aliquots using a microapplicator (Burkhard) fitted with a blunt- or sharp-tip 32-gauge needle (for oral and intrahemocoelic inoculation, respectively) mounted on a 1-ml tuberculin syringe (13).

Occlusions and ODV were administered orally by inserting the blunt-tip needle through the mouth until the tip was well within the midgut lumen. BV was injected into the hemocoel by inserting the sharp tip needle through the planta of one of the prolegs, as described previously (40). Larvae were orally inoculated within 15 min after molting to the fourth instar (i.e., newly molted) or intrahemocoelically inoculated with BV 24 ± 6 h postmolt. After inoculation, test larvae were maintained individually in 25-ml plastic cups and fed with diet ad libitum in a growth chamber at 28 ± 2°C. When larvae failed to liquefy, tissues were isolated and examined for the presence of occlusions by light microscopy to confirm polyhedrosis disease. For viral binding and fusion experiments, no diet was provided to larvae after inoculation.

Construction of *pif* deletion and repair viruses. Briefly, the strategy used to make viral mutants with deletions in *pif1*, *pif2*, or *pif3* was to insert a cassette containing the β-galactosidase reporter gene driven by the *Drosophila hsp70*

promoter into the relevant ORFs *Ac119* (*pif1*), *Ac022* (*pif2*), and *Ac115* (*pif3*), respectively, of parental strain Ac*M*NPV E2 (32). Specifically, subfragments of the Ac*M*NPV E2 genome were cloned, each containing one of the *pif* genes. Conveniently, each of the *pif* genes within the cloned subfragments contained a pair of unique restriction sites reasonably close to the 5′ and 3′ termini of each ORF (Fig. 1). The appropriate pairs of restriction enzymes were used to cut out the central region of each ORF: EcoRV and NruI for *Ac022*, BstEII and StuI for *Ac115*, and BsaBI and NruI for *Ac119* (see Fig. 1). In each case, the newly cut ends of the plasmids were first blunted and then ligated with the *hsp70*/*lacZ* cassette so that the cassette would occupy the central region of each of the targeted ORFs. Subsequently, the plasmids containing the *hsp70*/*lacZ* inserts were cotransfected with Ac*M*NPV E2 DNA into Sf9 cells. Viruses produced from these transfections were used to generate plaques that were selected by their blue color. Following three rounds of plaque purification and one round of end-point dilution for each virus, BV was amplified and its DNA was isolated. Subsequently, the genetic structure of each mutant was confirmed by restriction endonuclease and PCR analysis (not shown). The control virus used in all of the binding and fusion studies was Ac*hsp70*/*lacZ*, which is Ac*M*NPV E2 plus the same *hsp70*/*lacZ* reporter cassette used to construct the *pif* mutants described above (13). Notably, Ac*hsp70*/*lacZ* has the same virulence and infectivity as the laboratory strain of Ac*M*NPV E2, also used as a control in some experiments (13, 40).

Repair viruses were generated by cotransfecting mutant viral DNA with the relevant genomic subfragments shown in Fig. 1. The repair viruses, recognized by white plaques, were generated, selected, and processed through three rounds of purification plus one round of end-point dilution. The fidelity of the DNA of each repair virus was confirmed by restriction endonuclease and PCR analysis (not shown).

Virus amplification and preparation. Viral occlusions were generated in *T. ni* and *S. exigua* larvae by intrahemocoelic injection of BV. Cadavers were collected and processed, and occlusions were purified by sucrose density gradient centrifugation, rinsed to remove residual sucrose, and then stored at 4°C until use. To generate ODV, occlusions were suspended in dilute alkaline solution (DAS; 100 mM $NaCO_3$, 100 mM NaCl) for 30 min on ice and then neutralized with 1 M Tris-HCl buffer (pH 7.6). After shaking for 1 h for maximal release of ODV from the surrounding calyces, the empty calyxes and undissolved occlusions were pelleted by centrifugation (2,000 × *g*, 20 min). The ODV in the supernatant fraction was banded on sucrose step gradients (25%, 35%, 57%, and 63% [wt/vol] ultrapure sucrose) by centrifugation (100,000 × *g*, 1 h, 4°C), and the ODV bands were extracted using an 18-gauge needle (affixed to a syringe) inserted through the side of each Beckman Ultra Clear tube. The concentration of ODV obtained in this manner typically was between 0.3 and 1.3 μg/μl. More highly concentrated ODV (for use in competition assays) was generated by pelleting gradient-purified ODV diluted 1:3 with water and then resuspending the pellet in a minimal volume of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4). Concentrated ODV was stored at 4°C for no more than 2 days before use. The protein concentration of these and other samples was determined using Pierce bicinchoninic acid protein assay. Ac*M*NPV BV was harvested from the medium of infected Sf9 cells at 3 days postinfection, and the titers were determined by immunoplaque assay using Sf9 cells (36).

R18 labeling of ODV. For binding, fusion, and competition assays, ODV was labeled with the self-quenching fluorescent probe octadecyl rhodamine B chloride (R18; Molecular Probes), hereafter ODV_R, according the procedure outlined by Haas-Stapleton et al. (17), with modification. Occlusions (at 6.25 mg/ml) were incubated with one-fourth volume of 5× DAS for 30 min on ice and then treated as described above to generate supernatant containing ODV. Immediately prior to layering on sucrose step gradients, a range of volumes (19 to 230 μl) of R18 stock solution (5 μg/μl ethanol) were added to 5-ml aliquots of the ODV supernatant. For each batch, one gradient was layered with supernatant without label. Protein assays were conducted on the isolated ODV and ODV_R as outlined above, and stocks were stored in the dark at 4°C until use. All subsequent manipulations involving ODV_R, including dissection of ODV_R-inoculated larvae and quantifying ODV_R binding and fusion, described below, were conducted under no- or low-light conditions to avoid photobleaching.

Specific fluorescence of ODV_R (fluorescence/μg ODV_R protein) was determined by placing 5 μl ODV_R into a cuvette containing 2 ml of separation buffer (SB; 100 mM $NaCO_3$, 100 mM KCl, 100 mM EGTA, pH 9.5) and adding 220 μl of 1% Triton X-100. ODV_R was solubilized overnight at 4°C and then measured for fluorescence (also at 4°C) at excitation/emission of 560/583 using a Fluorolog-3 fluorescence spectrophotometer fitted with a temperature-controlled cuvette holder (Instruments S.A.). While the specific fluorescence varied among preparations, within preparations there was a strong correlation between the amount of R18 added to each 5-ml sample and the specific fluorescence of the

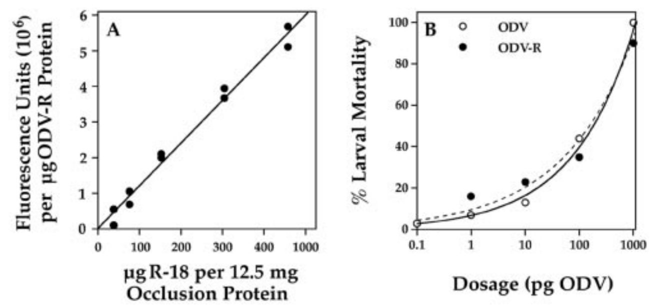

FIG. 2. (A) Correlation between specific fluorescence of Ac*M*NPV E2 ODV_R and concentration of R18 added to ODV preparations prior to gradient centrifugation. The points represent the average of two samples taken from each treatment. The regression line was fitted by the method of least squares ($y = 0.05x + 0.247$; $r^2 = 0.98$). (B) Mortalities of *H. virescens* larvae following oral inoculation as newly molted, fourth instars with various doses of Ac*M*NPV E2 ODV and ODV_R. The specific fluorescence of ODV_R was 1.1×10^6 fluorescence units/μg for this experiment. Each point represents the final mortality from a cohort of between 30 and 32 insects. Regression lines were fitted by the method of least squares (ODV, $y = 6.78x^{0.384}$, $r^2 = 0.99$; ODV_R, $y = 9.52x^{0.329}$, $r^2 = 0.92$).

resulting ODV_R (Fig. 2A). Importantly, we also found that when specific fluorescence exceeded 2.0×10^6 fluorescence units/μg ODV_R, virulence was significantly reduced (not shown). Therefore, we used preparations of ODV_R ranging from 6.25×10^5 to 1.0×10^6 fluorescence units/μg because at this level of label, ODV_R virulence was the same as that of the unlabeled control ODV (Fig. 2B).

Dissection of ODV_R-inoculated larvae. Newly molted, fourth instar *H. virescens* insects were inoculated orally with 2 μl containing 0.4 to 2.8 μg ODV_R or PBS (negative control) and dissected 60 ± 15 min later in ice-cold SB under minimal illumination (see reference 17). To isolate intact midgut epithelia, incisions were made along the length of the midgut and at the fore- and hindgut junctions within each midgut. Using forceps, the peritrophic membranes were removed and discarded, and the excised midguts were incubated for an additional 10 min in ice-cold SB. The intact midgut epithelia were then separated from their adherent basal lamina and associated muscles and tracheal elements, rinsed twice more in ice-cold SB, transferred in 40 μl of SB to 100 μl SB in chilled methacrylate four-clear-sided cuvettes (Fisher), and then frozen in liquid nitrogen. Cuvettes containing frozen midgut epithelia were stored in the dark at −80°C until fluorescence analyses were performed.

Quantification of ODV_R binding and fusion. To quantify ODV_R fusion levels, 1,860 μl ice-cold SB was added to each cuvette containing a frozen midgut to bring the final volume to 2.0 ml. Then, immediately before measuring fluorescence using the Fluorolog-3 fluorescence spectrophotometer as described above, a suspension of cells and cell fragments was generated from each midgut by pipetting 2 to 4 strokes using a P1000 pipette. Because R18 is self-quenching, the level of fluorescence measured at this step is indicative of the amount of ODV_R fused to the midgut (17).

To quantify the amount of ODV_R bound to each midgut epithelium, Triton X-100 (1% final concentration) was added to solubilize membranes during an overnight incubation at 4°C, and then fluorescence measurements were taken again (17). Fluorescence values were corrected both for the increased volume that resulted from the addition of detergent and for the background fluorescence associated with the midgut epithelial tissue of control larvae inoculated with PBS.

In competition experiments, newly molted, fourth instar *H. virescens* were inoculated orally with 0.4 or 0.5 μg of Ac*hsp70*/*lacZ* or AcΔPIF3 ODV_R, with or without an excess (up to 100×) of unlabeled ODV. Larvae were incubated at 28°C for 1 h in the dark prior to sampling, and viral binding and fusion were quantified as described above.

RESULTS

The first task was to confirm that all the mutants had the biological profiles of true *pif* mutants. To confirm that the BV of the deletion mutants had wild-type infectivity, we injected feeding, fourth instar larvae of *T. ni*, *S. exigua*, and *H. virescens* intrahemocoelically with 1 PFU of AcΔPIF1, AcΔPIF2,

TABLE 1. Larval mortalities following oral inoculation of occlusions or intrahemocoelic inoculation of BV[a]

Virus and dosage	Final mortality (%) in host species[b]:		
	T. ni	*S. exigua*	*H. virescens*
1 PFU			
AcΔPIF1	100	96	100
AcΔPIF2	88	92	100
AcΔPIF3	100	80	100
A*chsp70/lacZ*	100	82	100
10^4 occlusions			
AcΔPIF1	0	0	0
AcΔPIF2	0	0	5
AcΔPIF3	0	0	0
10 occlusions			
AcΔPIF1R	—	—	41
AcΔPIF2R	—	—	63
AcΔPIF3R	—	—	60
A*chsp70/lacZ*	—	—	54

[a] Each assay consisted of between 18 and 32 insects.
[b] —, not tested.

AcΔPIF3, or A*chsp70/lacZ*. The mortality levels for the *pif* mutants ranged from 80 to 100%, the same as for the control, suggesting there was no difference in BV virulence (Table 1). Next we compared the control and mutant viruses in oral bioassays using the same three permissive host species. For these hosts, the 50% lethal dose of Ac*M*NPV E2 is between 4 and 10 occlusions (38, 40, 44). As expected, 10 occlusions of each of the repair viruses and the control virus yielded mortality levels between 41 and 63% in orally inoculated, newly molted, fourth instar larvae of *H. virescens* (Table 1). In contrast, 10,000 occlusions of each of the three deletion mutants generated no mortality, with the exception of AcΔPIF2, which generated 5% mortality in *H. virescens*. LacZ expression was notably absent in larvae inoculated with 1,000 occlusions of any of the *pif* mutants but readily observed in midgut epithelia of insects orally inoculated with 100 occlusions of control A*chsp70/lacZ*, indicating that the critical events mediated by the three *pif* gene products were early (data not shown). These results confirmed the classification of *Ac119*, *Ac115*, and *Ac022* as authentic *pif* genes.

In order to determine whether the products of *Acpif1*, *Acpif2*, and/or *Acpif3* affected binding and/or fusion with midgut epithelia of newly molted, fourth instar *H. virescens*, we compared the R18-labeled ODV_R of the mutant viruses with the ODV_R of A*chsp70/lacZ* in our in vivo assay. The data presented in Fig. 3 are representative of at least three iterations of the assay for each *pif* mutant using dosages ranging from 0.4 to 2.8 μg ODV_R per insect. Among replicates, between 4 and 22% of control ODV_R administered to larvae at these dosages bound to the midgut (not shown). The results in Fig. 3 demonstrated that, relative to control, only about one-third of the administered ODV_R bound for both the PIF1 and PIF2 deletion mutants (38 and 33%, respectively). In contrast, there was no difference between the amounts of wild-type control ODV_R and PIF3-deficient ODV_R that bound. For all viruses, over half of ODV_R that bound also fused in each iteration. Specifically, for the results shown in Fig. 3, the proportions that fused were 75% for A*chsp70/lacZ*, 62% for AcΔPIF1, 100% for AcΔPIF2, and 74% for AcΔPIF3 ODV_Rs. Hence, the results obtained with AcΔPIF1 and AcΔPIF2 ODV_Rs were almost identical to the results reported for the AcMNPV *p74* deletion mutant, AcLP4 (14, 17). The binding and fusion profiles obtained with AcΔPIF3 ODV_R, however, were strikingly different and indistinguishable from those of the control virus (Fig. 3).

The lack of any significant quantifiable differences in the binding and fusion assay between control and AcΔPIF3 ODV_Rs suggested that PIF3 had no role during initial binding or fusion with the midgut. To confirm this conclusion, we tested A*chsp70/lacZ* and AcΔPIF3 ODV_Rs in reciprocal competition experiments (Fig. 4). If the ODVs of AcΔPIF3 and *Achsp70/lacZ* bound to the same midgut cellular receptors, the ODV_R of either should be displaced progressively by unlabeled ODV of the other (as well as unlabeled ODV of its own kind) (17). Indeed, the results of these experiments showed that the control and mutant ODV_Rs were displaced by excess unlabeled control ODV or AcΔPIF3 ODV with similar effectiveness. For example, a 100-fold excess of A*chsp70/lacZ* ODV and a 96-fold excess of AcΔPIF3 ODV reduced A*chsp70/lacZ* ODV_R binding by 65 and 75%, respectively (Fig. 4A and B). In contrast, competition experiments between control ODV_R and unlabeled ODV of either AcΔPIF1 (Fig. 5A) or AcΔPIF2 (Fig. 5B) showed that neither of the two mutant ODVs in excess quantities could interfere with binding of control ODV_R. These results confirmed that PIF1 and PIF2, like P74 and unlike PIF3, were involved in specific binding. Notably, no defects in fusion were observed in the absence of any of these three proteins.

DISCUSSION

In this study, we demonstrated that PIF1 and PIF2, the products of *Ac119* and *Ac022*, respectively, have a role in specific binding of Ac*M*NPV ODV to midgut cells of fourth instar *H. virescens* larvae. Hence PIF1, PIF2, and P74 have all been assigned the same function—specificity in ODV binding. Previous studies have shown that PIF1 and P74 are ODV

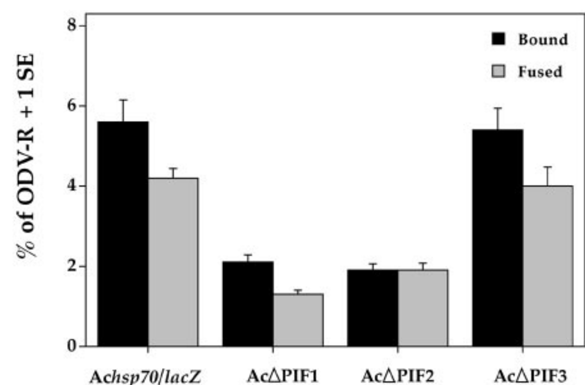

FIG. 3. Percentages of Ac*M*NPV ODV_R that bound and fused with midgut epithelia of newly molted, fourth instar *H. virescens* orally inoculated with 1.25 μg A*chsp70/lacZ* ODV_R, 2.1 μg AcΔPIF1, 2.4 μg AcΔPIF2 ODV_R, and 2.1 μg AcΔPIF3 ODV_R. Each histogram bar represents the mean (+1 standard error [SE]) of 10 to 15 larvae.

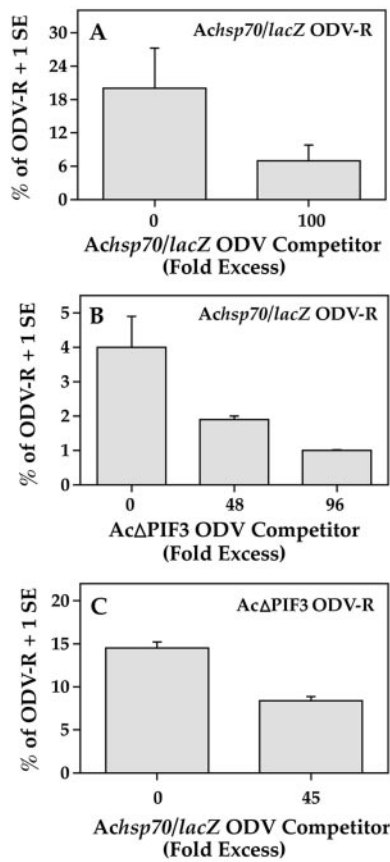

FIG. 4. (A) Percentages of Ac*hsp70/lacZ* ODV_R that bound and fused with midgut epithelia of newly molted, fourth instar *H. virescens* orally inoculated with 0.5 μg ODV_R alone and in the presence of 100× unlabeled homologous ODV competitor. Each histogram bar represents the mean (+1 standard error [SE]) of 15 larvae. (B) Percentages of Ac*hsp70/lacZ* ODV_R that bound and fused when larvae were inoculated with 0.4 μg Ac*hsp70/lacZ* ODV_R alone and in the presence of various amounts of unlabeled AcΔPIF3 ODV competitor. Each histogram bar represents the mean (+1 standard error) of 13 to 16 larvae. (C) Percentages of AcΔPIF3 ODV_R that bound and fused with midgut epithelia of newly molted, fourth instar *H. virescens* orally inoculated with 0.4 μg AcPIF3 ODV_R alone and in the presence of various amounts of unlabeled Ac*hsp70/lacZ* ODV competitor. Each histogram bar represents the mean (+1 standard error) of 13 larvae.

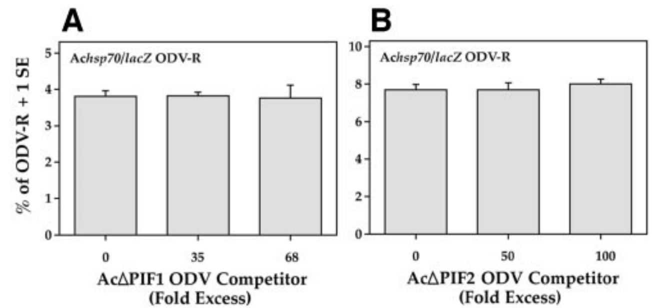

FIG. 5. Percentages of Ac*hsp70/lacZ* ODV_R that bound and fused when newly molted, fourth instar *H. virescens* were inoculated with 0.4 μg Ac*hsp70/lacZ* ODV_R alone and in the presence of various amounts of unlabeled AcΔPIF1 ODV competitor (A) or unlabeled AcΔPIF2 ODV competitor (B). Each histogram bar represents the mean (+1 standard error [SE]) of 13 to 16 larvae.

envelope components (14, 22), but PIF2 has not been localized to date. The data presented here, however, predict that PIF2 also is present within the ODV envelope.

Available evidence suggests that entry of ODV involves fusion of the ODV envelope with the apical microvillar membrane of midgut cells. ODV does not have apparent spikes, and because of the alkalinity of midgut lumen fluids (16), fusion presumably takes place at a high pH. Together, these details exclude ODV from the current paradigms for functional entry of enveloped viruses. Two types of entry are recognized based on mode of fusion (12, 31). Type I pertains to those viruses with obvious homotrimeric glycoprotein spikes that mediate binding and fusion at neutral or low pHs (e.g., influenza virus, herpesvirus, and Ac*M*NPV BV). Type II describes viruses with fusion proteins assembled as heterodimers and packed longitudinally close to the viral envelope (e.g., flaviviruses and alphaviruses). Type II heterodimers are triggered to dissociate when exposed to low pH, and three of the fusion protein partners reassociate to form fusion-competent homotrimeric spikes. ODV has no spikes, even when exposed to high pH. Moreover, deletion of the fusion proteins from any type I or type II virus would prevent fusion. In contrast, ODV fusion was not diminished by the deletion of any one of the PIF proteins (see also reference 17). It appears that either the Ac*M*NPV ODV fusion protein has yet to be identified or a novel viral fusion mechanism is at work.

ODV functional entry also presents a challenge to a paradigm of cell biology because nucleocapsids (30 to 60 nm by 250 to 300 nm) (3) are large compared to microvilli (1 μm by 100 nm) (9), and nucleocapsids entering microvilli apically would have to be transported basally to establish infection. At present, the cell biology literature predicts that bulky microvillar core and cross filaments would prevent cargo the size of nucleocapsids from being transported efficiently within microvilli (9). Moreover, dynamic membrane trafficking in gut cells is thought to be restricted to the base of microvilli and the basal invaginations intervening between them (9). Indeed, Lehman et al. (25) reported observing murine leukemia virus "surfing" toward these basal regions after engaging its receptor on filopodia and retraction fibers of fibroblasts in culture. There are no reports of ODV nucleocapsids entering midgut cells basally, however. Moreover, brush border microvilli are known to play a central role in cell fusion events during embryogenesis and after, and they even have been described as cell-fusion organelles (42). It is possible then, that in the case of ODV, it is the microvillus that provides the fusogenic apparatus.

Microvilli are rich in lipid rafts—membrane microdomains containing cholesterol/sphingolipids that recruit signaling molecules, including integrins (9, 10, 30). Integrins are heterodimeric molecules with quite large subunits (α and β) of 90 kDa or more. Typically, both subunits are transmembrane glycoproteins with large extracellular domains and short cytoplasmic tails (4). The extracellular domains of both subunits contribute to the binding site for ligands, and ligand binding can trigger changes in cytoskeletal organization (19). It is conceivable that such a signaling mechanism could play a role in ODV entry. Integrin binding is important for entry of rotaviruses (which infect human gut epithelial cells apically) and

several other nonenveloped and enveloped viruses (8, 27, 35). Unfortunately, little is known about integrins in lepidopteran larvae. An α integin subunit that recognizes the RGD motif, αPi2, has been described from hemocytes of *Pseudoplusia includens* larvae, and transcript levels of αPi2 are higher in the midgut than in hemocytes, suggesting αPi2 is synthesized in the midgut as well (24). While there are no reports of β subunits in lepidopteran midguts to date, two have been described in *Drosophila*: widely distributed βPS and midgut-specific βν, both of which are expressed throughout larval development and are essential for migration of primordial midgut cells (11, 43). In addition, Loeb et al. (26) have described cultured *Heliothis virescens* midgut cells with columnar cells clustered around a goblet cell and the cell borders stained with antibody against the human β1 subunit. In humans, the β1 subunit participates in RGD binding.

It would make good sense for a virus that targets only midgut cells to use specialized cellular machinery (such as signaling pathways through midgut-specific receptors) to gain functional entry from remote sites imposed by peritrophic membrane barriers. Integrins are full-fledged signal transduction receptors, and they afford immediate direct signaling to the cytoskeleton (19), and baculoviruses are known to regulate the cytoskeleton (6, 20). Powerful ODV binding partners capable of signaling the cytoskeleton would be needed to transport nucleocapsids (that occupy at least one-third the cross-sectional space along their length) down the full length of a microvillus. Indeed, electron micrographs have captured instances of microvilli distended with three nucleocapsids abreast (34). Considering the "squeeze" problem that ODV nucleocapsids face within microvilli, it seems odd that lepidopteran-specific NPVs have evolved a midgut-specific phenotype that delivers multiple nucleocapsids to the same microvillus at one time and place. Empirical evidence has shown, however, that ODV-containing multiple nucleocapsids are more efficient in establishing systemic infection than genetically identical ODV containing single nucleocapsids and that there is no difference in transport time (41). The most parsimonious explanation for rationalizing these contradictions is that the actin cytoskeleton that forms the microvillar core must actually be exploited by nucleocapsids for transport, but it's far from clear how.

Identification of the microvillar binding partners for P74, PIF1, and PIF2 should help elucidate the mechanisms involved in nucleocapsid entry and transport. Toward this end, Zhou et al. (45) recently used pull-down assays to detect a 35-kDa binding partner for Ac*M*NPV P74 in extracts of brush border membrane vesicles from host *Spodoptera exigua* larvae but absent in brush border extracts from non-host *Helicoverpa armigera* larvae. The identity of this 35-kDa protein has not been established to date, but it is unlikely to be an integrin subunit based on its relatively small size (18). Sequence analysis suggests that the best hope for an integrin ligand among the known *pif* gene products is PIF1. PIF1 not only has an RGD minimal integrin recognition motif but also has a region of homology with laminin, the same region of homology shared by tenascins, a major group of connective tissue ligands that signal through RGD motifs (7). If, in the future, the mutation of the RGD motif in PIF1 is found to destroy ODV infectivity, then integrin signaling will have been implicated in the Ac*M*NPV ODV entry process.

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