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Surface Display of *Ac*MNPV Occlusion-Derived P74 Does Not Enhance Oral Infectivity of Budded Viruses

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Key Words

Baculovirus · P74 · Pseudotyping · Budded virus · *Autographa californica* multiple nucleopolyhedrovirus

Abstract

Baculovirus occlusion-derived viruses (ODVs) and budded viruses (BVs) are morphologically and functionally distinct. ODVs are responsible for primary infection in insect hosts because of their high per os infectivity. On the contrary, BVs poorly infect endothelial gut cells, but propagate the infection in the tissues of insects with a high efficiency. P74 is one of the most important proteins from ODVs, and it participates in the attachment of this viral phenotype to endothelial cells in the midgut. We evaluated the possibility of pseudotyping BVs of *Autographa californica* multiple nucleopolyhedrovirus with two versions of P74 and its effect on their oral infectivity. Both recombinant BVs contained P74 and replicated similarly to wild-type viruses. Nevertheless, the presence of P74 on the BV's surface does not enhance the oral infectivity of this phenotype, suggesting that the presence of P74 in the membrane of budded virions interferes with their mechanism of infecting midgut cells.

Baculoviruses belong to a broad family of virus pathogens of insects. Two distinct infectious phenotypes exist in their life cycle: occlusion-derived viruses (ODVs) and budded viruses (BVs) [1]. ODVs are embedded within a polyhedrin matrix, forming the baculovirus occlusion bodies or polyhedra. When larvae feed on contaminated leaves, polyhedra dissolve in the midgut and release ODVs which infect the epithelial cells initiating primary infection [2]. The uptake of ODVs is mediated by specific factors of attachment and fusion to cell membranes, although the mechanisms involved in this process remain poorly understood.

In *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), the most deeply studied member of the *Baculoviridae* family, it is known that some ODV proteins, like P74 and other per os infectivity factors or PIFs, are essential to infect larvae by the oral route [3–5]. The p74 gene is expressed at the late stage of infection [4] and encodes a 645-amino acid protein that is located exclusively on the ODV lipid bilayer envelope and is exposed on the ODV surface [6].

Yao et al. [7] and Zhou et al. [8] showed that a soluble form of P74 is capable of rescuing the infectivity of P74-*null* ODVs, indicating that this protein has an essential role in viral adsorption or fusion with the target cell. Nevertheless, how the P74 protein interacts with host midgut proteins still remains unknown. BVs bud from the baso-

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lateral side of the infected midgut cells and infect tissues in the hemocoel. A baculovirus envelope glycoprotein called GP64 is involved in BV cell attachment and is required for adsorptive endocytosis followed by the fusion of the viral and endosomal membranes [9]. It has been reported that BVs have a very low infectivity in endothelial cells of the larval midgut [10]. Keddie and Volkman [11] showed that BVs are 10^6-fold less infectious in the midgut than in the hemocoel; for comparison, ODVs are 10^4-fold more orally infectious than BVs.

Pseudotyping is a successful strategy, used mainly in gene therapy, and consists of modifying viral tropism through the surface display of foreign proteins or peptides that interact with receptors present in certain cell types [12]. The display of antigens or other proteins on budded baculovirus envelopes, mainly fused with GP64, has been extensively studied and has become a powerful tool for modifying viral tropism, displaying antigens or studying the role of heterologous membrane proteins in the baculovirus context [13–19].

In many laboratories, the baculovirus-insect cell system is broadly used to express antigens of medical and veterinary interest. Recombinant viruses in which genes of interest are introduced into the polyhedrin locus do not produce occlusion bodies (occ-). We hypothesized that BV oral infectivity could be improved by bringing budded virions displaying P74 closer to the midgut cells, taking advantage of the interaction of P74 with its cellular receptor. This approach would contribute to the development of a method for the production of antigens expressed by occ- recombinant baculoviruses delivered by the oral route.

With the purpose of displaying P74 protein on BV, two recombinant baculoviruses were designed: *Ac*P74exSup and *Ac*P74Sig. In these constructs, the strong very late polyhedrin promoter drove the expression of a second copy of P74 inserted into the polyhedrin locus. In order to expose a portion of P74 in the BV's peplomers, *Ac*P74exSup virus was constructed by fusing a truncated form of P74 (P74ex) externally located in wild-type (wt) ODVs (*Ac*MNPV complete genome NC 001623, nucleotides 119,794 to 121,069) with a second copy of the viral glycoprotein GP64. The other strategy consisted of distributing P74 on the viral envelope, anchoring it to the cell membrane by its own transmembrane domain, taking advantage of baculoviruses passively acquiring P74 during the budding process. To obtain *Ac*P74Sig, the complete sequence of p74 omitting the start codon (*Ac*MNPV nucleotides 119,135 to 121,069) was fused with the gp64 signal peptide. Recombinant *Ac*P74exSup and *Ac*P74Sig were obtained using BaculoGold™ methodology (Pharmingen). Immunoblotting of cell lysates demonstrated the expression of P74 in cells infected with both recombinant baculovirus, and time course analysis of BV production showed similar virus yields to wt *Ac*MNPV (data not shown).

The exposure of both recombinant forms of P74 on the surface of cell membranes was evidenced by immunofluorescence assays (fig. 1a). Upon infection with either recombinant baculovirus, *Ac*P74Sig or *Ac*P74exSup, nonpermeabilized cells displayed positive staining for the recombinant P74s on cell surfaces with a specific antibody against P74 (N25 8c [6]) and a FITC-conjugated goat anti-mouse antibody (BD Biosciences). In contrast, fluorescence staining was absent on cells infected with wt *Ac*MNPV. A control with anti-GP64 (AcV5 [20]) was included to show that viral proteins can be detected at the cell surface. This analysis suggests not only the presence, but also the display, of P74 in BV membranes. Consequently, preparations of each purified BV [21] were examined by immunoblotting to determine the association of P74 to the virions.

As shown in figure 1b, an antibody against P74 revealed bands of 110 and 74 kDa, according to the case of P74 fused to GP64 or P74, respectively. P74 was not detected in wt *Ac*MNPV BVs. Western blot analysis using an antibody against GP64 revealed the 110-kDa GP64-P74 fusion product in *Ac*P74exSup and wt GP64 in wt *Ac*MNPV BVs and in *Ac*P74exSup. Therefore, as expected, P74 was readily detected in BVs purified from cells infected with recombinant baculoviruses, although at least 200 ml of culture supernatants (5×10^7 plaque-forming units, pfu/ml) had to be processed to visualize immunoblotting bands, suggesting that P74 was poorly represented in the virions. Likewise, wt p74 is a late gene weakly transcribed [4], and the low quantity of P74 has made it difficult to investigate. Besides, although P74 plays a crucial role in the oral route of infection, the overexpression of P74 protein does not enhance the pathogenicity of viral occlusion bodies [8].

It is known that viral attachment and fusion of membranes are processes that need the participation of more than one protein. At least PIFs 1, 2 and 3 and P74 are all required for ODV oral infection. Slack et al. [22] have shown that a truncated mutant of P74 lacking its transmembrane anchor is able to keep its functionality, and it has been suggested that P74 could form a complex with other envelope proteins. Recently, Peng et al. [23] have demonstrated that PIFs form a complex and that P74 is associated with it. Although budded baculoviruses lack

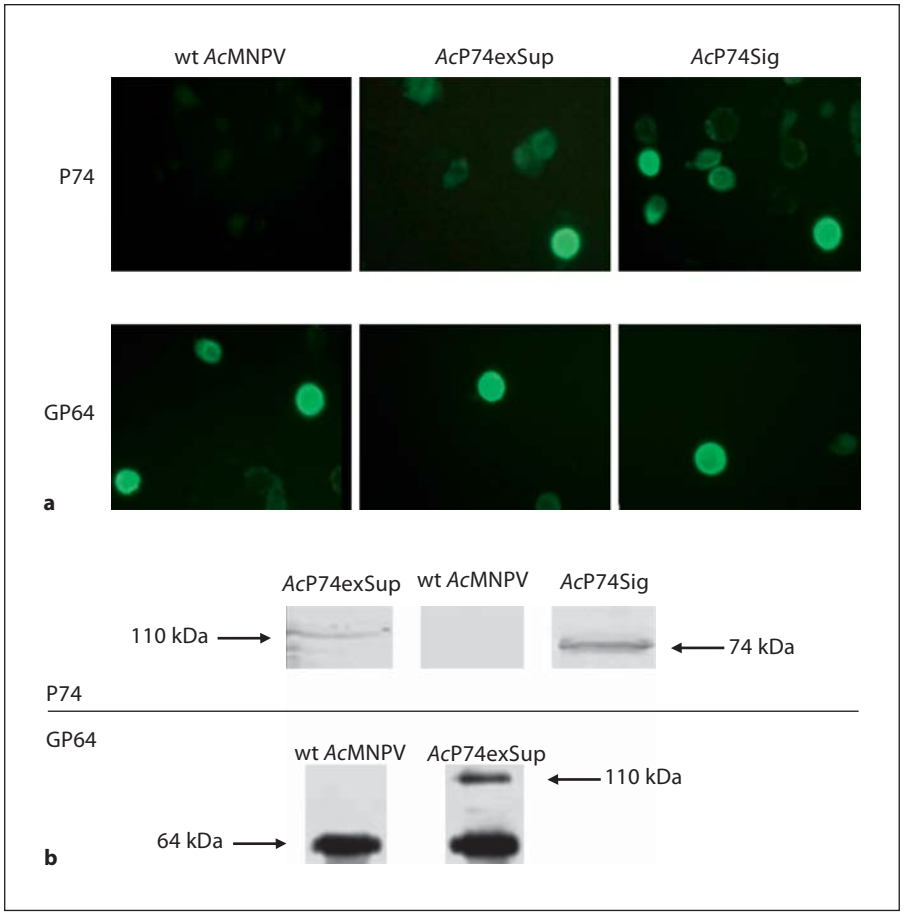

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Fig. 1. a Detection of recombinant P74 by immunofluorescence in infected cells. Sf-9 cells were infected with *Ac*P74exSup, *Ac*P74Sig or wt *Ac*MNPV at a multiplicity of infection of 10 pfu. At 2 days postinfection, cells were probed with anti-P74 antibody or anti-GP64 antibody and visualized using FITC-conjugated goat anti-mouse IgG. **b** Analysis of recombinant P74 in purified budded virions. *Ac*P74exSup BVs, *Ac*P74Sig BVs and wt *Ac*MNPV BVs were purified by a sucrose gradient and analyzed by SDS-PAGE and Western blot. Blots were probed with anti-P74 and anti-GP64 antibodies.

PIFs, it has been shown that they are able to initiate an infection by the oral route in the larval hosts with low efficiency. Thus, P74-pseudotyping could improve the binding of BVs to insect gut cells since the interaction of P74 to brush border membrane vesicles has previously been proven [24].

With the purpose of determining whether displaying recombinant P74 or P74ex on BVs was able to induce changes in *Ac*MNPV oral infectivity, virus yields were measured in a bioassay of oral infection of *Rachiplusia nu* and compared against nonrecombinant BV infectivity. Bioassay was not carried out by scoring mortality because most larvae do not die from BV feeding. Groups of 27 third instar *R. nu* larvae fed individually with a diet containing 6×10^6 pfu of *Ac*P74exSup BV, *Ac*P74Sig BV, wt *Ac*MNPV BV or PBS were daily monitored.

Twenty-four hours or 4 days postinfection viral DNA was extracted from hemolymph and quantified by qPCR using SYBR Green PCR Master Mix (Applied BioSystems) and the qPCR primers VP39forN 5′ cgacaaatgagagttaatcgctgc 3′ and VP39RCuan 5′ gtcgtcttcgtcgaaaatgggcaa 3′, which were designed to amplify a 183-bp genomic fragment of the vp39 gene. Samples were analyzed in a 7500 Real Time PCR System using the 7500 System Software (Applied BioSystems). Viral DNA was not detected in either experimental group at 24 h postinfection. However, as depicted in figure 2, viral DNA was detected in samples taken at the 4th day postinfection and a high dispersion was noticed in qPCR values among individual larvae from each experimental group. Analysis of the data revealed, on the contrary to what we expected, that recombinant viruses were not more infectious than wt.

The presence of P74 in BVs was associated with a high number of samples with values of viral DNA in hemolymph below quantification limit ($p = 0.0041$ *Ac*P74Sig vs. wt *Ac*MNPV, $p = 0.0108$ *Ac*P74exSup vs. wt *Ac*MNPV; Fisher's exact test). As expected, larvae fed with a PBS-contaminated diet reached DNA values below the quantification limit. Similar results were obtained when viral titers in hemolymph were determined by plaque assays

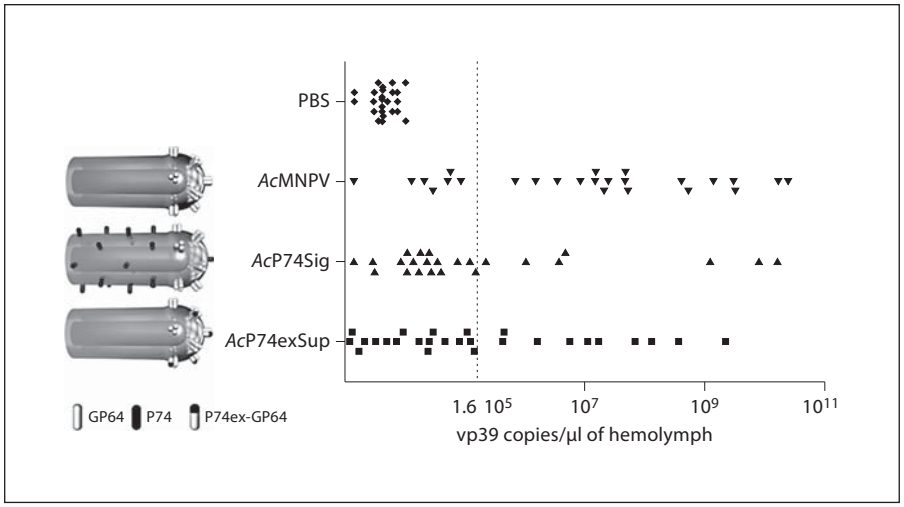

Fig. 2. Quantitative real-time PCR analysis of viral DNA. *R. nu* larvae were fed with a diet containing PBS or 6×10^6 pfu of *Ac*P74exSup BV, *Ac*P74Sig BV or wt *Ac*MNPV BV. At 4 days postinfection, DNA was extracted from hemolymph and analyzed by qPCR. The dotted line represents the assay's quantification limit, calculated as the lowest DNA concentration in the standard curve multiplied by the dilution factor.

(data not shown). These results demonstrated that the presence of P74 in BVs does not improve the oral infectivity in *R. nu* larvae, suggesting that a major proximity of the virus to the target cells is not the only condition to increase the natural infectivity of this viral phenotype and that P74 by itself is not capable of modifying BV tropism. However, we cannot rule out the incorrect folding of P74 in both recombinants, and the spatial conformation of P74 present in BV may not be adequate for achieving a biological role.

Moreover, a diminished infectivity was observed in recombinant BVs compared with wt BVs. Since growth curves performed in cultured cells with *Ac*P74exSup BV, *Ac*P74Sig BV and wt viruses revealed that P74 protein present on the surface of BV does not interfere with BV entry into cells, modified viruses retain the ability to infect cells in a secondary infection on the same level as wt. Nevertheless, it is possible that P74 modifies virus entry to midgut cells, altering the interaction between an unknown cell receptor and the virus ligand. On the other hand, this steric impediment would be also capable of disturbing ODV attachment to endothelial cells. Perhaps BVs are not able to infect cells of the midgut, and the per os infection of larvae with wt BVs is due to the presence of a pre-occluded virus or POVs in supernatants of infected cells as a product of lysis that co-purify in small quantities with BVs. Therefore, a plausible explanation could be that the decrease in infectivity observed for *Ac*P74exSup and *Ac*P74Sig BVs is due to the interference of pseudotyped BVs to contaminating POVs.

In this paper, our results demonstrate that the surface display of P74 protein on budded baculoviruses is not an adequate tool to raise their per os infectivity, and it is possible that it interferes with the mechanism used by this phenotype to infect midgut cells. Further investigation is required to understand in more detail the different pathways used by the two baculoviral phenotypes to orally infect lepidopteran larvae.

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