

Production of GP64-free virus-like particles from baculovirus-infected insect cells

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Abstract

The retroviral Gag protein is frequently used to generate 'virus-like particles' (VLPs) for a variety of applications. Retroviral Gag proteins self-assemble and bud at the plasma membrane to form enveloped VLPs that resemble natural retrovirus virions, but contain no viral genome. The baculovirus expression vector system has been used to express high levels of the retroviral Gag protein to produce VLPs. However, VLP preparations produced from baculovirus-infected insect cells typically contain relatively large concentrations of baculovirus budded virus (BV) particles, which are similar in size and density to VLPs, and thus may be difficult to separate when purifying VLPs. Additionally, these enveloped VLPs may have substantial quantities of the baculovirus-encoded GP64 envelope protein in the VLP envelope. Since VLPs are frequently produced for vaccine development, the presence of the GP64 envelope protein in VLPs, and the presence of *Autographa californica* multicapsid nucleopolyhedrovirus BVs in VLP preparations, is undesirable. In the current studies, we developed a strategy for reducing BVs and eliminating GP64 in the production of VLPs, by expressing the human immunodeficiency virus type 1 *gag* gene in the absence of the baculovirus *gp64* gene. Using a GP64null recombinant baculovirus, we demonstrate Gag-mediated VLP production and an absence of GP64 in VLPs, in the context of reduced BV production. Thus, this approach represents a substantially improved method for producing VLPs in insect cells.

INTRODUCTION

Virus-like particles (VLPs) are assemblies that are similar to and mimic virus particles. VLPs are typically assembled from one or a few viral proteins and may be enveloped by a membrane derived from the cell, or they may be non-enveloped, depending on the virus and method of production. Typically, VLPs do not contain a viral genome, cannot replicate, and are infection incompetent, which makes VLPs excellent models for studying viral structure and immune system activation during viral infection [1, 2]. VLPs are also used as vaccines, as surface display platforms, and as vehicles for gene delivery [3–5]. VLPs are capable of inducing B and T cell-mediated immune responses as well as neutralizing antibody production. In addition to their capacity for robust immune system activation, VLPs are safer and often lower cost alternatives to live or attenuated vaccines [6, 7]. A number of VLP-based vaccines have been commercialized worldwide. Examples include Engerix-B and Recombivax HB (against the hepatitis B virus) which are produced in yeast cells, and Gardasil and Cervarix (against the human papilloma virus) produced in yeast cells and

baculovirus-infected insect cells, respectively [8]. The baculovirus expression vector system (BEVS) is a powerful eukaryotic expression system, capable of extremely high levels of protein production, combined with complex eukaryotic post-translational protein modifications, which may be important for the correct self-assembly and release of some VLPs. As such, the BEVS is an attractive and valuable platform for the production of VLPs [9–11].

The mechanism of VLP assembly and VLP composition depends on many factors, including the type of virus particle (enveloped or non-enveloped). Typically, the final steps in the assembly of an enveloped VLP involve the matrix and/or capsid proteins interacting with cellular protein complexes at the plasma membrane, followed by budding to produce the VLP. In this process, the envelope of the VLP may incorporate viral and cellular membrane proteins found at the cell surface [12]. While this is advantageous for some applications, it can also be problematic as cellular membrane proteins and the envelope protein from a viral vector (such as the baculovirus envelope glycoprotein

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Abbreviations: AcMNPV, *Autographa californica* multicapsid nucleopolyhedrovirus; BEVS, baculovirus expression vector system; BV, budded virus; HIV-1, human immunodeficiency virus type 1; ODV, occlusion-derived virus; TEM, transmission electron microscopy; TNMFH, *Trichoplusia ni* medium Fred Hink; VLP, virus-like particle.

GP64) may be abundantly displayed on the cell surface, and subsequently displayed on the VLP surface [13, 14].

During *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) infection of insect cells, two types of virions are produced: occlusion-derived virus (ODV) and budded virus (BV). Both contain the same genome and nucleocapsid but ODV and BV differ in morphogenesis, envelope composition, and function within the viral infection cycle. The major envelope protein of the AcMNPV BV particle is GP64, a type I integral membrane glycoprotein that is required for viral entry and is important for efficient budding of BV during virion egress [15–17]. The GP64 protein is immunogenic in mammals and can trigger a specific immune response in BALB/c mice [18]. Enveloped VLPs produced using BEVS may contain GP64 that is acquired during particle assembly (budding) and it was reported previously that this occurs when human immunodeficiency virus type 1 (HIV-1) Gag is used to generate VLPs in the BEVS [14].

Expression of the retroviral Gag protein is an efficient method for producing VLPs. The HIV-1 *gag* ORF encodes a precursor polyprotein of 55 kDa (Pr55) that is processed into a series of proteins including the matrix protein (p17 or MA), the capsid protein (p24 or CA), the nucleocapsid protein (p7 or NC) and a p6 protein. Myristoylation of the HIV Gag precursor (Pr55^{Gag}) is essential for its localization to the cytoplasmic side of the plasma membrane [19–21]. Expression of Pr55^{Gag} alone is sufficient for the assembly and budding of VLPs from the plasma membrane of the cell [22–24]. Because VLPs are important for vaccine development, and the BEVS is one of the most attractive systems for producing VLPs, a system for producing VLPs free of baculovirus particles and for eliminating the baculovirus envelope protein from VLPs could be of great value in vaccine development and production.

In the current study, we generated recombinant baculoviruses expressing the HIV-1 Gag protein in either the presence or absence of the baculovirus GP64 protein. When a recombinant baculovirus expressing both the Gag and GP64 proteins was examined, we found that VLP preparations contained BV particles that were difficult to separate from VLPs. However, when a recombinant virus expressing the Gag protein but lacking the *gp64* gene was examined, BVs appeared to be substantially reduced and no GP64 was detected. Thus, these approaches should facilitate development of improved VLP-based vaccines using BEVS.

RESULTS

Analysis of AcMNPV BV and HIV-1 VLP production

To initially examine and compare the production of VLPs and BVs from a recombinant baculovirus, Sf9 cells were infected at an m.o.i. of 0.1 with WT AcMNPV or vGAGHIV-1, a recombinant AcMNPV virus that expresses the HIV-1 Gag protein under the polyhedrin promoter (Fig. 1a, b). Supernatants were collected and BVs and VLPs from

each infection were purified, first by pelleting through a 25 % sucrose cushion, then by fractionating on a 30–60 % sucrose gradient. Although the HIV-1 Gag VLPs and baculovirus BVs differ somewhat in size and shape (around 100 nm diameter vs 30–60×250–300 nm, respectively), only a single diffuse band was visible in sucrose density gradients of particles isolated from AcMNPV- or vGAGHIV-1-infected cell supernatants (data not shown). After fractionation of each gradient, a portion of each fraction was loaded onto a lane of an SDS-PAGE gel, proteins were stained with Coomassie Brilliant Blue, and quantified by densitometry (Figs 2 and 3). Fractions containing AcMNPV BVs were identified by the combined presence of the GP64 and VP39 proteins, the most abundant structural proteins of AcMNPV BVs. Fractions containing HIV-1 VLPs were identified by the presence of the Gag protein. Proteins GP64, VP39 and Gag were confirmed by Western blot analysis and the approximate quantities of the proteins in each lane were determined by Coomassie Brilliant Blue staining and interpolation from a standard curve of BSA.

AcMNPV BVs from cells infected with WT AcMNPV were identified as a major peak at a density of approximately 1.19 g ml⁻¹, as determined by maximal detection of GP64 (green line) and VP39 (red line) (Fig. 2a, dashed lines). A second (minor) peak of AcMNPV BVs was also identified at a density of approximately 1.20 g ml⁻¹. The identities of GP64 and VP39 were confirmed by Western blot analysis (Fig. 2b, right panel). Purified particles from the fraction containing peak levels of AcMNPV BVs were analysed by negative staining and transmission electron microscopy

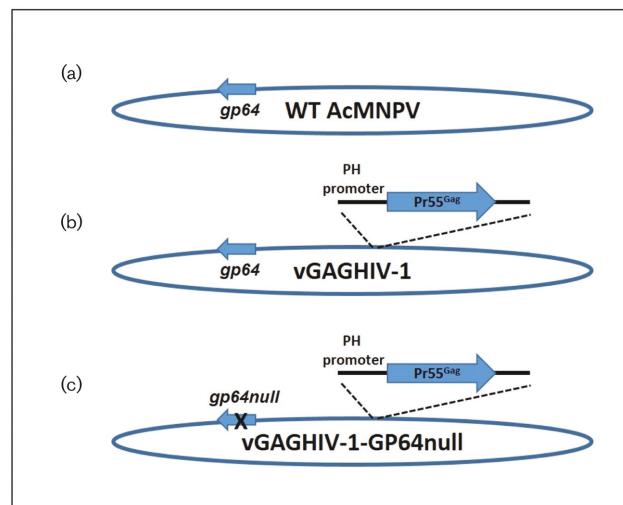


Fig. 1. WT AcMNPV and recombinant AcMNPV viruses (vGAGHIV-1 and vGAGHIV-1-GP64null) are represented as diagrams. Presence or absence of the AcMNPV *gp64* gene and the polyhedrin (PH) promoter driven HIV-1 *gag* gene (Pr55^{Gag}) is indicated for each virus: WT AcMNPV (a), vGAGHIV-1 (b) and vGAGHIV-1-GP64null (c). Virus vGAGHIV-1-GP64null was generated from a *gp64*null AcMNPV virus that was previously described in detail [26, 45].

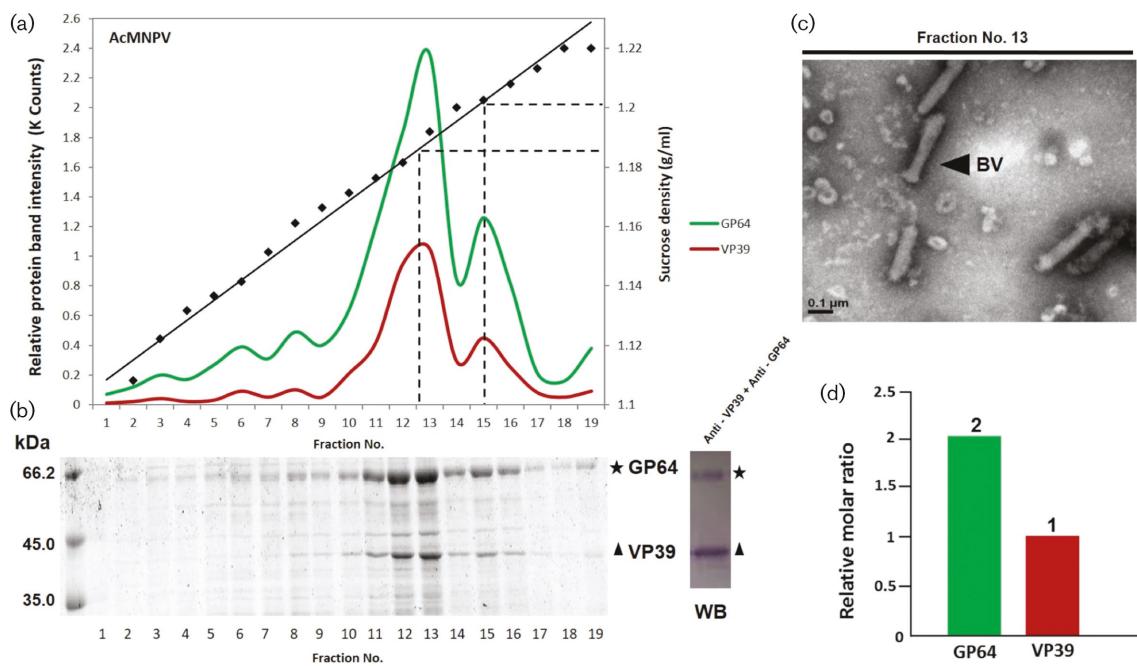


Fig. 2. Analysis of AcMNPV BV particles by sucrose density gradient fractionation. (a, b) BVs were isolated on 30–60 % sucrose density gradients from supernatants of AcMNPV-infected Sf9 cells. Lanes 1–19 represent fractions (top to bottom) from the sucrose gradient, analysed by 12 % SDS-PAGE (b, left panel). AcMNPV GP64 and VP39 proteins were identified by Western blot analysis of fraction 13 using an anti-GP64 Mab (star) and an anti-VP39 antiserum (triangle) (b, right panel). Relative quantities of GP64 and VP39 proteins were determined by measuring fluorescence intensities of bands from Coomassie Blue-stained gels by infra-red imaging. Band intensities were plotted as a red line for VP39 and a green line for GP64 in (a). The density of sucrose in each fraction is indicated by a black diamond and a solid black regression line indicates the linearity of the gradient. Dashed lines show densities of peak fractions of BVs. (c) Negatively stained virion preparations from fraction 13 were examined by a transmission electron microscope (TEM). The arrow indicates an example of the rod-shaped baculovirus BV. (d) The relative molar ratios of GP64 and VP39 proteins present in fraction 13 are indicated. Protein quantities were estimated by interpolation from a standard curve of BSA protein (not shown), and molar ratios were estimated based on the molecular weight of each protein.

(TEM), and the presence of BVs was confirmed (Fig. 2c, fraction 13). The approximate molar ratio of GP64 and VP39 proteins in the major BV fraction (2:1) is also indicated (Fig. 2d).

In parallel, we examined the production of VLPs from Sf9 cells infected with virus vGAGHIV-1, which expresses the HIV-1 Gag protein (Fig. 3). Proteins in each gradient fraction were separated, stained and analysed as above. Two major peaks of the Gag protein were observed. The largest peak of Gag was identified at a density of approximately 1.17 g ml^{-1} (Fig. 3a, b, fractions 10–11), and a second slightly less intense peak of Gag was coincident with the major peaks of GP64 and VP39 at approximately 1.19 g ml^{-1} (fraction 14). The larger peak of Gag protein at 1.17 g ml^{-1} is indicative of VLPs. The presence of relatively low quantities of both GP64 and VP39 in the same peak suggests that either these proteins may be found within VLPs or more likely, that a low quantity of BVs was co-purifying with VLPs. It is expected that VLPs might acquire GP64 during the budding process but because VP39 should only be found in the cytoplasm as assembled nucleocapsids, it

would seem less likely that VP39 could be incorporated into VLPs budding at the surface. To address these questions, particles purified from fraction 11 were analysed by negative staining and TEM. As expected, we detected numerous spherical structures consistent with previously described HIV-1 VLPs. In addition, occasional rod-shaped structures that appear to be AcMNPV BVs or nucleocapsids were also present in this fraction (Fig. 3c, fraction 11, closed arrow). Thus, AcMNPV BV particles were not clearly separated from VLPs. The other peak of Gag (Fig. 3a, b, fraction 14, 1.19 g ml^{-1}) contains maximal levels of GP64 and VP39, indicating that Gag VLPs may also be co-purified with the BV fraction. The approximate molar ratios of Gag, GP64 and VP39 (3.7:1.4:1) in the major VLP fraction are also indicated (Fig. 3d). Thus, VLPs were highly enriched in the 1.17 g ml^{-1} fraction but that fraction also contained AcMNPV BVs.

Production of GP64-free VLPs

It was previously shown that GP64 is an essential baculovirus protein, required for efficient budding and for production of infectious BVs. The production of an infectious

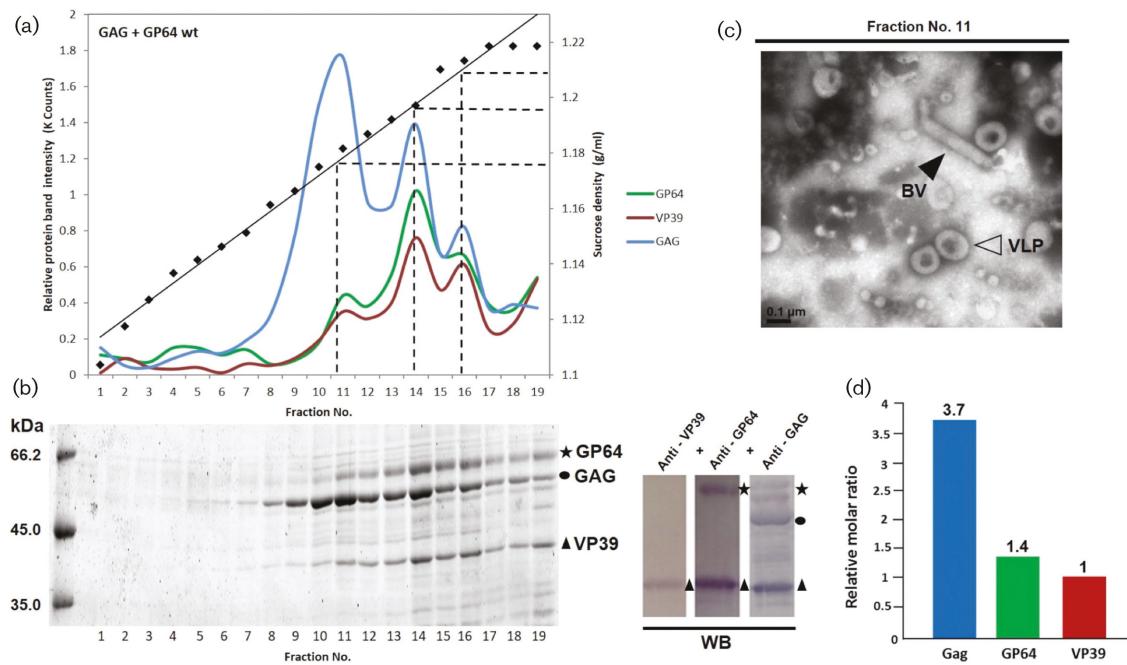


Fig. 3. Analysis of AcMNPV BVs and HIV-1 Gag VLPs by sucrose density gradient fractionation. Gradient fractions were prepared and analysed as described in the legend to Fig. 2. (a, b) Proteins GP64 (green line), VP39 (red line) and HIV-1 Gag (blue line) were detected and quantified by Coomassie Blue staining and infra-red imaging. Proteins were identified by Western blot analysis of fraction 11 using an anti-GP64 monoclonal antibody (star), an anti-Gag monoclonal antibody (closed circle) and an anti-VP39 polyclonal antibody (triangle). (c) Negative staining of fraction 11 showing BVs (closed arrowhead) and VLPs (open arrowhead) in the fraction. (d) Quantification and relative molar ratios of the GP64, VP39 and Gag proteins present in fraction 11 were determined as described above.

recombinant baculovirus containing a *gp64* gene deletion (a GP64null AcMNPV virus) can be accomplished using a complementing cell line that constitutively expresses a heterologous GP64 protein [16, 25]. Because of the difficulty in separating VLPs and BVs, a GP64null recombinant baculovirus expressing Gag (Fig. 1c) was constructed (vGAGHIV-1-GP64null) with the goals of: (a) producing VLPs with no GP64 in the envelope and (b) generating few baculovirus BV particles. To produce and propagate this Gag-expressing GP64null virus, we used cell line Sf9^{Op1D} (which constitutively expresses the GP64 protein from baculovirus OpMNPV [26]). A virus preparation produced in Sf9^{Op1D} cells was then used to infect Sf9 cells (m.o.i. 1) for analysis of VLP production in the absence of GP64 production. First, infected Sf9 cells were collected at 72 h post infection (p.i.) and examined by Western blot analysis for the presence of GP64. We detected no GP64 protein in Sf9 cells infected with vGAGHIV-1-GP64null, confirming the absence of detectable GP64 protein (Fig. 4, centre panels, lane 4). In contrast, GP64 was detected in control cells infected with WT AcMNPV or vGAGHIV-1 (Fig. 4, centre panels, lanes 2–3). In addition, the baculovirus capsid protein VP39 was detected in all infected cells, and Gag protein was detected in both vGAGHIV-1 and vGAGHIV-1-GP64null virus-infected Sf9 cells.

Characterization of GP64-free HIV-1 VLPs

We next examined the envelopes of VLPs produced in Sf9 cells infected with either vGAGHIV-1 or vGAGHIV-1-GP64null. After infecting cells at an m.o.i. of 1 and incubating for 72 h p.i., infected cells were prepared for immunogold labelling and TEM. The GP64 protein was readily detected on the surfaces of VLPs budding from Sf9 cells infected with vGAGHIV-1 (Fig. 5a, c), but was not detected on VLPs budding from Sf9 cells infected with vGAGHIV-1-GP64null (Fig. 5b, d). VLPs produced from cells infected with vGAGHIV-1 and vGAGHIV-1-GP64null were also purified by ultra-centrifugation through a sucrose cushion and analysed by negative staining and TEM (Fig. 5e, f). The observation of reduced BVs (by electron microscopy) in purified particles isolated from cells infected with the GP64null virus expressing HIV-1 Gag (Fig. 5f) is consistent with prior studies [16] showing dramatic quantitative reductions of baculovirus BVs in GP64null infections.

DISCUSSION

The production of VLPs for use as recombinant vaccines or gene therapy vectors represents a powerful tool in medical biotechnology. However, the production of enveloped VLPs can be costly and inefficient when produced in mammalian cell lines, and the presence of adventitious agents from

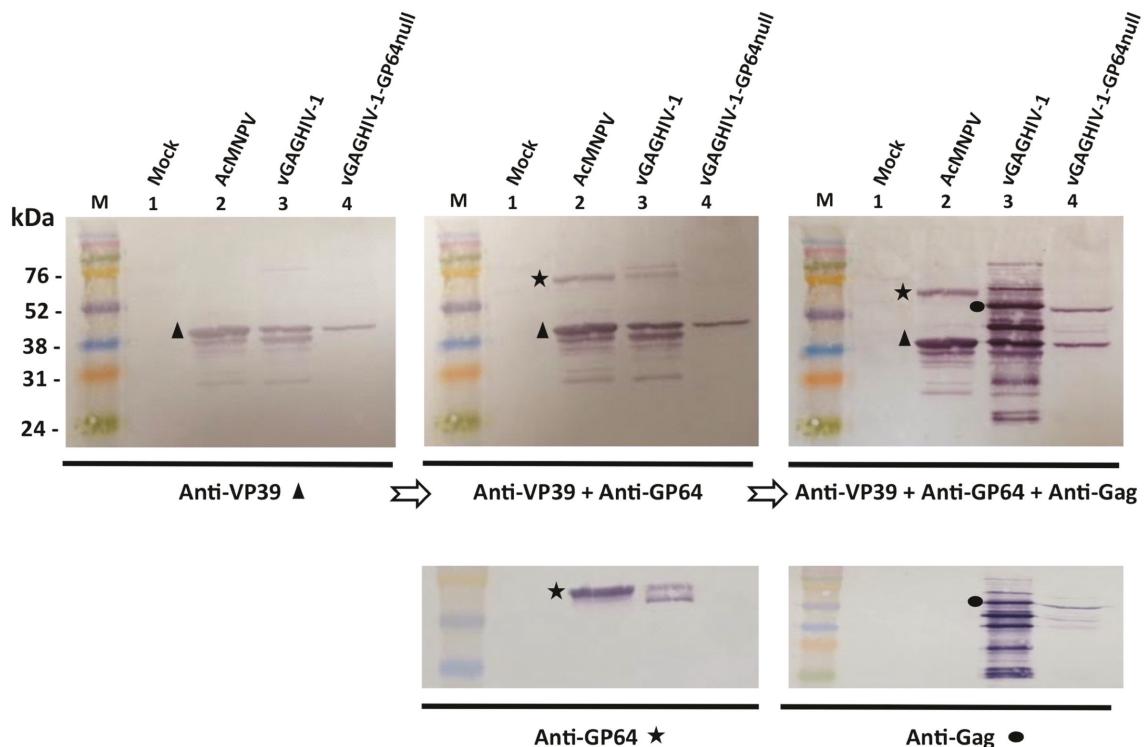


Fig. 4. GP64 detection in insect cells infected with viruses WT AcMNPV, vGAGHIV-1 and vGAGHIV-1-GP64null. Sf9 cells were infected with WT AcMNPV (lane 2), vGAGHIV-1 (lane 3) or vGAGHIV-1-GP64null (lane 4) baculoviruses, or mock-infected as a negative control (lane 1). At 72 h p.i., cells were collected and proteins separated by SDS-PAGE, and analysed by Western blot analysis for the presence of proteins VP39 (triangle), GP64 (star) and Gag (closed circle). Upper panels: the membrane was first incubated with a polyclonal anti-VP39 antibody, followed by detection, then washed and incubated with an anti-GP64 MAb. After detection, the blot was again washed, incubated with an anti-GAG MAb and bound antibody detected. Note, while the Gag antibody is directed against the Gag Pr55 precursor, proteolytic cleavage products can also be detected (p55, p47, p41 and p24). Lower panels: membranes with the same samples shown above were also individually incubated with each antibody separately (as indicated), to confirm the identity of each protein. M represents the molecular weight marker (Amersham RPN800E) and molecular weights are indicated in kDa on the left panel.

mammalian cells may be a concern. Because the BEVS represents a non-vertebrate heterologous expression system, and expression levels can be exceptionally high, baculovirus expression represents a highly attractive system for VLP production in terms of both safety and production costs [27, 28]. Another important feature of the baculovirus-insect cell system is the presence of complex eukaryotic post-translational modifications such as glycosylation, acylation, phosphorylation, etc. on proteins expressed in this system [29, 30].

The expression of retroviral Gag proteins as a means of producing VLPs in the BEVS has been explored in some detail [31–34]. Lynch *et al.* [35] produced Pr55^{Gag} VLPs using the BEVS and examined the stability of these particles after storage. They found that such VLPs can be stored for up to 12 months, with VLPs retaining stability. Pillay *et al.* [36] optimized HIV-1 Gag-mediated VLP production by monitoring conditions such as cell density at infection, baculovirus dose and timing of infection. While a variety of such studies have examined HIV-1 Gag-based VLPs, no study to date has effectively separated the large quantities of baculovirus BV

particles from the VLPs produced in the viral infection cycle [37, 38]. Although separating VLPs and BV particles by techniques such as size exclusion or affinity chromatography may be possible, such techniques are typically expensive, inefficient, and often difficult steps in VLP preparation. Thus, separation of VLPs and BVs remains a substantial problem. While the presence of the baculovirus particles (which are not infectious to mammals) does not represent a significant problem for many applications, the presence of the major BV envelope glycoprotein, GP64, may be problematic as GP64 is highly immunogenic [18]. When HIV-1 Gag VLPs bud from AcMNPV-infected insect cells, the envelope is derived from the host cell membrane that typically contains large quantities of the GP64 protein. A recent study showed that each HIV-1 VLP produced using the BEVS, has approximately 1 GP64 molecule for every three Gag molecules [14] and we observed similar molar ratios in the current study (Fig. 3d). It is of special note that in some applications like gene therapy, the presence of GP64 on VLPs has been shown to enhance the efficiency of transduction of mammalian cells. Indeed, lentiviral (HIV-1 and other) vectors pseudotyped with GP64 have

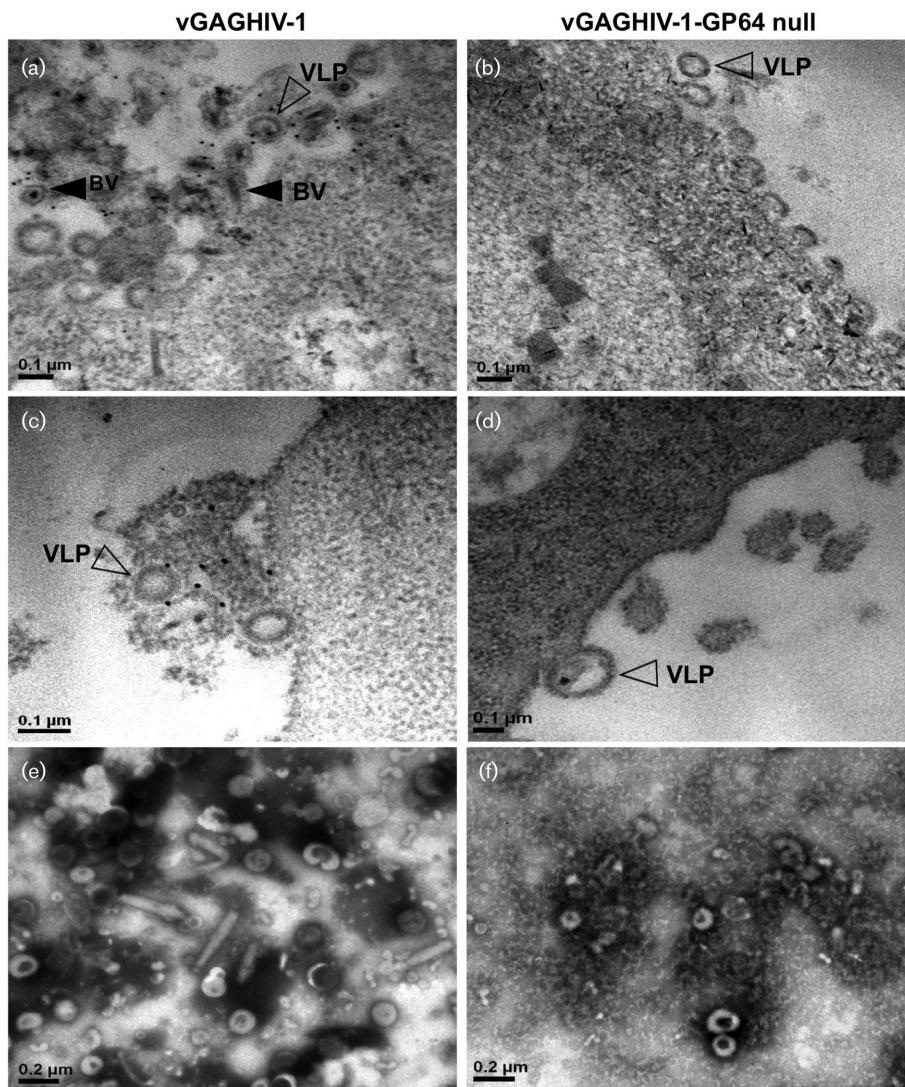


Fig. 5. Transmission electron microscopic analysis of HIV-1 VLPs produced in Sf9 cells. Sf9 cells infected at m.o.i. 1 with either virus vGAGHIV-1 or vGAGHIV-1-GP64null were harvested at 72 h p.i. and prepared for TEM. For immunogold labelling (a–d) a monoclonal antibody against GP64 (AcV1) was used, followed by incubation with an anti-mouse IgG conjugated with 10 nm gold particles. Supernatants from infected cells were also used for isolation of BVs and VLPs by ultracentrifugation. The two lower panels show negative staining of BVs and VLPs prepared from supernatants of Sf9 cells infected with either vGAGHIV-1 (e) or vGAGHIV-1-GP64null (f).

an increased transduction efficiently in numerous cell types and in animal models [39–41]. However, when VLPs are used as human vaccine candidates to display immunogens, the presence of GP64 may not be desirable [14] and indeed, the presence of GP64 in the cell membrane may compete with the target immunogen for sites on the VLP surface, thus reducing the effectiveness of the VLP vaccine.

Since (a) it is not simple to efficiently separate BVs from VLPs and (b) GP64 on VLPs can be problematic for some applications, we constructed a GP64null recombinant baculovirus expressing the HIV-1 Pr55^{Gag} precursor in order to produce VLPs that are free of the baculovirus GP64 protein, and with reduced BV levels. Prior studies showed that

deletion of the *gp64* gene decreases the quantity of BVs produced by approximately 90–95 % [16]. Because the residual BVs contain no GP64 protein and are not infectious, this provides an additional safety factor for VLP preparations produced in this manner. A limitation of this system however, is the fact that the GP64null virus cannot amplify except in the producer (Sf9^{Op1D}) cells, and this often leads to lower yields during VLP production. Improvements in producer cell lines or very high m.o.i. infections could possibly address these issues. More recently, a method for producing recombinant products free of AcMNPV BVs was reported [42] and that method also used a knockout in a gene important for BV production, *vp80*. The *vp80* gene is essential for viral replication and the *vp80* knockout results

in the absence of normal nucleocapsids, ODV, and BV. A similar strategy (a stable cell line expressing VP80) was used to complement the *vp80null* virus. While the *vp80null* strategy likely results in the absence of BVs (as compared with only a reduction in BVs using the *gp64null* strategy), the GP64 protein remains in the *vp80null* system and when budded VLPs are produced, GP64 may be found decorating subsequent VLPs. Thus, for VLPs from enveloped viruses, some combination of these strategies may be desirable in order to achieve optimal reduction of AcMNPV BVs and the absence of AcMNPV GP64 on enveloped VLPs. In the current study we combined for the first time, HIV-1 Gag-mediated VLP production with a GP64null system for producing VLPs in the absence of GP64. The use of a GP64null baculovirus represents an important advance in BEVS production of enveloped VLP-based vaccines, and a technology with potential for use in the development of other applications in biotechnology and medicine.

METHODS

Insect cells

Spodoptera frugiperda Sf9 cells [43] were maintained in suspension shaker cultures at 100 rpm at 27 °C in *Trichoplusia ni* medium Fred Hink (TNMFH) insect medium [44] supplemented with 10 % fetal bovine serum (FBS), 1 % antibiotic/antimycotic (100 units ml⁻¹ of penicillin, 100 µg ml⁻¹ of streptomycin and 0.25 µg ml⁻¹ of amphotericin B) and 0.1 % (w/v) Pluronic F-68 (Gibco). *Spodoptera frugiperda* Sf9^{Op1D} [26, 45] and Sf9-ET cells [46] were maintained as monolayers at 28 °C in TNMFH insect medium supplemented with 10 % FBS and 1 % antibiotic/antimycotic. Sf9^{Op1D} cells constitutively express the GP64 protein from the *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) [45] which complements the deletion of the *gp64* gene in various recombinant baculoviruses. The presence of functional GP64 at the cell surface of Sf9^{Op1D} cells was confirmed by a syncytium formation assay as described previously [26, 47, 48].

Wild-type and recombinant viruses

For construction of baculoviruses expressing the HIV-1 Gag protein, recombinant baculoviruses were generated using the pFastBac plasmid pFB-HIV-2gb that contains the full-length HIV-1 Pr55^{Gag} (Gag) gene under control of the polyhedrin promoter. pFB-HIV-2gb was kindly provided by Volker Vogt (Cornell University) and was used to construct the recombinant baculovirus vGAGHIV-1 using the Invitrogen Bac-to-Bac system [49]. For the production of the recombinant baculovirus vGAGHIV-1-GP64null, we used the same pFastBac plasmid (pFB-HIV-2gb), but with a bacterial strain carrying a *gp64null* AcMNPV bacmid (*gp64*⁻ bacmid-DH10B+pMON7124) [45]. To propagate the WT AcMNPV virus (strain E2) [50] and the vGAGHIV-1 recombinant virus, Sf9 cells were infected (m.o.i. 1) and the supernatant was collected at 72 h p.i. and titred. The same procedure was used to propagate the vGAGHIV-1-GP64null recombinant virus, but using Sf9^{Op1D} cells. For

virus titrations, end-point dilution assays were performed as described previously using Sf9-ET cells [46] and scoring for eGFP positive cells at 3–7 days p.i. [51, 52].

Production and purification of VLPs

To produce VLPs, Sf9 cells were infected with viruses (AcMNPV, vGAGHIV-1 and vGAGHIV-1-GP64null) under different conditions. For infections with AcMNPV or vGAGHIV-1 viruses, cells were infected at a density of 5 × 10⁵ cells ml⁻¹ and m.o.i. 0.1. For infections with virus vGAGHIV-1-GP64null (a virus that does not propagate infection from cell to cell in Sf9 cells), Sf9 cells were infected at a density of 1 × 10⁶ cells ml⁻¹ and m.o.i. 1. After 72 h p.i. the supernatant was collected and clarified by centrifugation at 2000 g for 5 min at 4 °C. VLPs were pelleted from the supernatant by ultra-centrifugation at 100 000 g for 2 h through a 25 % sucrose cushion, then resuspended in 1 × PBS (100 mM Na₂HPO₄, 17 mM KH₂PO₄, 1.4 mM NaCl and 27 mM KCl, pH 7.4). The semi-purified VLPs were loaded onto a 30–60 % (w/v) sucrose gradient and centrifuged to equilibrium (18 h at 90 000 g). Fractions of 600 µl were collected from the 12 ml gradient. From each fraction, 20 µl was used for density determination using a refractometer, 280 µl was used for microscopic analysis and 300 µl was used for virion and VLP analysis by SDS-PAGE. To each 300 µl fraction, 900 µl of 1x PBS (pH 7.4) was added and mixed, then virus particles or VLPs were pelleted for 2 h at 100 000 g. Virions or VLPs in each fraction were resuspended in 2x Laemmli buffer [53] and incubated for 5 min at 95 °C, then stored at –20 °C.

Analysis of VLPs

Proteins from purified virions and VLPs from all fractions were analysed by SDS-PAGE and quantified [53]. After electrophoresis, proteins were stained with Coomassie Brilliant Blue (40 % methanol, 10 % acetic acid and 0.1 % Coomassie Brilliant Blue R-250) for 18 h at room temperature, destained in a destain solution (30 % methanol, 7 % acetic acid) for 18 h at room temperature, and the protein profile of each fraction was analysed by densitometry using infrared detection with a Li-Cor Biosciences Odyssey Infrared Imaging System (Li-Cor Biosciences). For quantification, varying quantities of BSA (8.8, 4.4, 2.2, 1.1 and 0.5 µg) were loaded onto the gel and measured to generate a standard curve. Sample fractions were also loaded onto a parallel 12 % SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane (GE Healthcare) for Western blot analysis. Membranes were blocked in 3 % skim milk in 1x PBS for 1 h at room temperature (RT), or overnight at 4 °C. Membranes were then washed three times (5 min per wash) with a 1x PBS solution containing 0.05 % Tween and incubated for 1 h at RT with the primary antibody. Primary antibodies consisted of AcV5, a mouse monoclonal anti-GP64 antibody [54, 55] diluted 1 : 2000; a rabbit polyclonal anti-VP39 antibody diluted 1 : 5000, and anti-GAG HIV-1 p24 (NIH AIDS Reagent Program 6457) diluted 1 : 10 000. Membranes were washed three times (5 min per wash) as above, then incubated with an alkaline phosphatase (AP)

conjugated secondary antibody (goat anti-mouse IgG, Sigma) diluted 1:10 000, for 1 h at RT. After incubation, the membranes were washed three times as above and specific proteins were detected with NBT/BCIP (Invitrogen) diluted in distilled water, as described by the manufacturer.

Analysis of infected cells

Sf9 cells in suspension (5×10^5 cells ml $^{-1}$) were infected with each of the viruses (m.o.i. 1) and collected at 72 h p.i. by centrifugation at 2000 g for 5 min at 4 °C. The cell pellet was resuspended in 2× Laemmli buffer [53], proteins were denatured at 95° for 5 min, and the proteins were separated by electrophoresis in a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (GE Healthcare) for Western blot analysis using anti-VP39, anti-GP64 and Anti-Gag antibodies, as described above. The membrane containing AcMNPV-, vGAGHIV-1- and vGA-GHIV-1-GP64null-infected cell extracts was challenged with one primary antibody (followed by the appropriate secondary antibody and substrate) and imaged as described above. Shortly after development, the membrane was washed, blocked and incubated with the second primary antibody (followed by the appropriate secondary antibody and substrate), and imaged. After development the membrane was then washed, blocked and incubated with the third primary antibody (followed by the appropriate secondary antibody and substrate). Thus, the same membrane was challenged and imaged sequentially with three different primary antibodies (Anti-VP39, Anti-GP64 and Anti-Gag). The same experiment was also performed using membranes incubated with only a single primary antibody.

Electron microscopy

Purified (sucrose gradient) or semi-purified (sucrose cushion) VLPs were pipetted onto glow-discharged carbon-coated grids and incubated for 30 min. The grids were then washed three times with 1x PBS, negatively stained for 2 min with 2% phosphotungstic acid (PTA) [56], and samples analysed on a JEOL 1011 TEM. For immunogold staining, Sf9 cells were infected (m.o.i. 1) with either vGAGHIV-1 or vGAGHIV-1-GP64null. At 72 h p.i., the cells were collected by centrifugation at 2000 g for 5 min at 4 °C, washed in 1x PBS and fixed in a solution of 4% paraformaldehyde, 0.5% glutaraldehyde and 0.01% picric acid in 0.1 M sodium cacodylate buffer pH 6.4 with 5% sucrose. After fixation, cells were washed twice in 0.1 M sodium cacodylate buffer (pH 6.4) followed by 1 h incubation in 50 mM ammonium chloride (pH 7.2). Samples were washed twice in distilled water and contrasted by incubation overnight with 2% uranyl acetate in the dark at 4 °C. The samples were washed twice in distilled water then dehydrated in increasing concentrations of acetone (30–90%) for 10 min at each concentration. Finally, samples were embedded in LR gold resin and incubated for 72 h at –20 °C under ultraviolet light. Thin sections were generated with a Leica EM UC7 ultramicrotome, placed on nickel grids and incubated 30 min in 80 mM ammonium chloride diluted in 1x PBS. The nickel grids containing the samples were incubated 1 h with blocking solution (0.01% Tween 20, 1.5% BSA and

1x PBS) at RT, then incubated for 1 h with primary antibody (anti-GP64 monoclonal antibody AcV1, which recognizes a conformational epitope on GP64) [55, 57] in blocking solution (1:50). Afterwards, the grids were washed three times in 1x PBS followed by incubation with a secondary antibody (anti-mouse IgG conjugated with colloidal gold particles of 10 nM, 1:20 dilution; Sigma Immuno Chemicals). Samples were then washed twice in 1x PBS, once in distilled water, and analysed by TEM.

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Conflicts of interest

LCSC and BMR declare that there are no conflicts of interest. GWB is a coinventor on a US patent based on concepts described in this work.

References

1. Naskalska A, Pyrc K. Virus like particles as immunogens and universal nanocarriers. *Pol J Microbiol* 2015;64:3–13.
2. Yan D, Wei YQ, Guo HC, Sun SQ. The application of virus-like particles as vaccines and biological vehicles. *Appl Microbiol Biotechnol* 2015;99:10415–10432.
3. Koho T, Ihalainen TO, Stark M, Uusi-Kerttula H, Wieneke R et al. His-tagged norovirus-like particles: a versatile platform for cellular delivery and surface display. *Eur J Pharm Biopharm* 2015;96: 22–31.
4. Kushnir N, Streatfield SJ, Yusibov V. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* 2012; 31:58–83.
5. Roy P, Noad R. Virus-like particles as a vaccine delivery system: myths and facts. *Hum Vaccin* 2008;4:5–12.
6. Chackerian B, Lenz P, Lowy DR, Schiller JT. Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J Immunol* 2002;169:6120–6126.
7. Kirchmeier M, Fluckiger AC, Soare C, Bozic J, Ontsouka B et al. Enveloped virus-like particle expression of human cytomegalovirus glycoprotein B antigen induces antibodies with potent and broad neutralizing activity. *Clin Vaccine Immunol* 2014;21:174–180.
8. Roldão A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM. Virus-like particles in vaccine development. *Expert Rev Vaccines* 2010;9:1149–1176.
9. Liu F, Wu X, Li L, Liu Z, Wang Z. Use of baculovirus expression system for generation of virus-like particles: successes and challenges. *Protein Expr Purif* 2013;90:104–116.
10. Ren Z, Ji X, Meng L, Wei Y, Wang T et al. H5N1 influenza virus-like particle vaccine protects mice from heterologous virus challenge better than whole inactivated virus. *Virus Res* 2015;200:9–18.
11. Thompson CM, Aucoin MG, Kamen AA. Production of virus-like particles for vaccination. *Methods Mol Biol* 2016;1350:299–315.
12. Buonaguro L, Tagliamonte M, Tornesello ML, Buonaguro FM. Developments in virus-like particle-based vaccines for infectious diseases and cancer. *Expert Rev Vaccines* 2011;10:1569–1583.

13. Oomens AG, Monsma SA, Blissard GW. The baculovirus GP64 envelope fusion protein: synthesis, oligomerization, and processing. *Virology* 1995;209:592–603.
14. Valley-Omar Z, Meyers AE, Shephard EG, Williamson AL, Rybicki EP. Abrogation of contaminating RNA activity in HIV-1 Gag VLPs. *Virol J* 2011;8:462.
15. Hefferon KL, Oomens AG, Monsma SA, Finnerty CM, Blissard GW. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. *Virology* 1999;258:455–468.
16. Oomens AG, Blissard GW. Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. *Virology* 1999;254:297–314.
17. Rohrmann GF. *Baculovirus Molecular Biology*. Maryland: Bethesda; 2013.
18. Luo WY, Lin SY, Lo KW, Lu CH, Hung CL et al. Adaptive immune responses elicited by baculovirus and impacts on subsequent transgene expression in vivo. *J Virol* 2013;87:4965–4973.
19. Wagner R, Fliessbach H, Wanner G, Motz M, Niedrig M et al. Studies on processing, particle formation, and immunogenicity of the HIV-1 gag gene product: a possible component of a HIV vaccine. *Arch Virol* 1992;127:117–137.
20. Provitera P, El-Maghrabi R, Scarlata S. The effect of HIV-1 Gag myristylation on membrane binding. *Biophys Chem* 2006;119:23–32.
21. Morikawa Y, Hinata S, Tomoda H, Goto T, Nakai M et al. Complete inhibition of human immunodeficiency virus Gag myristylation is necessary for inhibition of particle budding. *J Biol Chem* 1996;271: 2868–2873.
22. Jouvenet N, Bieniasz PD, Simon SM. Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature* 2008;454:236–240.
23. Gheysen D, Jacobs E, de Foresta F, Thiriart C, Francotte M et al. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* 1989;59:103–112.
24. Dick RA, Vogt VM. Membrane interaction of retroviral Gag proteins. *Front Microbiol* 2014;5:187.
25. Monsma SA, Oomens AG, Blissard GW. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J Virol* 1996;70:4607–4616.
26. Plonsky I, Cho MS, Oomens AG, Blissard G, Zimmerberg J. An analysis of the role of the target membrane on the Gp64-induced fusion pore. *Virology* 1999;253:65–76.
27. van Oers MM. Vaccines for viral and parasitic diseases produced with baculovirus vectors. *Adv Virus Res* 2006;68:193–253.
28. Metz SW, Pijlman GP. Arbovirus vaccines; opportunities for the baculovirus-insect cell expression system. *J Invertebr Pathol* 2011; 107:S16–S30.
29. Kost TA, Condreay JP, Jarvis DL. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol* 2005;23:567–575.
30. Emery VC. Baculovirus expression vectors : choice of expression vector. *Methods Mol Biol* 1992;8:287–307.
31. Nermut MV, Hockley DJ, Bron P, Thomas D, Zhang WH et al. Further evidence for hexagonal organization of HIV gag protein in prebudding assemblies and immature virus-like particles. *J Struct Biol* 1998;123:143–149.
32. Luo L, Li Y, Kang CY. Expression of gag precursor protein and secretion of virus-like gag particles of HIV-2 from recombinant baculovirus-infected insect cells. *Virology* 1990;179:874–880.
33. Luo L, Li Y, Dales S, Kang CY. Mapping of functional domains for HIV-2 gag assembly into virus-like particles. *Virology* 1994;205: 496–502.
34. Yamshchikov GV, Ritter GD, Vey M, Compans RW. Assembly of SIV virus-like particles containing envelope proteins using a baculovirus expression system. *Virology* 1995;214:50–58.
35. Lynch A, Meyers AE, Williamson AL, Rybicki EP. Stability studies of HIV-1 Pr55gag virus-like particles made in insect cells after storage in various formulation media. *Virol J* 2012;9:210.
36. Pillay S, Meyers A, Williamson AL, Rybicki EP. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. *Biotechnol Prog* 2009;25:1153–1160.
37. Chua AJ, Vituret C, Tan ML, Gonzalez G, Boulanger P et al. A novel platform for virus-like particle-display of flaviviral envelope domain III: induction of Dengue and West Nile virus neutralizing antibodies. *Virol J* 2013;10:129.
38. Li W, Jin H, Sui X, Zhao Z, Yang C et al. Self-assembly and release of peste des petits ruminants virus-like particles in an insect cell-baculovirus system and their immunogenicity in mice and goats. *PLoS One* 2014;9:e104791.
39. Kataoka C, Kaname Y, Taguwa S, Abe T, Fukuhara T et al. Baculovirus GP64-mediated entry into mammalian cells. *J Virol* 2012;86: 2610–2620.
40. Kumar M, Bradow BP, Zimmerberg J. Large-scale production of pseudotyped lentiviral vectors using baculovirus GP64. *Hum Gene Ther* 2003;14:67–77.
41. Oakland M, Maury W, McCray PB, Sinn PL. Intrapulmonary versus nasal transduction of murine airways with GP64-pseudotyped viral vectors. *Mol Ther Nucleic Acids* 2013;2:e69.
42. Marek M, van Oers MM, Devaraj FF, Vlak JM, Merten OW. Engineering of baculovirus vectors for the manufacture of virion-free biopharmaceuticals. *Biotechnol Bioeng* 2011;108:1056–1067.
43. Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In Vitro* 1977;13:213–217.
44. Hink WF. Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature* 1970;226:466–467.
45. Lung O, Westenberg M, Vlak JM, Zuidema D, Blissard GW. Pseudotyping *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. *J Virol* 2002;76:5729–5736.
46. Hopkins R, Esposito D. A rapid method for titrating baculovirus stocks using the Sf-9 easy titer cell line. *Biotechniques* 2009;47: 785–788.
47. Blissard GW, Wenz JR. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J Virol* 1992;66:6829–6835.
48. Yu Q, Blissard GW, Liu TX, Li Z. *Autographa californica* multiple nucleopolyhedrovirus GP64 protein: analysis of domain I and V amino acid interactions and membrane fusion activity. *Virology* 2016;488:259–270.
49. Luckow VA, Lee SC, Barry GF, Olins PO. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol* 1993;67:4566–4579.
50. Smith GE, Summers MD. Restriction maps of five *Autographa californica* MNPV Variants, *Trichoplusia ni* MNPV, and *Galleria mellonella* MNPV DNAs with endonucleases smal, Kpnl, BamHI, SacI, Xhol, and EcoRI. *J Virol* 1979;30:828–838.
51. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493–497.
52. O'Reilly DR, Miller LK, Luckow VA. *Baculovirus Expression Vectors, a Laboratory Manual*. New York: W. H. Freeman and Co; 1992.
53. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
54. Monsma SA, Blissard GW. Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. *J Virol* 1995;69:2583–2595.
55. Hohmann AW, Faulkner P. Monoclonal antibodies to baculovirus structural proteins: determination of specificities by Western blot analysis. *Virology* 1983;125:432–444.

56. Brenner S, Horne RW. A negative staining method for high resolution electron microscopy of viruses. *Biochim Biophys Acta* 1959;34: 103–110.

57. Zhou J, Blissard GW. Mapping the conformational epitope of a neutralizing antibody (AcV1) directed against the AcMNPV GP64 protein. *Virology* 2006;352:427–437.

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