**Bombyx mori** nucleopolyhedrovirus-based surface display system for recombinant proteins

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We describe here the development of a ‘eukaryotic display system’ for heterologous proteins on the viral and host cell surfaces using *Bombyx mori* nucleopolyhedrovirus (BmNPV). The reporter gene gfp (green fluorescent protein) was fused to either the gp64 gene encoding the full-length BmNPV envelope protein GP64 or to its 5′ region encoding only the N-terminal domain harbouring the signal sequence, and recombinant viruses expressing the corresponding fusion proteins under the strong viral polyhedrin promoter were generated. On infection of the host insect *B. mori* or the host-derived BmN cells with the full-length GP64–GFP virus, abundant expression of the recombinant protein and its display on the cell surface were achieved. The fusion protein was also a component of the budded virions. Thus, the BmNPV-based display system provides an alternative to the previously established Autographa californica multinucleocapsid nucleopolyhedrovirus display system. The recombinant virus expressing GFP has also been used in preliminary pathological investigations on virus infection in *B. mori* and provides a simple method for screening for antiviral agents.

**INTRODUCTION**

Viruses belonging to the family *Baculoviridae* have gained importance as expression vectors for heterologous genes in insect-derived cells as well as in the host caterpillar (Luckow & Summers, 1988; Fraser, 1992; King & Possee, 1992). The application mostly hinges on the extremely high expression levels obtained from promoters of the viral very late genes, polyhedrin (polh) and p10. These genes are dispensable for virus propagation *in vitro* and therefore can be replaced with foreign genes to be expressed from these loci.

Host specificity of baculoviruses was thought to be restricted to cells derived from arthropods. More recently, however, *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) has been demonstrated to infect mammalian cell lines such as hepatocytes, non-hepatic cells and nerve cells non-productively and to maintain stably the expression cassette within the cells (Hofmann et al., 1995; Boyce & Bucher, 1996; Shoji et al., 1997; Sarkis et al., 2000). These studies have opened up the additional possibility of exploiting the virus as a gene delivery system, with the major advantages that it accommodates large sizes of foreign DNA and expresses glycoproteins as well as multimeric eukaryotic proteins, which show fastidious folding requirements.

In the conventional baculovirus expression system, the foreign DNA inserted at the polh or p10 or any other convenient locus on the viral genome is expressed in the cellular cytoplasm or secreted out into the medium if the signal sequences are provided. AcMNPV has also been developed as a vector to display foreign proteins on the viral or host cell surfaces, where the foreign gene sequence is fused in frame with a gene encoding one of the virus surface proteins and the hybrid fusion protein that is synthesized is incorporated into virus particles (Boublik et al., 1995; Grabherr et al., 1997; Mottershead et al., 1997; Ernst et al., 1998, 2000; Lindley et al., 2000; Tami et al., 2000; Ojala et al., 2001). The recent developments in the use of baculoviruses for the surface display of complex eukaryotic proteins have been reviewed by Grabherr et al. (2001). The only baculovirus that has been exploited for surface display so far is AcMNPV. In this report we describe the successful development of another baculovirus, *Bombyx mori* nucleopolyhedrovirus (BmNPV), as an alternative eukaryotic display system.

BmNPV is a major pathogen of the mulberry silk worm *B. mori* and is second in popularity only to AcMNPV as a baculovirus expression system. A BmNPV-based expression system is an economic alternative for large-scale synthesis of commercially important biomolecules, since the silkworm larvae, which are easy to rear on a synthetic or natural diet, can be used instead of the cultured cell lines (Maeda, 1989; Palhan et al., 1995; Sumathy et al., 1996; Siriram et al., 1997; Sehgal & Gopinathan, 1998; Acharya et al., 2002). Like its counterpart AcMNPV, BmNPV also encodes a virion surface glycoprotein, GP64, which is responsible for virus entry into the host cells and efficient virion budding. GP64 is a type I transmembrane glycoprotein possessing an N-terminal signal peptide and a C-proximal transmembrane...
domain (Monsma & Blissard, 1995). The virally encoded GP64 protein is incorporated into the host cell membrane, and during the budding process the emerging virus nucleocapsid particles pick up the protein as a constituent of the viral envelope (M. M. Rahman & K. P. Gopinathan, unpublished data). Since gp64 is essential for the virus infection process, a second copy of the gene was introduced at the polh locus of BmNPV for manipulations and to achieve high levels of expression. On infection of the insect hosts or the host-derived cell lines, recombinant viruses with the foreign gene inserted in the gp64 copy at the polh locus lead to the synthesis of substantial amounts of the fusion protein and display it on the infected cell surface as well as on the budded virions. The availability of a GFP-harbouring recombinant virus also provides a simple and direct means of analysing the virus infection process.

METHODS

Baculovirus and host cells. B. mori-derived BmN cells were grown at 27°C in TC-100 insect medium, supplemented with 10% foetal bovine serum and 50 μg gentamicin ml⁻¹, BmNPV-BGL, a local isolate of BmNPV (Palhan & Gopinathan, 1996), was propagated in BmN cells. The budded virions were purified by a series of high- and low-speed centrifugations and banding on sucrose gradients (Palhan & Gopinathan, 1996) and were used for the generation of recombinant virus or for the analysis of viral proteins and genomic DNA.

Transfer plasmids. Full-length gp64 was amplified by PCR from BmNPV genomic DNA (nt 99732–101436; Genbank accession no. L33180) using the primers 5'-CGGAATTCCATGAGTAAAGGAGAAGAA-3' (underlined sequences represent EcoRI and BamHI site within gp64) and genomic DNA. From pBSKS-GP64, the insert was mobilized into the plasmid vector pTSKs-GFP64. From pBSKS-GP64, the insert was mobilized into the plasmid vector pTZ18R as a KpnI–PstI fragment in order to make the BamHI site within gp64 unique. This BamHI site within gp64 was used for in-frame fusion of gfp cDNA. GP64 has an N-terminal domain of 100 amino acids containing the signal sequence. From the full-length gp64, the 5' region (300 bp) encoding the N-terminal domain was released by digestion with EcoRI and BamHI and cloned into the plasmid vector pMAL-c2 to generate pMAL-c2-GP64N.

The gfp gene was PCR-amplified from the plasmid construct pVL1393-GFP (Seghal & Gopinathan, 1998) with or without the translational stop codon, using appropriate oligonucleotide primers to make it in frame with the target gene gp64 (PCR forward primer 5'-CGGAATTCCATGAGTAAAGGAGAAGAA-3' and reverse primer 5'-CGGAATTCCATGAGTAAAGGAGAAGAA-3'; the underlined nucleotides generated a BamHI site). The gfp gene was cloned at the BamHI site of either full-length gp64 or the 5’ terminal (300 nt) region of gp64 to generate GP64GFP and GP64NGFP respectively. The fusion cassettes were released by digestion with EcoRI (for full-length) or EcoRI and PstI (for the 5’ region encoding the N-terminal domain) and mobilized into the baculovirus transfer vector pVL1393, under the control of the AcMNPV polh promoter.

Generation of recombinant virus. For generating the recombinant BmNPVs, the transfer plasmids pVL1393Gfp64GFP and pVL1393Gfp64NGFP (5 μg DNA) were individually transfected into 1 x 10⁶ BmN cells followed by BmNPV infection at 27°C (Stirn et al., 1997). The recombinant viruses were selected based on GFP expression with concomitant loss of polyhedral body formation (gfp⁺, acc phenotypes) and purified by three rounds of plaque purification. Purified recombinant virus was titered by plaque assay, and high-titre stocks (10⁵ p.f.u. ml⁻¹) were used for infecting cells and larvae.

SDS-PAGE and Western blots. The budded virus samples for immunoblot analysis were prepared from 30 ml infected cell culture supernatants by ultracentrifugation (100,000 g for 90 min at 4°C). The virus pellet was solubilized in SDS-PAGE sample buffer containing β-mercaptoethanol, boiled for 5 min and 1/20 of the sample was used for electrophoresis. The protein was transferred on to PVDF membranes using semi-dry electric transfer and probed with anti-GP64 or anti-GFP rabbit antisera. Polyclonal antibody to BmNPV GP64 was raised in rabbits using the bacterially expressed purified protein. Mouse polyclonal antibodies to total BmNPV proteins were raised by administering the purified budded BmNPV virions to mice by repeated injections (three times) and this antisem showed positive reactions with the viral structural proteins including GP64. Rabbit polyclonal anti-GFP antibody was a gift from Dr S. Das of our department. Antibody reactions were detected using anti-rabbit IgG conjugated to horseradish peroxidase (HRP), followed by an ECL kit (Amersham Pharmacia Biotech).

The expression of GP64–GFP and N-terminal GP64–GFP fusion proteins and their subcellular distribution in cytosolic or cell membrane fractions were also detected by Western blots using anti-GFP or anti-GP64 polyclonal antibodies. For this purpose, the recombinant virus-infected cells (either vBmGP64GFP or vBmGP64NGFP; m.o.i. 10) were harvested at 72 h post-infection (p.i.), washed with PBS and lysed by lysis buffer (50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol and 1% Triton X-100). The cytosolic and membrane fractions of infected cells were prepared as described by Tamura et al. (2000). Briefly, the cells were washed twice with PBS and lysed in PBS containing 2 mM PMSF and 1% NP-40 for 1 h at 4°C. Nuclei and unbroken cells were removed by centrifugation for 5 min at 1000 g and the cytosolic and membrane fractions were separated by centrifugation for 30 min at 11,000 g. The samples were analysed on 0.1% SDS-polyacrylamide (8 or 10%) gels.

Confocal microscopy and flow cytometry. BmN cells were infected with the wild-type or recombinant BmNPV (m.o.i. 10). At 48 h p.i., the cells were stained with anti-GP64 or anti-GFP antibodies. For this purpose, mouse polyclonal antibodies to total BmNPV proteins were raised by administering the purified budded BmNPV virions to mice by repeated injections (three times) and this antisem showed positive reactions with the viral structural proteins including GP64. Rabbit polyclonal anti-GFP antibody was a gift from Dr S. Das of our department. Antibody reactions were detected using anti-rabbit IgG conjugated to horseradish peroxidase (HRP), followed by an ECL kit (Amersham Pharmacia Biotech).

Detection of GP64–GFP fusion protein on the virus particle by ELISA. Microtiter plates (96-well) coated with polyclonal anti-GFP antibody (1:500 dilution in PBS at 4°C overnight) were incubated with recombinant and control virus preparations (purified budded virus samples at a protein concentration ranging from 100 ng as the highest and serially diluted twofold) for 1 h at 37°C to allow binding, followed by washing with PBS containing 0.1% Tween-20. The presence of bound virus particles was detected using mouse polyclonal anti-GP64 antibodies (1:250 in PBS and incubation for 1 h at 37°C). Anti-mouse IgG conjugated to HRP (1:2500 in PBS) was allowed to react with this sample for 1 h at 37°C and detected by reaction with o-phenylene diamine dihydrochloride as substrate (A405).

Infection of B. mori larvae. B. mori larvae (strain NB4D2) in the first day of fifth instar were injected with 40 μl of wild-type or recombinant virus (10⁵ p.f.u. ml⁻¹) into the haemocoele at the third abdominal spiracles. The larvae were reared on mulberry leaves for the entire infection period (4–5 days).
RESULTS AND DISCUSSION

Construction of GFP fusion display viruses

A surface display system with BmNPV was developed using the gp64 gene encoding the surface glycoprotein GP64, located at the polh locus. Since gp64 is a single copy gene in BmNPV and is essential for virus propagation, all manipulations of gp64 were undertaken with a second copy of the gene that was PCR-amplified from the BmNPV genome and inserted at the polh locus. The functional domains of gp64 mapped in AcMNPV (Monsma & Blissard, 1995) are also conserved in BmNPV. To enable fusion of heterologous protein to the BmNPV gp64, we made use of a unique BamHI site 300 nucleotides from the +1 ATG of the gp64 ORF, while retaining the functional domains intact (Fig. 1). In-frame fusion of the cDNA for the GFP was made at the BamHI site in order to test the suitability of BmNPV GP64 to direct the incorporation of the resultant fusion protein into the baculovirus particle (designated vBmGP64GFP) and to display the recombinant protein on the host cell membrane. We also generated another recombinant in which GFP was fused with only the N-terminal signal sequence of GP64 (designated vBmGP64NGFP) to analyse the efficiency of cell membrane localization and secretion.

The GP64 fusion plasmids used to generate the recombinant baculoviruses vBmGP64GFP (full-length GP64 fused to GFP) and vBmGP64NGFP (N-terminal GP64 fused to GFP) contained the fusion cassettes under the control of the strong polyhedrin promoter (Fig. 1). The recombinant BmNPVs were made by homologous recombination (Siriram et al., 1997). Although the 5' upstream region of AcMNPV polh present in the transfer vector pVL1393 is not identical to the BmNPV polh upstream, it was still possible to generate recombinants at this locus and the AcMNPV polh promoter was fully functional in BmN cells. These recombinant viruses had the parental gp64, essential for viral infectivity, and a second copy of the gene with the gfp fusion (or only the 5’ region of gp64 encoding the N-terminal domain fused to gfp) under the control of the polyhedrin promoter. The presence of the gp64 fusion cassette in the recombinant virus genomes was confirmed by PCR amplification using appropriate primer combinations. Unlike the prototype baculovirus AcMNPV where different kinds of transfer vectors as well as improved methods of generating recombinants are available, in BmNPV the only method for generating recombinants is homologous recombination and still relies on the cumbersome method of occ- phenotype for recombinant selection. However, since the recombinants generated here should harbour the gfp gene, the selection of recombinants based on positive GFP expression (together with the occ- phenotype) made the process easier. The recombinant viruses were purified through three rounds of plaque isolation.

The titres of the recombinant viruses generated were similar to the wild-type virus and were stable in subsequent passages. Such recombinant viruses synthesized substantial amounts of the fusion protein and displayed it on the infected cell surface.

Expression and localization of GP64 fusion proteins

In BmN cells infected with vBmGP64GFP, the fusion protein was present in the cytoplasm and membrane of infected cells (Fig. 2a–c). However, the cells infected with the virus vBmGFP where the gfp gene was directly under the control of the polh promoter (Sehgal & Gopinathan, 1998) showed even more intense fluorescence with GFP being distributed all over the cell (Fig. 2g–i).

GFP fusion to GP64 did not affect virus maturation or the localization of GP64 on the cell membrane. Thus, this system permitted the expression of intact proteins, which were displayed on the cell surface. The fusion of the N-terminal GP64 and GFP was also expressed in the cytoplasmic regions (Fig. 2d–f). Although we had anticipated that the recombinant GFP would be secreted out of the cell due to the presence of secretory signal sequences and the lack of a transmembrane domain in this construct, there was no secretion of GFP into the medium, as judged visually and by analysis by spectrofluorometry and Western blots of the extracellular media, even after concentration of the medium. This suggested that, unlike AcMNPV, the BmNPV GP64 signal sequence alone may not be sufficient for protein secretion (Wang et al., 1995).

The membrane and cytoplasmic localization of the expressed GP64–GFP fusion protein was also confirmed by Western blot analysis of the subcellular fractions from cells infected with the recombinant virus using anti-GFP antibodies (Fig. 3A). In the cytosolic fraction, in addition to the 93 kDa band corresponding to the GP64–GFP fusion protein, protein bands around 66–70 and 56 kDa were also seen, arising due to partial degradation of the full-length fusion protein (Fig. 3A, lane 2). The degradation of the full-length GP64–GFP fusion protein was confirmed in Western blots using GP64 antibodies (data not shown). The membrane fractions, however, showed the presence of only the 93 kDa full-length fusion protein (Fig. 3A, lane 3). The specificity of the GFP antibody was clear from the absence of any interactive protein bands in the control lanes 1 and 4, containing the cytosolic and membrane fractions, respectively, resulting from the wild-type BmNPV-infected BmN cells. Extensive degradations of N-terminal GP64–GFP...
fusion protein in the cellular cytosolic fraction was also evident (Fig. 3A, lane 5; the predicted size of the intact fusion protein is 39–40 kDa). In addition, this N-terminal GP64–GFP fusion protein was not detected in the membrane fraction as anticipated, due to the absence of transmembrane anchoring signals in this fusion protein (Fig. 3A, lane 6).

There was no noticeable degradation of GFP expressed directly from the polh promoter in vBmGFP and the intrinsic fluorescence levels were much higher compared with the fusion protein. It is likely that fusion of GFP to GP64 resulted in the formation of sites susceptible to proteolytic degradation or that the fusion altered the conformation of the protein to expose proteolytic sites.

The mean intensity of fluorescence in cells infected with recombinant virus vBmGP64 (where gfp was directly under the polh promoter), when quantified by flow cytometry, showed a tenfold higher intensity than vBmGP64GFP.
and vBmGP64NGFP. In fact, the observed distribution of GFP throughout the cell including the nuclear region (see Fig. 2g–i) could be due to the diffusion of the protein within the cell, due to the very high concentrations of the expressed protein. Since the GFP expression in all these instances was achieved from the polh promoter, the apparent differences in GFP levels could be due to partial quenching of GFP fluorescence when fused to GP64 (full-length or the N terminus) or the relative instability of the fusion protein leading to its degradation (see earlier).

The full-length GP64–GFP fusion protein was also incorporated into the budded virus particles arising from the recombinant virus infection (Fig. 3B). The presence of both native GP64 (64 kDa) and the full-length GP64–GFP fusion protein (93 kDa) was evident in the purified virus particles (Fig. 3B, lane 3 compared with lane 1 showing only the parental GP64 in wild-type BmNPV infection). The presence of the 93 kDa fusion protein associated with the purified budded virions was also confirmed using anti-GFP antibodies (lane 6). Clearly no fusion protein of N-terminal GP64–GFP was associated with the budded virions of vBmGP64NGFP (harbouring only the N-terminal domain of GP64 fused to GFP) nor wild-type BmNPV showed binding to the GFP antibodies, thus confirming the absence of the expressed recombinant protein on these virus particles.

Expression and localization of GP64–GFP fusion proteins in insect tissues infected with recombinant viruses

The gfp-tagged virus was successfully exploited to express the displayed protein in different tissues of B. mori larvae. The recombinant BmNPV generated here was as efficient as the wild-type BmNPV in infectivity in cell culture, as well as in different tissues of the host larvae. The display of the recombinant proteins on the cell surface of the infected animals seen here is novel and has not been reported in the previously established AcMNPV-based display system.

When the larvae were infected with the recombinant budded virus of vBmGP64GFP by direct injection into the haemocoel, the haemocytes showed expression of GFP in the cytoplasm and membrane by 24 h p.i. (Fig. 4A, top panels). The nuclear region (stained by DAPI) was totally free of the expressed protein (Fig. 4A, panels b and c) unlike infection with vBmGFP where the entire cell structure including the nuclear region was covered with the expressed GFP (Fig. 4A,
Fig. 3. Intracellular localization of GP64–GFP fusion proteins. (A) BmN cells infected with recombinant viruses vBmGP64GFP or vBmGP64NGFP (m.o.i. 10) were harvested at 72 h p.i. The cytosolic and membrane fractions were subjected to electrophoresis on 0-1% SDS-polyacrylamide (8 or 10%) gels, Western blotted and probed using rabbit polyclonal anti-GFP antibody. The antibody reactions were monitored by HRP-conjugated secondary antibodies and an ECL detection kit. Lanes 1 and 4, cytosolic and membrane fractions, respectively, from wild-type BmNPV-infected BmN cells included as control; lanes 2 and 3, full-length GP64–GFP recombinant virus-infected cell fractions; lanes 5 and 6, N-terminal GP64–GFP virus-infected cell fractions. (B) Virus association of the GP64–GFP fusion protein. The budded viruses produced by infection of BmN cells with vBmGP64GFP or vBmGP64NGFP were purified by ultracentrifugation. The virus preparations were solubilized in lysis buffer containing 2% SDS and subjected to 0-1% SDS-8% PAGE. As a control, wild-type BmNPV was also included. The samples were Western blotted and probed using anti-GP64 antibody (lanes 1–3) or anti-GFP antibodies (lanes 4–6). The titres of recombinant budded viruses were generally higher than the budded virus titres in wild-type infection, where a part of the viral progeny is packaged into polyhedral bodies. The difference in band intensities in different lanes was due to variations in virus concentrations. Lanes are as marked and molecular size markers are indicated. (C) Surface localization of GFP tested by ELISA. The budded viruses were purified by ultracentrifugation, serially diluted twofold from an initial concentration of ~5 μg ml⁻¹ (100 ng protein per well) and added to ELISA plates that had been precoated with polyclonal rabbit anti-GFP antibodies (diluted 1:500). The anti-GP64 mouse antibody was used to locate virus bound to the anti-GFP antibody. Detection was carried out using an anti-mouse IgG–HRP conjugate. Values on the y-axis show A₄₉₀ readings and numbers on the x-axis correspond to reciprocal dilutions of virus samples.

Fig. 4. Detection of virus infection in larval tissues. (A) Haemocytes from larvae infected with vBmGP64GFP (a–c) or vBmGFP (d–f) included as control were collected after 24 h p.i., stained with DAPI (for nuclear staining) and examined under a confocal microscope. Scale bars, a–c, 42 μm; d–f, 30 μm. (B) Fat body from vBmGP64GFP-infected larvae after 36–48 h p.i. (b) and nuclear staining of the same area with DAPI (c); an overlay of panels (b) and (c) is shown in (d); fat body cells from larvae infected with vBmGFP are shown in (a). Scale bars, a, 50 μm; b–d, 25 μm. (C) Trachea showing widespread infection after 48 h p.i. with infected tracheoblasts (a) on the surface of the midgut (b). Scale bars, a, b, 75 μm.
panels d–f) as in BmN cells (Fig. 2). At 24 h p.i., the other larval tissues did not show virus infection. However, by 36–48 h p.i., the virus infection had spread to the fat bodies and the associated tracheal system (Fig. 4B, C). Fat bodies, which are mainly composed of connective tissues, showed the presence of GFP predominantly in the cell membrane and cytoplasm, while the nuclear region was completely free (Fig. 4B, panels b–d). Once again, infection with vBmGFP
showed the presence of protein throughout the entire cell (Fig. 4B, panel a). By 48 h p.i., the fat bodies showed very high levels of virus multiplication and also widespread infection of the tracheal system (Fig. 4C, panel a). In tracheae, the infections originated from tracheoblast cells and proceeded linearly along the tracheal branches. This was also evident from the examination of midgut by about 72 h p.i. (Fig. 4C, panel b). Midgut cells themselves were not showing infection at this time but extensive local infection of tracheoblast and tracheal epidermal cells associated with midgut was evident. Similarly the gonads were infected through the tracheal system (not shown). Our preliminary studies suggested that haemocytes were the primary targets when the larvae were infected through the haemocoel. Similar observations and the spread of viral infection through tracheal cells have been previously reported in the case of AcMNPV infection in *Trichoplusia ni* larvae (Engelhard et al., 1994). No virus multiplication was seen in the gut in BmNPV infection achieved through direct injection of the budded virions to the haemolymph, although this tissue is considered to be the primary target for infection when the viral polyhedra are ingested by the larvae (Keddie et al., 1989; Engelhard et al., 1994). Likewise, no fluorescence was seen in the silk glands and the cuticle, presumably due to the absence of virus multiplication in these tissues. The whole larvae appeared greenish at 5 days p.i., and on exposure to long-range UV emitted bright-green fluorescence.

The economic loss due to BmNPV infection is of major concern in tropical sericulture. Thousands of people in silk-growing countries make use of sericulture as a means of livelihood and at present there are no simple diagnostic or control methods available for virus infections. The generation of tagged virus displaying GFP is helpful in the pathological investigation and screening of antiviral agents for better control of BmNPV infections. Since only the budded virus form is infective to the cells in culture, the availability of GFP-tagged budded viruses provides an easy and quantitative means of screening for antiviral agents in cell culture by directly visualizing the infection (i.e. monitoring GFP expression) rather than the cumbersome plaque assays. In addition, for screening of the antiviral activity in host insects, direct injection of the tagged budded virions into the haemocoel provides a more quantitative description than using the polyhedral bodies, which are orally administered by mixing with feed (and consequently the infective dose can vary substantially between animals).

The ability of BmNPV to display GP64–GFP fusion proteins also offers the possibility of using this baculovirus as vector for the production and display of a wide range of antigenically important proteins on the cell or virus surface. Using *B. mori* larvae rather than the cell lines as a host for recombinant virus amplification and recombinant protein production has advantages in terms of their large size and easiness to rear. Since their expression through the insect larvae allows the avoidance of the more demanding cell culture system once the recombinant virus is generated, it is also cost effective. The purified virus particles displaying the cloned foreign protein on the viral envelope could be used directly for interaction studies without isolating the proteins. Since the presence of GP64 fusion protein did not alter the growth and yield of the virus, the availability of the *gfp*-tagged virus will also help us gain insight into the virus–host cell interactions.

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