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# Baculovirus as Versatile Vectors for Protein Display and Biotechnological Applications

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## Abstract

The baculovirus–insect cell system has long been deployed for a variety of applications including for use as biopesticides, for recombinant protein production, transient transgene expression, tissue therapy, and for vaccine production. Apart from the advantage of large-scale heterologous protein production with appropriate eukaryotic post-translational modification, foreign proteins can also be displayed on the viral envelope. This surface-display technology preserves the native multimeric structure of the protein, thereby expanding the clinical and pharmaceutical utility of the baculovirus system. Recombinant baculoviruses displaying major antigens for human or animal viruses can serve as appropriate vaccines. They can also serve as effective diagnostic platforms and various cell-based assay systems. In this review, we discuss progress in applying baculovirus surface-display, including protein display on the envelope, capsid, and occlusion bodies of baculoviruses, as well as on cells. We will also describe strategies for improvement of this biotechnological approach.

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## Introduction

### Surface display of foreign proteins

Display of foreign proteins or peptides on the surface of a cell or virus, which we refer to as ‘surface-display’, is one of the most valuable techniques for protein engineering. Surface display has been used for basic research for the characterization of protein function and identification of protein counterparts (Hartmann *et al.*, 2018; Nguyen *et al.*, 2018), in addition to applied research such as for the establishment of diagnostic tools for infectious disease and development of gene therapies (Rothe *et al.*, 2006; Sergeeva *et al.*, 2006; Brown, 2010; Aghebati-Maleki *et al.*, 2016; Goulart and Santos, 2016; Lee *et al.*, 2017). Thus far, the most widely applied surface-display technique is phage display (Smith *et al.*, 2015; Yang *et al.*, 2017), whereby a library of billions of bacterial phages displaying foreign peptides can be used for screening (Liu *et al.*, 2017a). However, being limited to bacterial hosts, phage surface-display systems fail to express eukaryotic proteins with post-translational

modifications or proteins with complex folding or branched structures (Mäkelä and Oker-Blom, 2008; Grabherr and Ernst, 2010; Liu *et al.*, 2017a). Other surface-display techniques have been established using yeast (Kuroda and Ueda, 2011; Tanaka *et al.*, 2012), lentivirus (Taube *et al.*, 2008; Lei *et al.*, 2010), adenovirus (Meulenbroek *et al.*, 2004; Vujanovic and Vellinga, 2018), and adeno-associated virus (AAV) (Adachi and Nakai, 2010; Varadi *et al.*, 2012; Münch *et al.*, 2013) to encompass post-translational modifications of expressed eukaryotic proteins. However, most of these systems can only express short peptide sequences (e.g. the yeast system and AAV) or are infectious to host cells and hazardous to researchers (Buchholz *et al.*, 2015; Levin *et al.*, 2016; Lee *et al.*, 2017). Surface-display using the baculovirus expression vector system (BEVS) does not have the limitations associated with these other systems, allowing for eukaryotic post-translational modifications and expression of large properly folded heterologous proteins, whilst not being infectious or a threat to human health. Hence BEVS has become an attractive platform for protein surface-display.

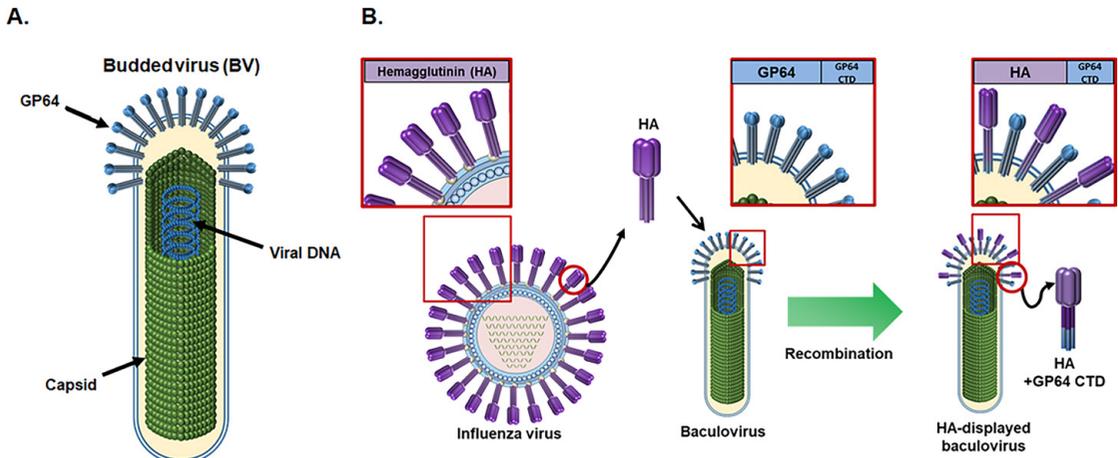
### Characteristics of the baculovirus-insect cell system

The BEVS uses insect viruses from the family *Baculoviridae* and their hosts, allowing for large-scale recombinant protein expression (Palomares *et al.*, 2015). As discussed in Chapter 9, the *Baculoviridae* is a family of double-stranded DNA viruses that infect the larvae of Lepidoptera, Hymenoptera, and Diptera. These large, rod-shaped and enveloped viruses contain circular DNA genomes of approximately 80–180 kilobase pairs (kbp) (van Oers and Vlak, 2007). Based on the type of occlusion bodies (OBs) they produce, baculoviruses can be further divided into two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). NPVs produce polyhedral-shaped OBs (the polyhedra) in the nucleus to generate multiple NPV virions, whereas GVs produce ovoid cylindrical OBs (granules) and typically generate a single virion that is usually found in infected cells with a disrupted nuclear membrane (Ikeda *et al.*, 2014). Both NPVs and GVs exhibit a biphasic life cycle, producing two forms of viral progeny, the intracellular occlusion-derived virus (ODV) and extracellular budded virus (BV). ODVs are the major forms for transmitting

viruses between insects. ODV-containing OBs are dissolved in the insect host midgut, releasing the ODVs to initiate primary infection. BVs are produced in infected insect cells and are responsible for cell-to-cell secondary infection and ultimate systemic infection within the infected host (Clem and Passarelli, 2013). The BVs are typically used in insect cell cultures in the BEVS.

*Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is the type species of the *Baculoviridae*. AcMNPV expresses the major glycoprotein GP64 on the rod-shaped envelope of its budded virions (Fig. 11.1A). Capsid proteins inside the envelope enclose its circular DNA (134 kbp). A total of 156 open reading frames (ORFs) have been identified for AcMNPV, and each ORF is expressed at either early, late, or very late stages of infection (Ayres *et al.*, 1994). Early genes (e.g. *ie2*, *dnapol*, and *lef1–12*) are expressed 6–9 hours post-infection (hpi) to turn on viral replication and to stimulate the expression of late genes. Late genes mainly code for structural proteins such as *vp39*, *p24*, *p6.9* and *e25* and are expressed 6–12 hpi. Very late genes, including *p10* and *polyhedrin* (*polh*), are strongly expressed approximately 18–76 hpi, so their promoters are extensively used for recombinant protein expression in the BEVS. Although some genes are expressed at specific stages of infection, several genes are expressed from early to late stages, such as *ie1*, *pp31*, and *gp64* (Friesen, 2007).

The BEVS has long been applied as an efficient tool for protein expression and gene delivery in both insect and mammalian cell systems. The baculovirus genome allows for insertion of a large foreign DNA (at least 38 kbp) (Airenne *et al.*, 2013). Insertion of a foreign gene that is driven by the *p10* or *polh* promoter results in strong expression and the resulting recombinant protein production can be at the scale of milligrams per litre (Ikonomou *et al.*, 2001; Furuta *et al.*, 2010; de Pinheiro *et al.*, 2016). Moreover, the BEVS accommodates extensive eukaryotic post-translational modification and enables appropriate oligomerization of complex proteins, neither of which are possible through a bacterial expression system. Following the discovery that the AcMNPV glycoprotein, GP64, mediates entry of these insect viruses into mammalian cells, the BEVS has been further modified to transduce foreign genes into mammalian cells such as hepatocyte, Vero, CHO, and U-2OS (Hu, 2006;



**Figure 11.1** Baculovirus and display of foreign protein on the baculoviral surface envelope. (A) Schematic of the rod-shaped AcMNPV BV particle. GP64 is the major glycoprotein on the envelope responsible for cell entry and budding of the virus. Capsid proteins cover the circular viral DNA genome. (B) Display of HA from influenza virus on the baculovirus surface. The CTD of HA from influenza virus is truncated and fused with the CTD of GP64. The resulting recombinant virus displays the trimeric HA on the viral surface as well as the native GP64.

Liu *et al.*, 2010). Foreign genes in these transduced viruses are expressed with the additional insertion of mammal-appropriate promoters, e.g. *CMV*, *SV40*, and *RSV* promoters (Spenger *et al.*, 2004).

### Principles and advantages of baculoviral surface-display techniques

Baculoviruses are typically used to display foreign proteins either on the viral surface or on the infected cell surface through fusion with viral glycoprotein GP64. In these cases, the foreign proteins [e.g. the haemagglutinin (HA) of *Influenza A virus*] are usually fused with the GP64 signal peptide (SP) and either its transmembrane (TMD), cytoplasmic tail (C-terminal domain, CTD), or both the TMD and CTD domains of GP64 (Fig. 11.1B) (Yang *et al.*, 2007). The resulting recombinant virus thus displays the foreign proteins on the envelope alongside wild-type (WT) GP64. WT GP64 allows the virus to propagate normally, so that recombinant viruses expressing foreign genes are readily amplified.

The BEVS surface-display technique has many advantages, such as allowing for post-translational modification, large insert capacity, preservation of the native multimeric structures of the fusion protein, the relatively easy purification process for recombinant virus compared to recombinant protein, and a safer operating system. Unlike other

surface-display technologies using mammalian viruses (e.g. AAV and lentivirus), surface-display via BEVS is not hazardous to human health. In this chapter, we provide an overview of the surface-display technology using BEVS. We describe different platforms for surface-display, provide examples of current applications, suggest strategies for potential improvement, and present a final summary and future outlook for this versatile system.

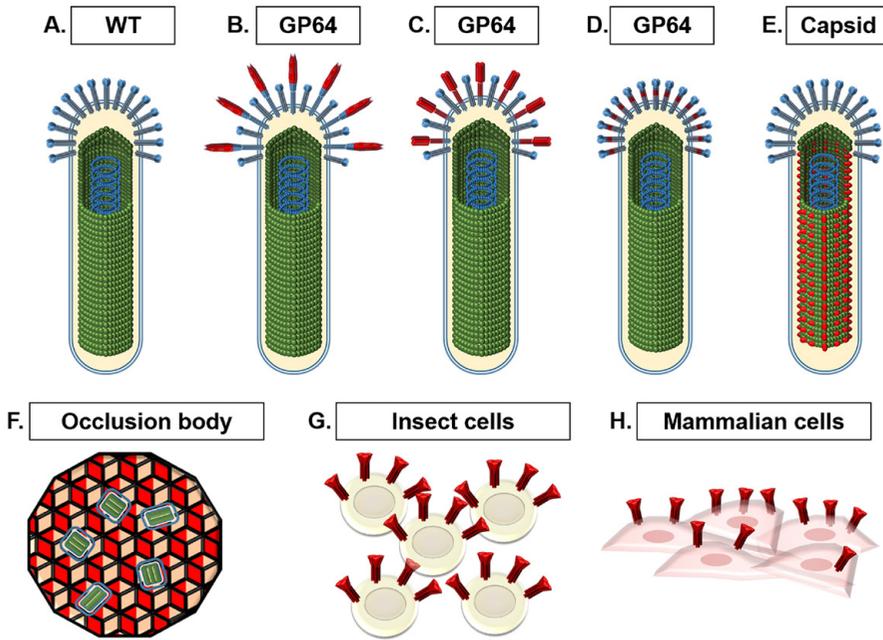
### Surface-display platforms

Several different strategies can be employed using BEVS to display a protein on a viral or cell surface (Fig. 11.2). Here, we discuss surface-display of foreign proteins on either baculovirus particles or infected/transduced cells.

### Virus surface-display strategies

#### GP64 glycoprotein

The envelope glycoprotein GP64 is the major glycoprotein on the envelope of group I baculoviruses, including AcMNPV and *Bombyx mori nucleopolyhedrovirus* (BmNPV), and is the major fusion partner in most BEVS surface-display techniques. GP64 is a type I integral membrane protein consisting of 512 amino acids with a molecular mass of 64–67 kDa. GP64 is essential for AcMNPV infection as it



**Figure 11.2** Different platforms for surface-display technologies. Schematic of different baculovirus surface-display technologies. The target foreign proteins for display are shown in red. (A) WT baculovirus with native GP64. (B) Baculoviral virion surface-display system with entire GP64 as a fusion partner. (C) Baculoviral virion surface-display system using the CTD or TMD+CTD of GP64. (D) Baculoviral virion surface-display system whereby the peptide for display is inserted into native GP64. (E) Baculoviral virion surface-display system using capsid fusion. (F) Occlusion body display. (G) Surface display on infected lepidopteran insect cells. (H) Surface display on baculovirus-transduced mammalian cells.

facilitates viral entry into host cells and membrane fusion upon endocytosis at the early stage of infection, as well as budding of BVs from infected cells at the final stage of infection. GP64 localizes to the envelope of both budded virions and infected insect cells, exhibiting a trimeric oligomeric structure thereby providing an outstanding fusion partner for heterologous proteins in the BEVS.

Several different forms of GP64 fusion can be deployed in baculovirus surface-display techniques. The first is to use the entire GP64 protein to fuse with the display targets (Fig. 11.2B). The target protein coding sequences are cloned into the baculovirus-expression vector, to produce N-terminal fusion to the GP64 SP and C-terminal fusion to GP64 (Grabherr and Ernst, 2010; Mäkelä *et al.*, 2010). Heterologous proteins as large as HIV-1 GP120 have been successfully displayed using this system and retained their protein bio-activities (Boublik *et al.*, 1995). Proteins displayed using this approach are highly accessible for immune-recognition using viral particles as

immunogens for vaccine or monoclonal antibody production (Lindley *et al.*, 2000).

Another technique, now the most common approach, is to fuse the displayed target proteins with the SP, CTD or TMD+CTD of GP64 (Fig. 11.2C). This baculovirus surface-display strategy was first applied to HIV-1 envelope protein GP41 by fusing its ectodomain to the SP and the C-terminal 43 amino acids (covering the TMD and CTD) of GP64, resulting in successful display on the viral surface (Grabherr *et al.*, 1997). This strategy reduces the size of the transfer vector and simplifies the cloning procedure. However, whether a foreign protein can be displayed efficiently, either via fusion with the entire GP64 or partial GP64, is highly dependent on the properties of the target protein, size in particular (Oker-Blom *et al.*, 2003). It is generally easier to display shorter target proteins on the surface of virions or cells, and they are more likely to retain their biological activities. In some instances, the GP64 TMD may not incorporate the target

protein on the surface as well as the native TMD (Tang *et al.*, 2010). In contrast, replacement of the GP64 TMD with the heterologous TMD and CTD of vesicular stomatitis virus glycoprotein (VSVG) has proven to be a promising alternative approach that increases both protein incorporation and production of virus progeny (Chapple and Jones, 2002; Zhou and Blissard, 2008). We provide a detailed discussion of peptide domain selection below.

In both of these above-described strategies, the fusion protein is expressed alongside the WT GP64 on the virion or cell envelope. As the virus randomly selects between the WT or engineered GP64 protein, the fusion target protein may not be efficiently incorporated onto virus particles (Mäkelä and Oker-Blom, 2008). An alternative baculovirus surface-display strategy is to insert the target peptide sequence directly into the native GP64 (Fig. 11.2D). The HIV-1 GP41 amino acid motif ELDKWA has been inserted into the naturally occurring *NotI* restriction site at residue 278 of GP64 (Ernst *et al.*, 2000), and another 13 insertion sites on GP64 have been described (Spenger *et al.*, 2002). However, as these recombinant baculoviruses lack WT GP64 to facilitate membrane fusion and virus budding, the insertions must be small to prevent dysfunction of the modified GP64. Thus far, the largest inserted peptide is the 23-amino-acid VP1 protein of *Foot-and-mouth disease virus* (FMDV), but the resulting recombinant virus exhibited decreased infectivity and low GP64 incorporation compared to virus with WT GP64 (Spenger *et al.*, 2002).

### Capsid protein

The capsid protein of baculovirus has also been designed as a fusion partner in baculovirus surface-display systems and is called the baculovirus capsid display (BCD) system. VP39, the major and most abundant nucleocapsid protein of AcMNPV, is the main fusion partner used in BCD. VP39 is a 39-KDa protein monomer arranged in stacked rings around the baculoviral nucleoprotein core, so heterologous proteins can be displayed along the capsid surface by fusing them to VP39 (Fig. 11.2E). Kukkonen *et al.* (2003) first reported this strategy using VP39 to display enhanced green fluorescent protein (EGFP). EGFP display did not impact virus titre and virions displayed high quantities of EGFP,

estimated at 590–860 EGFP molecules per virus particle.

The greatest advantages of BCD are the lack of interference with virus assembly and the high levels of protein displayed (Molinari *et al.*, 2011). Moreover, the protein displayed by BCD is uniformly distributed along the capsid surface (Fig. 11.2E), unlike the distribution of GP64-foreign protein constructs that are restricted to one end of the virus particle (Fig. 11.2B–D). Heterologous proteins as large as 41 kDa have been successfully displayed using BCD and display of even larger foreign proteins is possible as the capsid protein enables relatively unlimited length extension. BCD appears to accommodate both N- and C-terminal protein fusions, which may be due to the flexibility of the capsid protein (Kukkonen *et al.*, 2003).

VP39 has been linked to actin polymerization during viral entry into the cytoplasm and nucleus of infected insect cells (Charlton and Volkman, 1993; Ohkawa *et al.*, 2002), and is therefore a good candidate for anchoring target proteins onto the baculovirus surface for viral imaging in live cells. Liu *et al.* (2014) used an EGFP–VP39 fusion virus in co-localization and live-cell imaging experiments to study the transduction route of baculovirus in mammalian cells from the cell surface into the nucleus. Direct visualization of the BCD system in this way has helped elucidate the internalization and intracellular trafficking pathways of baculovirus in both insect and vertebrate cells.

Beyond imaging studies, Molinari *et al.* (2011) displayed ovalbumin (OVA) peptides on the capsid of baculovirus and found that they were delivered to MHC-I molecules in dendritic cells, which elicited a potent cytotoxic immune response in mice strong enough to reject implantation of tumour cells expressing OVA. Song *et al.* (2010) fused ZnO binding peptide to the N-terminus of VP39 and found that the virus not only retained its infectivity, but also gained the ability to bind nano-sized ZnO powders. A theoretical strategy for using capsid display to augment delivery of foreign proteins into the nuclei of mammalian cells has also been proposed (Kukkonen *et al.*, 2003). Unlike most viruses, baculoviruses transport their intact nucleocapsids through the nuclear pore into the cell nucleus. Accordingly, from a therapeutic point of view, the BCD system may enable nuclear delivery of therapeutic proteins to achieve the desired

treatment effects as effectively as current protein transduction methods (Ford *et al.*, 2001).

### Occlusion body

During the late phase of baculovirus infection, the ODVs are produced. The ODVs are embedded with polyhedrin (29 kDa), which forms the crystalline protein matrix of polyhedral occlusion bodies (Harrap, 1972; Rohrmann, 1986). Polyhedrin accumulates in infected insects to very high levels, up to 30–50% of total protein (Seo *et al.*, 2003). The polyhedrin matrix, generated via extensive cross-linking of polyhedrin trimers to form cubic crystals (Coulibaly *et al.*, 2007), protects the viral particle and genome from damage and environmental threats, and assists in delivery of virus to the next target insect. Although abundant in infected cells, polyhedrin is not essential for the life cycle of budded baculovirus (Hu *et al.*, 1999).

Surface display of foreign proteins using baculoviral polyhedrin as a fusion partner (Fig. 11.2F), termed the occlusion body display (OBD) system, was first proposed by McLinden *et al.* (1992). In that study, recombinant polyhedrin proteins displaying a peptide with an influenza HA epitope were expressed on the surface of baculovirus, as revealed by immunoprecipitation with an anti-influenza monoclonal antibody (mAb) (McLinden *et al.*, 1992). Je *et al.* (2003) generated recombinant virus expressing a polyhedrin–GFP fusion construct and found that the recombinant polyhedrin only formed occlusion bodies upon co-expression with native polyhedrin. Lee *et al.* (2005) demonstrated that incorporation of foreign proteins does not require co-infection of WT virus or co-expression of native polyhedrin, as their polyhedrin–GFP and polyhedrin–VP1 constructs could both assemble occlusion bodies without co-infection with WT virus. Bae *et al.* (2013) determined that amino acids 19–110 and 32–110 of AcMNPV polyhedrin could localize the EGFP fusion in the nucleus with a 10-fold increase in protein yield, and Sampieri *et al.* (2015) identified polyhedrin residues 58–110 as being the minimal sequence necessary for efficient incorporation of foreign protein.

Polyhedrin from BmNPV has also been applied in the OBD system. Immobilization of EGFP and LacZ protein with fluorescence and  $\beta$ -galactosidase activity respectively into BmNPV polyhedral

occlusion bodies has been demonstrated (Xiang *et al.*, 2012). The N-terminal 50 amino acids of polyhedrin represents the recognition signal necessary for foreign protein incorporation, and the N-terminal 100 amino acids of polyhedrin allowed for the most efficient protein incorporation into polyhedral occlusion bodies (Chen *et al.*, 2013). The protein encoded by ORF 134 of BmNPV has also been shown to be a suitable fusion partner for OBD (Guo *et al.*, 2017).

The major application of baculoviral OBD might be as a carrier of insecticidal toxins. Polyhedral occlusion bodies carrying such toxins could be delivered directly into the midgut of an insect host. This technology has been used to incorporate the *Bacillus thuringiensis* insecticidal toxins Cry1Ac (Chang *et al.*, 2003), Cry1–5 (Jung *et al.*, 2012; Shim *et al.*, 2013), and Cry1Ab (El-Menofy *et al.*, 2014) into host cells, resulting in enhanced speed of action and pathogenicity. An insect-specific cyto-insectotoxin (Cit1a) from the venom of the central Asian spider *Lachesana tarabaevi* has been fused to the polyhedrin of both AcMNPV and BmNPV, with improved insecticidal activity (Ali *et al.*, 2015). The OBD carrier can protect both the displayed toxin and the toxin-expressing baculovirus from ultraviolet destruction under field conditions (Mäkelä and Oker-Blom, 2008).

### Cell surface-display strategies

#### Infected insect cells

Apart from displaying foreign protein on the surface of budded virions, baculoviruses can be used to display heterogeneous proteins on the surfaces of infected insect cells (Fig. 11.2G). Membrane-bound proteins with an appropriate SP and TMD can be expressed and translocated to the surfaces of infected host cells, such that foreign proteins are displayed on the surfaces of insect cells infected with the appropriate recombinant baculovirus. To do this, the coding sequence for the target protein for display can be cloned into an expression cassette with its full-length coding region (including the native SP and TMD), a truncated GP64-fused form (e.g. Fig. 11.2B–D), or a truncated form fused to foreign anchor domains (e.g. the C-terminus of VSVG).

Insect cell lines that are commonly used in BEVS include Sf21 (IPLB-Sf21AE) (Vaughn *et*

*al.*, 1977) derived from *Spodoptera frugiperda* pupal ovarian tissue, and its subclone Sf9 (Smith *et al.*, 1983), High Five (BTI-TN-SB1-4) isolated from *Trichoplusia ni* embryos (Granados *et al.*, 1986, 1994), and Bm5 from *Bombyx mori* ovarian tissue. These insect cell lines are semi-adherent and can be adapted easily to serum-free media or suspension cultures for large-scale production. Sf9 cells are more resistant to physical shear stresses, and so are more suitable for suspension culture than Sf21 cells (Drugmand *et al.*, 2012). Both cell lines are commonly used to amplify recombinant AcMNPV and for the production of recombinant proteins. High Five cells are not used for virus propagation because these cells generate low titres of virus progeny, typically 100-fold lower than *S. frugiperda* cells (Kelly *et al.*, 2008; Wilde *et al.*, 2014), and rapidly accumulate mutations in the *fp25k* gene (Kelly *et al.*, 2006, 2008), which reduces the polyhedrin-based expression (Harrison *et al.*, 1996). However, High Five cells have a 50% larger cell volume than Sf9 cells, and recombinant protein expression by High Five cells can be 5- to 10-fold higher than either Sf21 or Sf9 cells (Saarenpää *et al.*, 2015). Hence High Five cells are excellent for large-scale protein production in some cases. While the *S. frugiperda* and *T. ni* cell lines are frequently used for AcMNPV protein production, they are not permissive to BmNPV, the virus used to infect the silkworm larvae or pupae for producing recombinant proteins. BmNPV only replicates in *B. mori* cell lines, such as Bm5 cell (Xu *et al.*, 2012b).

Apart from involving simpler culture conditions, a major advantage for use of insect cells for foreign protein expression is that these cells perform appropriate eukaryotic post-translational modifications such as glycosylation, phosphorylation, and fatty acid acylation. Moreover, complex mammal-derived proteins are folded and assembled in insect cells in a comparable way to mammalian cells. Although some differences between insect and mammalian expression systems remain, such as N-glycosylation (insect cells tend to produce simpler N-glycans with terminal mannose residues, whereas mammalian cells produce more complex N-glycans with terminal sialic acids), insect cells are still highly suited for mammalian protein expression (James *et al.*, 1995; Jarvis and Finn, 1995). Insect cell lines have

been engineered with enzymes to produce sialic acid-terminating glycans for improved mammalian post-translational modification (Hollister *et al.*, 2002; Harrison and Jarvis, 2006; Aumiller *et al.*, 2012; see also Chapter 10).

As a baculovirus surface-display system, insect cells have proven ideal for over-expression of pharmaceutically important membrane proteins, particularly for functional studies and screening of interacting molecules. Membrane proteins, receptors, and transporters that are usually expressed in low amounts in mammalian cells can be over-expressed on insect cell surfaces through recombinant virus infection before being assayed for their substrates or inhibitors by cell-based platforms. The insect cell surface-display system circumvents tedious purification and immobilization processes and is easily optimized for high-throughput approaches. For study of protein function, display of foreign proteins by insect cells provides a system that prevents interference from homologous proteins in mammalian cells when mammalian proteins or mammalian virus proteins are being analysed. This approach has been used to study the pharmacological properties of G protein-coupled receptors (GPCR) for example (Schneider and Seifert, 2010; see below).

#### Mammalian and other non-lepidopteran cell surface display

Although baculoviruses do not replicate in mammalian cells, they can enter a variety of mammalian and several non-lepidopteran cells, and express heterologous target genes following insertion of mammal-specific or other appropriate promoters (such as *CMV* or *SV40*) in the expression cassette. These 'BacMam'-type viruses (Boyce and Bucher, 1996) are a powerful tool for transgene delivery into mammalian systems due to the ability of the baculovirus BV to enter diverse cell types. Baculovirus can transduce human, rabbit, non-human primate, rodent, porcine, bovine, and ovine mammalian cells (Kost and Condreay, 2002), as well as non-mammalian cells from fish (Kost *et al.*, 2005), fly (Lee *et al.*, 2000), and mosquito (Naik *et al.*, 2018), albeit with variable transduction efficiencies. Baculovirus transduction has been studied in many cell types, including hepatic cells (Hofmann *et al.*, 1995), A549 and HFL-1 (Chang *et al.*, 2004), chondrocytes (Ho *et al.*, 2004), kidney

cells (Liang *et al.*, 2004), osteosarcoma cells (Song and Boyce, 2001), mesenchymal stem cells (Ho *et al.*, 2005), amongst many others. Uptake of baculovirus particles into mammalian cells greatly depends on the interaction of baculovirus GP64 with phospholipids on the mammalian cell surface (Tani *et al.*, 2001), but co-display of VSVG on the baculovirus surface has been shown to increase BacMam transgene expression efficiencies (Bar-soum *et al.*, 1997).

The BacMam system provides additional attractive characteristics in that it has little cytopathic impact on the transduced cells and is safe for use by researchers. The transduction of baculovirus does not cause significant cellular toxicity or alter the gene expression profiles in mammalian cells (Kenoutis *et al.*, 2006). Although transduction of BacMam cells usually requires a high multiplicity of infection (MOI) (e.g. MOI of 100–1000), baculovirus can easily be amplified to high titres in insect cells. Several strategies have been developed to improve transgene efficiency (for details, see below).

The use of non-lepidopteran host cells for protein display (see Fig. 11.2H) is especially suited to pharmacological cell-based assays for cellular receptors, transporters, ion-channels, and viral targets (Condreay *et al.*, 2006). The combination of transient expression (e.g. transfection via a baculovirus expression cassette) and automatic assay platforms can further aid high-throughput screening of surface-display systems using non-lepidopteran host cells. Many pharmacological target proteins require co-expression of cellular components, and this requirement can be met by co-transducing multiple baculoviruses expressing the individual components (Condreay *et al.*, 1999). Co-transduction of different recombinant baculoviruses may limit reproducibility because of an uneven distribution of baculoviruses among cells. Consequently, the ‘MultiBacMam’ system was established to deliver multiexpression cassettes via a single baculovirus into mammalian cells. With the advantage of defined expression ratios (Trowitzsch *et al.*, 2011; Mansouri *et al.*, 2016), this technology has shown considerable potential for protein–protein interaction studies in mammalian and non-lepidopteran cells, such as in combination with bimolecular fluorescence complementation (BiFC) assays (Bellón-Echeverria *et al.*, 2018).

## Applications for baculoviral surface-display systems

### Viral-based assay

Baculovirus display of eukaryotic protein for functional study

The BEVS surface-display technology has long been favoured for functional studies of eukaryotic proteins including GPCR, the largest integral membrane protein family in the human genome (Cherezov *et al.*, 2010). GPCRs are important drug targets in the pharmaceutical industry, with 30–50% of currently marketed drugs acting on GPCRs (Gruber *et al.*, 2010). Display of GPCRs on Sf9 cell surfaces through the BEVS has proven an excellent system for GPCR studies (for details, see ‘Insect cell surface display’). However, several GPCRs are expressed in relatively low amounts or are largely unglycosylated on Sf9 cell membranes (Masuda *et al.*, 2003). Unexpectedly, the surface of baculovirus virions was demonstrated to be a good alternative for GPCR display. The  $\beta_2$ -adrenergic receptor was expressed on the surface of recombinant baculovirus with homogenous receptor function and post-translational modification in contrast to display on the surface of the infected insect cell (Loisel *et al.*, 1997). This baculovirus virion display technique was subsequently applied to the study of target binding by the human leukotriene B<sub>4</sub> receptor (BLT1) (Masuda *et al.*, 2003), serotonin 1A receptors (5-HT<sub>1A</sub>) (Töntson *et al.*, 2014), melanocortin 4 receptors (MC4) (Veiksina *et al.*, 2014; Link *et al.*, 2017), and dopamine D<sub>1</sub> receptors (Sakihama *et al.*, 2008a; Allikalt and Rincken, 2017). The baculovirus particles were found in all of these studies to be a homogenous and far more stable system for membrane-bound GPCRs, allowing for their use in different experimental procedures and maintaining signal for long periods of time (Veiksina *et al.*, 2014; Allikalt and Rincken, 2017).

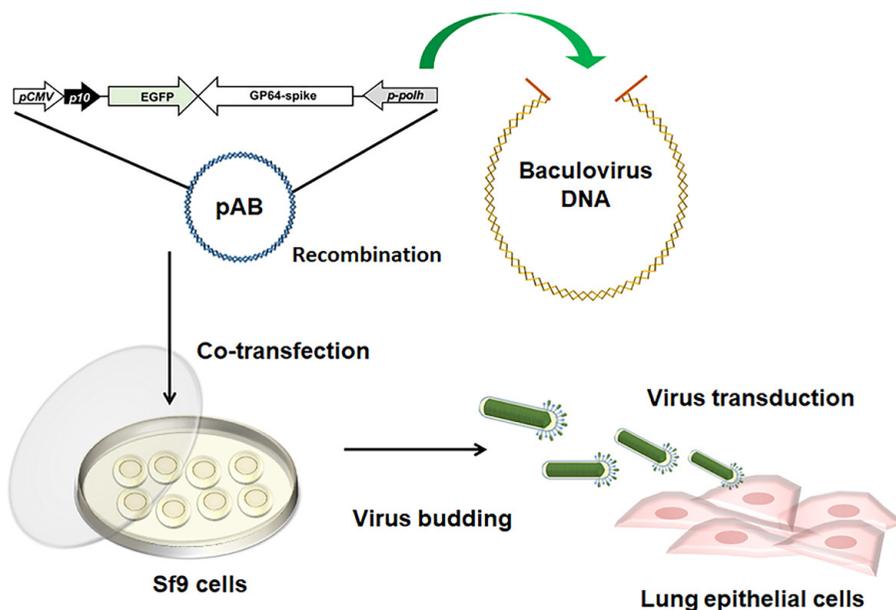
Apart from GPCRs, the  $\gamma$ -secretase complex implicated in Alzheimer’s disease has also been displayed on the surface of baculovirus virions (Hayashi *et al.*, 2004). This complex requires at least four transmembrane proteins for protease function. Co-infection into Sf9 cells of four recombinant baculoviruses expressing the individual proteins achieved exclusive complex display on

the budded virus particles while the Sf9 cell membranes retained the non-functional protein components. Consequently,  $\gamma$ -secretase activity was nearly 2.5-fold higher on budded baculovirus than on Sf9 cell membranes (Hayashi *et al.*, 2004). Similarly, display of human (pro)renin receptor (hPRR) on BmNPV virus particles resulted in easy amplification in silkworm larvae, and the virus particles could be subjected to an enzyme-linked immunosorbent assay (ELISA) system to detect protein–protein interactions and to screen for protein inhibitors (Kato *et al.*, 2009, 2011, 2012). Given the strong reconstitution of active forms of proteins and protein stability, surface-display systems using baculovirus virion particles are an attractive technology for studying protein–protein interactions and binding partners of complex eukaryotic transmembrane proteins (Sakihama *et al.*, 2008b; Kakutani *et al.*, 2011).

#### Baculovirus-mediated display of infectious viral proteins as pseudoviruses

A promising application for the baculovirus surface-display technique is the display of structural proteins from human-infectious viruses or

pseudoviruses. Baculovirus vectors are safe and powerful tools in the study of such viruses. The first such pseudoviruses involved baculovirus virion display of HIV-1 glycoproteins GP120 (Boublik *et al.*, 1995) and GP41 (Grabherr *et al.*, 1997). The virion-displayed GP120 exhibited CD4-binding activity equivalent to WT HIV-1 (Boublik *et al.*, 1995). These baculovirus-based pseudoviruses are especially useful for functional studies of particular viral proteins as they eliminate cross-activities from other viral components. In a study of herpes simplex virus 1 (HSV-1) glycoprotein D (gD) (Zhou and Roizman, 2002), baculoviruses displaying different forms of gD were generated as pseudotyped HSV-1 and transduced into SK-N-SH cells to investigate how specific forms of gD block apoptosis and the reason for low efficiency of cell entry (Zhou and Roizman, 2002). Chang *et al.* (2004) used BEVS to display Spike (S) protein from SARS coronavirus (SARS-CoV) on the baculovirus and without interference from other SARS proteins, proved that the S protein triggers release of interleukin-8 (IL-8) in different lung cells to induce a cytokine storm (Fig. 11.3). Display of S protein fragments on virions further aided identification of specific functional regions, including one novel functional site.



**Figure 11.3** Baculovirus surface-display of spike (S) protein from SARS-CoV as a pseudovirus. SARS-S was fused with the baculovirus GP64 TMD and CTD and was then co-transfected with baculovirus DNA to generate recombinant virus that displayed SARS-S on its surface. These recombinant viruses can serve as pseudoviruses for the study of SARS-CoV.

These findings may support the development of vaccine immunogens and disease therapies (Chang *et al.*, 2004). Apart from their use in functional studies, baculovirus-based pseudoviruses could also serve as safe and efficient vaccine antigens for human pathogens (see below).

#### Baculovirus-mediated display of immunogens as vaccine antigens

Display of immunogenic proteins on baculovirus surfaces as vaccine antigens is one of the most popular ways of applying the BEVS technology (Premanand *et al.*, 2018). Immunogenic determinants (usually the membrane or capsid proteins) from infectious pathogens are displayed on the surface of the baculovirus envelope to serve as vaccine immunogens that can be administered to animal hosts. Many studies have also reported the immune responses induced by baculovirus-displayed antigens. Together, these studies (described further below) demonstrate the potential of generating next-generation vaccines.

#### Baculovirus as an immunizing reagent

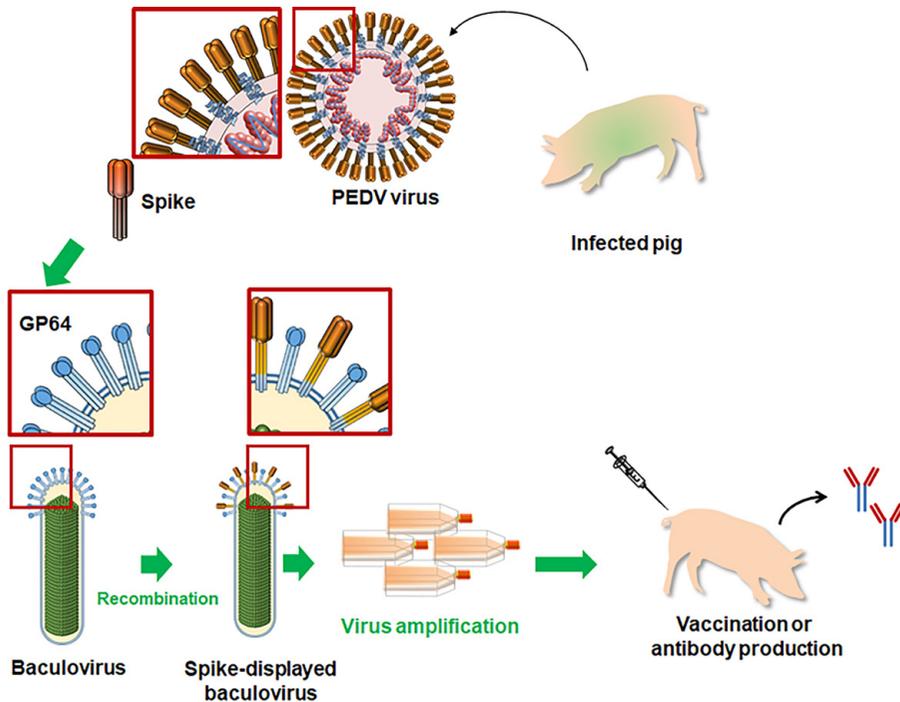
Although baculovirus transduction does not have significant cytopathic impact on transduced mammalian cells, the internalization of live baculovirus still stimulates anti-viral responses. These responses probably result from the recognition of unmethylated CpG dinucleotides in viral DNA in either immune cells (e.g. macrophages or dendritic cells) or non-immune cells (e.g. fibroblasts) of mammals (Abe *et al.*, 2005; Ono *et al.*, 2018). The strong innate immune response triggered by inoculation with a WT baculovirus could sustain lethal challenge by influenza virus (Abe *et al.*, 2003). These responses on the other hand can be exploited as a valuable adjuvant activity in vaccination using baculovirus as immunogen. In addition to innate immunity, baculovirus can also induce B cell and T cell activation to improve adaptive immunity, with the removal or inactivation of baculovirus abolishing this adjuvant effect (Suzuki *et al.*, 2010; Heinimäki *et al.*, 2017).

#### Examples of using surface-display strategies in vaccination

HA protein, the major glycoprotein of influenza virus, is probably the best-studied immunogen

that has been displayed on the surface of baculovirus. HA frequently mutates, resulting in new influenza outbreaks annually, and hence is an important target of influenza vaccines. Since HA is also a type I membrane protein (Veit and Thaa, 2011), it is particularly suited for display on the surface of baculovirus as a vaccine. Different HA subtypes have been displayed on baculovirus. These subtypes include H1N1 (Prabakaran *et al.*, 2011; Sim *et al.*, 2016), H5N1 (Yang *et al.*, 2007; Jin *et al.*, 2008; Prabakaran *et al.*, 2008; Wu *et al.*, 2009; Tang *et al.*, 2010; Hu *et al.*, 2012; Ge *et al.*, 2016), H6N8 (Musthaq *et al.*, 2014), H7N7 (Rajesh Kumar *et al.*, 2013), H7N9 (Prabakaran *et al.*, 2014), and H9N2 (Lin *et al.*, 2011). Other type I membrane proteins from human pathogens displayed on baculovirus as vaccine immunogens include envelope (E) glycoprotein from *Japanese encephalitis virus* (JEV) (Xu *et al.*, 2011) and Spike (S) protein from SARS-CoV (Feng *et al.*, 2006). A novel strategy has been applied to the display of VP1 capsid protein from enterovirus 71 (EV71) on baculovirus. As VP1 is not a membrane protein, it was fused to the SP, TMD, and CTD of baculovirus GP64 for display on baculovirus surface (Meng *et al.*, 2011; Premanand *et al.*, 2012; Kiener *et al.*, 2013; Premanand *et al.*, 2013). A series of human malaria vaccines have also been successfully developed using baculovirus surface-display technology (Yoshida *et al.*, 2003; Strauss *et al.*, 2007; Mlambo *et al.*, 2010; Iyori *et al.*, 2017).

Baculovirus surface-display techniques have also been applied to pathogens affecting non-human animals. Chang *et al.* (2018) displayed spike protein from *Porcine epidemic diarrhoea virus* (PEDV) on the surfaces of baculovirus to evaluate the immune responses and protective effects in mice and pig models (Fig. 11.4). In that study, recombinant viruses displaying spike protein elicited systemic anti-PEDV spike-specific IgG. Piglets immunized with spike-displayed baculovirus exhibited almost no or only mild diarrhoea after being challenged with PEDV, demonstrating the potential of spike-displayed baculovirus as an effective vaccine against PEDV infection. Similar approaches have been applied to generate vaccines against other swine fevers. These include the display of GPs and glycoprotein ORFs of porcine reproductive



**Figure 11.4** Baculovirus surface-display of spike protein from PEDV as a vaccine antigen. PEDV was isolated from infected pigs, and the DNA sequences encoding spike protein were amplified for fusion with baculovirus GP64 CTD. Recombinant viruses displaying spike were amplified and immunized into pigs as vaccination immunogens.

and respiratory syndrome virus (PRRSV) (Wang *et al.*, 2007; Xu *et al.*, 2012a; Karuppanan *et al.*, 2013), E2, E<sup>rms</sup> and NS3 proteins of classical swine fever virus (CSFV) (Xu and Liu, 2008; Xu *et al.*, 2008, 2009), and ORF2, the major capsid protein of *Porcine circovirus 2* (PCV2) (Xu *et al.*, 2012a; Ye *et al.*, 2013). Although baculoviruses have not yet been approved for use as a vaccine antigen in humans, their use as animal vaccines is promising.

#### Improvements for baculovirus surface-display of immunogens

Chen *et al.* (2010) studied baculovirus as a vaccine vehicle by comparing the mouse immune responses induced by baculovirus that either displays the HA antigen on the surface or expresses the HA after entry into the host cell via a mammalian promoter. Baculovirus displaying HA on the viral surface triggered a stronger humoral and mucosal response upon intranasal and subcutaneous injection, whereas baculovirus expressing HA after cell entry triggered a more robust humoral

response upon intramuscular injection. Accordingly, a baculovirus that both displays and expresses the HA after cell entry elicited more robust immune responses via either administration route (Chen *et al.*, 2010). If designed to enhance the mucosal immune response, co-administration of the mucosal adjuvant cholera toxin B subunit (CTB) with the antigen-displaying baculovirus (Prabakaran *et al.*, 2008), or co-display of the heat-labile enterotoxin B subunit (LTB) on the baculovirus surface (Zhang *et al.*, 2016), have proven effective.

#### Cell-based assays

Baculovirus can be used to display foreign proteins on the surface of both infected insect cells and transduced mammalian and non-lepidopteran cells. These cell surface displays provide ideal platforms for protein functional studies and protein-binding target identification. Different cell systems have their own advantages when applied to foreign protein display.

## Insect cell surface display

### Functional study of eukaryotic transmembrane proteins

Insect cell surface display mediated by baculovirus infection offers a heterologous display system for mammalian proteins especially suited to the establishment of screening platforms for pharmacologically interesting targets such as GPCRs, transporters, and ion channels. As mentioned above, GPCRs are important targets for drug development due to their involvement in many physiological responses on ligand binding. However, functional studies for GPCRs are difficult as expression of endogenous GPCRs is low in native tissue (Allikalt and Rincken, 2017). Heterogeneous overexpression of GPCRs in a mammalian cell system is confounded by the expression of endogenous GPCRs and the various G proteins produced in mammalian cells that may interfere with functional analyses. The display of GPCRs on insect cell surfaces hence provides a solution for these problems. Insect cells (e.g. Sf9 cells) have no active GPCRs and express only one form of G protein (Schneider and Seifert, 2010). Insect cells can also be used to enhance the signal-to-noise ratio in GPCR functional assays due to high level expression of heterologous proteins. The expression and activity of 16 different human GPCRs have been characterized in three insect cell lines. Albeit under different infection conditions, many of these GPCRs were expressed at high levels (greater than 20 pmol/mg determined by activity) on insect cell surfaces (Akeroun *et al.*, 2005). Many GPCRs have been studied by use of Sf9 cell surface display strategies, including neurotransmitter receptors (e.g. histamine receptors, serotonin receptors, and muscarinic acetylcholine receptors) (Schneider and Seifert, 2010), and human olfactory receptors (ORs) (Matarazzo *et al.*, 2005; Matarazzo and Ronin, 2013). In addition to GPCRs, several ion transporters have also been characterized by use of the insect cell surface display system. Sf9 cells were used to display a human cardiac Na<sup>+</sup>/Ca<sup>2+</sup> exchanger for functional study. As Sf9 cells lack native Na<sup>+</sup>/Ca<sup>2+</sup> exchanger activity, there was no background interference with the activity of the displayed target exchanger (Egger *et al.*, 1999). A Na/Ca-K exchanger has also been displayed on High Five cells for stoichiometric analysis (Szerencsei *et al.*, 2001).

### Generation of protein library

The insect cell surface-display system has been exploited to generate an expression library in which the desired targets displayed on cells can be sorted by fluorescence-activated cell sorter (FACS) with specific antibodies. Granziero *et al.* (1997) reported a human placental cDNA library generated by baculovirus-infected Sf9 cells. Although display of cDNA products on the cells was not intended, the library allowed for identification of cDNAs encoding cell-surface molecules (Granziero *et al.*, 1997). Ernst *et al.* (1998) inserted a six amino-acid epitope (ELDKWA) from HIV-1 GP140 into the antigenic site of influenza virus HA and generated an insect cell surface display library expressing this peptide fusion adjacent to an additional three random amino acids. A single baculovirus clone was isolated by peptide-specific antibody from a pool harbouring approximately 8000 variants likely due to increased binding characteristics (Ernst *et al.*, 1998). Two display libraries were generated by Crawford *et al.* (2006) to display random peptides on insect cells by major histocompatibility complex (MHC) class I or class II fusion constructs and were screened to identify T-cell receptor mimotopes. Meller Harel *et al.* (2008) generated a maize cDNA library displayed on insect cells by fusing the cDNAs to SP of GP64 and TM/CTD of VSVG. Their library was estimated to contain  $2.5 \times 10^5$  independent clones (Meller Harel *et al.*, 2008). However, no other insect cell surface-display libraries have been established. A possible obstacle elucidated by Xu *et al.* (2013), is that insect cells are easily co-infected by different baculoviruses. Hence each insect cell may harbour a mixture of proteins and a mixture of baculoviruses, making it difficult to recover the virus expressing the gene of interest. Another limitation for use of baculovirus to generate a cDNA library is the relatively low diversity (only  $10^5$ – $10^6$  variants per library) compared to other display systems, e.g.  $10^{10}$  variants per library for the phage display system (Bazan *et al.*, 2012).

### Display of infectious viral protein

In addition to the baculovirus virion, the insect cell surface is a good platform to display infectious viral protein for functional study and diagnostic usage. Early in 1986, HA proteins of influenza virus were displayed on the insect cell surface by recombinant baculovirus infection (Kuroda *et al.*, 1986; Possee,

1986). In these studies, HA proteins were detected by antibody on the insect cell membrane, and exhibited HA activities such as hemadsorption and hemagglutination, indicating expression of the functional oligomeric conformation (Kuroda *et al.*, 1986; Possee, 1986). The insect cell surface-display technique of influenza HA has been modified slightly in expression constructs, with the replacement of the *polh* promoter with the *White spot syndrome virus* (WSSV) *ie1* promoter for example. The antigenicity of insect cell displayed HA was determined by hemagglutination and hemagglutination inhibition assays (Gadalla *et al.*, 2014). Apart from the influenza HAs, glycoprotein H (gH) of HSV-1 has also been displayed on the insect cell surface with authentic glycosylation. Intriguingly, gH expressed in mammalian cells requires other HSV-1 factors to be translocated to the cell surface, whereas in insect cells expression of gH alone was sufficient for transport (Ghiasi *et al.*, 1991). The insect cell surface-display of the haemagglutinin-neuraminidase (HN) glycoprotein of *Newcastle disease virus* (NDV) resulted in hemadsorption, hemagglutination, and neuraminidase activities (Niikura *et al.*, 1991; Murakami *et al.*, 1994; Ong *et al.*, 2000). Insect cells displaying HN have been deployed as immunogens to inoculate chickens and provided protection against NDV (Niikura *et al.*, 1991). As co-expression with NDV fusion (F) protein resulted in syncytium formation of the HN-displayed insect cells (Murakami *et al.*, 1994), these HN-displayed cells can be used for rapid detection of NDV infection. Similarly, Du *et al.* (2015) displayed the envelope (E) protein of JEV on the insect cell surface with retained fusion activity. The co-expression of JEV precursor membrane protein (prM) induced a considerable syncytial formation of infected insect cells applicable to the screening of neutralizing antibodies, or small antiviral molecules for JEV E protein (Du *et al.*, 2015).

Display on mammalian and other non-lepidopteran cells

### Display of pharmacologically important transmembrane proteins

Baculovirus-mediated mammalian cell display technology is superior in cell-based assays for pharmacologically relevant proteins such as GPCRs, nuclear receptors, ion channels, and transporters. In

these assays, target proteins are typically transiently expressed in case of cellular toxicity caused by over-expression (Su *et al.*, 2004; Mäkelä and Oker-Blom, 2008). The baculovirus transgene system (e.g. BacMam) meets the transient expression requirement as demonstrated by Pfohl *et al.* (2002). The expression of a multi-subunit ATP-sensitive K<sup>+</sup> (KATP) channel could be titrated by controlling the amount of BacMam virus added to mammalian cells. The co-display of two or more target proteins can be achieved by simply co-transducing with BacMam viruses expressing individual proteins (Hassan *et al.*, 2006). Assays conducted using the BacMam transduction system show high consistency relative to the standard DNA transfection approach, as reported by Clay *et al.* (2003) in their study to profile the agonists of an oestrogen receptor in human osteosarcoma cells. In a study of human ether-a-go-go-related gene (hERG) channel, Titus *et al.* (2009) developed a high-through-put screening assay using BacMam-transduced cells to profile the hERG inhibitors. The outcome IC<sub>50</sub> values for 10 known hERG inhibitors correlated well with results determined by traditional manual patch clamp assays.

In terms of cell-based, high-through-put assays, the BacMam transduction system has several advantages over the stable cell line system; only one cell line needs to be maintained routinely when using BacMam transduction, in which transient expression can be developed quickly (Su *et al.*, 2004; Davenport *et al.*, 2009). Moreover, the BacMam transduced cells may adapt to different assays as reported by Shukla *et al.* (2012) in their study of ATP-binding cassette (ABC) drug transporters. They applied the BacMam transduced HeLa cells in both transport function and biochemical property assays, which previously had to be conducted using two different cell lines resulting in data inconsistency. As the BacMam transduced HeLa cells were able to adapt to both assays, it would be suitable for establishment of a robust assay for ABC transporters during the preclinical drug development process (Shukla *et al.*, 2012). The broad host tropism of baculovirus BV may further facilitate the application of BacMam in cell-based assays. The choice of cell line in cell-based assays for ABC transporters used to be limited as the cells have to polarize, but such cells usually show low transfection efficiency. Fung *et al.* (2016) found that the BacMam virus expressing

the ABC transporters can efficiently transduce the polarized cell monolayers of LLC-PK1, MA104, and T84 cell lines, which is beneficial for establishment of ABC transporter drug screening methods (Fung *et al.*, 2016). As BacMam is such a versatile tool for pharmacologically relevant proteins for both characterization and drug discovery screening, the pharmaceutical company GSK has adapted BacMam to several of their GPCR cell-based assays (Davenport *et al.*, 2009).

### Display of viral protein for high-throughput screening

Apart from the cellular transmembrane proteins, BacMam also facilitates study of infectious viruses by efficiently displaying the viral membrane proteins or antigens. The BacMam virus displaying the S1 glycoprotein of infectious bronchitis virus (IBV) gained the ability to enter chicken primary cells and increased IBV vaccine efficacy (Zhang *et al.*, 2014). As for the cell-based assays, Jenkinson *et al.* (2003) applied BacMam to develop a surrogate assay for measuring HIV viral/cell fusion. As M-tropic HIV mediates its cell entry by binding the viral envelope protein GP120 to the cellular CCR5/CD4 coreceptor, inhibition of this interaction can reduce viral fusion and entry into the cells. Jenkinson *et al.* (2003) generated a BacMam virus to mediate the surface display of HIV GP120 on HEK-293 cells. As the cell fusion event could be quantified by luciferase reporter between the GP120 displayed cells and CCR5/CD4 displayed cells, a safe high-throughput assay for screening of fusion inhibitors could thereby be established (Jenkinson *et al.*, 2003).

In addition to the mammalian systems, baculovirus are able to transduce the cells of several non-lepidopteran hosts. Naik *et al.* (2018) established a 'BacMos' system using baculovirus as a vector to deliver genes of interest into mosquito cells, larvae, and adults. They introduced a neuraminidase (NA) from H5N3 influenza virus into the baculovirus vector, and then transduced mosquito C6/36 cells. The NA protein was successfully displayed with activity on the surface of the mosquito cells by the native NA transmembrane domain (Fig. 11.5). Because many mosquito cell lines adhere to culture plates more tightly than lepidopteran cells and require a lower MOI for baculovirus transduction compared to mammalian cells, BacMos

systems may prove to be a better approach for large-scale screens that involve intensive washing or physical treatments.

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## Strategies for improvements

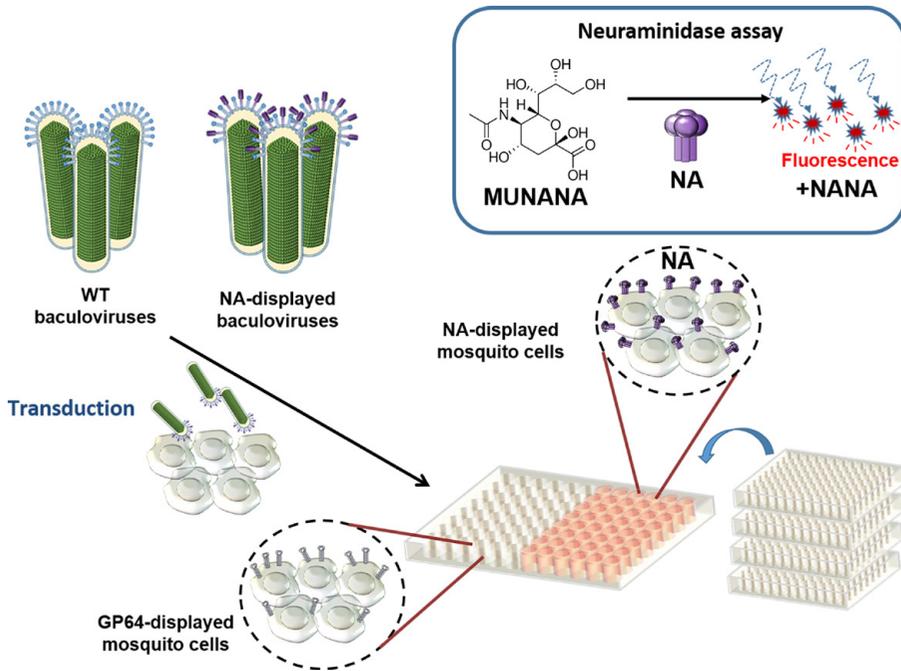
### Peptide domain selection

#### Signal peptide (SP)

Although the BEVS is suitable for the expression of complex secretory and membrane-bound glycoproteins, problems of insolubility or poor secretion may be encountered for some target proteins. Alteration of the baculovirus SP is one possible solution for overcoming this difficulty. SPs are required for protein entry into the cellular endoplasmic reticulum and subsequent secretion out of cells. Although many SPs possess similar amino acid compositions, heterologous SPs might be inefficiently recognized by the insect cell protein translocation machinery, thereby decreasing the yield of secretory protein (Jarvis *et al.*, 1993). The most frequently used native secretory SP from AcMNPV is GP64, but the SP from *Apis mellifera* prepromellitin has been shown to enhance secretion of plant propapain 5-fold in insect cells (Tessier *et al.*, 1991). The SPs derived from cecropin B (van Hofsten *et al.*, 1985) and GRP78/BiP (Gething and Sambrook, 1992) have also proved to be useful for BEVS. Finally, modifying the nucleotide sequence of SPs through codon optimization may also represent a feasible way of improving the expression and secretion of some proteins (Futatsumori-Sugai and Tsumoto, 2010).

#### Transmembrane (TMD) and cytoplasmic tail (CTD) domains

In a study by Yang *et al.* (2007) in which they displayed the influenza HA protein on the surface of baculovirus, they found that the CTD of GP64 in the expression construct increased the incorporation of HA protein on the viral surface. This also improved the transduction efficiency of the virus into mammalian cells and the immunogenicity of the HA-displayed baculovirus. It was later demonstrated that a construct comprising SP and CTD from GP64 with the TMD of HA resulted in the most efficient display of HA on baculoviral surfaces compared to other domain combinations



**Figure 11.5** Application of baculovirus-transduced mosquito cells as a cell-based platform for influenza NA activity assay. Baculoviruses displaying NA were transduced into mosquito cells to induce surface-display of NA on cell surfaces, with the insertion driven by a mosquito-specific promoter. Wild-type (WT) baculoviruses were transduced in parallel as a negative control, resulting in mosquito cells displaying baculoviral GP64. The NA-displaying mosquito cells exhibited NA activity, as determined by a high-throughput fluorescence-based neuraminidase inhibition assay using 2'-(4-Methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid (MUNANA) as substrate.

(Tang *et al.*, 2010). As the target peptide domains largely influence the efficiency of protein display, many studies have also examined the potential for use of the TMDs and CTDs of other proteins. For example, the combination of the GP64 SP and VSVG TMD and CTD resulted in increased display efficiency (Chapple and Jones, 2002). Zhou and Blissard (2008) generated a G-stem construct containing nearly 90 amino acids from the C-terminus of VSVG (including a partial ectodomain, as well as the predicted TMD and CTD), and found that it increased HA incorporation on baculovirus. Apart from replacement of the peptide domains of type I membrane proteins, peptide domains from type II membrane proteins such as the NA of *Influenza A virus* have also been studied. Borg *et al.* (2004) determined the possibility of fusing EGFP with the SP and TMD of NA. In their construct, both SP and the TMD are located at the N-terminus of the foreign genes, thereby minimizing problems such as elimination of stop codons and frame-shift errors.

## Promoters and enhancer elements used in the BEVS

### Promoters

Promoter selection is an important issue in the BEVS, as it affects protein quantity and quality, as well as whether or not the target protein can be expressed by the cross-phylum baculovirus transduction system. Strong promoters, such as the *p10* or *polh* promoters, promote extremely high levels of foreign gene expression in insect cell systems, so they would be suitable for functional assays using either virus or insect cells that require high protein expression. Several baculovirus immediate-early promoters, e.g. the *ie1* and *gp64* promoters, have also been applied in baculovirus surface-display systems. One of the advantages of using an immediate-early promoter is that they utilize the cellular transcription system, so are able to express in different organisms. A *Heliothis zea nudivir-1* (HzNV-1) early promoter (the *pag1* promoter) has been used in both Sf21 cells (Wu *et al.*, 2010)

and mosquito C6/36 cells (Naik *et al.*, 2018). The *ie1* promoter of WSSV is also frequently used in baculovirus surface-display systems, especially for display of influenza HA protein. The WSSV-*ie1* promoter operates in both insect and mammalian systems, and hence is well suited for the display of proteins on baculovirus surfaces for use as gene therapy vectors and vaccine antigens (He *et al.*, 2008). Apart from these cross-phylum promoters, composite promoters that include two or more promoters with different features are also widely used. For expression in mammalian systems, foreign genes inserted into the viral genome can be turned on by composite mammalian promoters, such as *CMV* or *SV40*. Expression from three viral promoters (the *CMV*, *SV40* and *RSV* promoters), as well as one cellular promoter (*ubiquitin C*), each carried individually by baculoviruses was compared in three mammalian cell lines (CHO, COS-1 and HEK293). In this study, the *CMV* promoter resulted in the strongest expression levels (Spenger *et al.*, 2004).

#### Enhancer factors and elements

Several enhancer elements can be incorporated into vectors to assist in either expression of or function of a heterologous protein. When baculovirus vector is applied in mammalian systems, surface display of human or pig IgG Fc could specifically target cells of the immune system expressing Fc receptors (FcRs), thereby enhancing transduction and vaccination efficacy (Martyn *et al.*, 2009; Liu *et al.*, 2017b). Display of synthetic IgG-binding domains target the viral vector to desired cell types with IgG antibody (Ojala *et al.*, 2004). In addition, surface display of a 23 amino acid arginine-glycine-aspartic acid (RGD)-motif from the FMDV VP1 increased the uptake of baculovirus in mammalian cells (Ernst *et al.*, 2006). Co-expression of the baculovirus IE1 and IE2 proteins have been reported to trans-activate the *CMV* and *SV40* promoters in mammalian Vero E6 and U-2OS cells through both transient transfection and recombinant baculovirus transduction (Murgess *et al.*, 1997; Liu *et al.*, 2007, 2009). This activation could be further augmented by the presence of a baculovirus enhancer element, i.e. the homologous region (*hr*) sequence (Lo *et al.*, 2002; Liu *et al.*, 2009). The *hr* sequence has been shown to activate *CMV* and *hsp70* promoters in several mammalian cell lines (Vero E6, COS-1

and HepG2); indeed, the *hr* sequence enhanced the *CMV* promoter activity more than 3-fold and over 122-fold upon co-expression of IE2 (Liu *et al.*, 2009). The Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) can enhance baculovirus-mediated transgene expression and has been incorporated as an element of BacMam systems (Mähönen *et al.*, 2007). Apart from co-display or co-expression of enhancer elements, histone deacetylase (HDAC) inhibitors such as sodium butyrate (NaBu) and trichostatin A (TSA) enhanced baculovirus transgene expression (Condreay *et al.*, 1999; Spenger *et al.*, 2004), and could be added to mammalian culture systems during baculovirus transduction to increase protein expression. Wang *et al.* (2017) performed a comprehensive screen of 176 known antiviral innate immune genes in mammalian cells and identified 43 host restriction factor genes that suppress baculovirus gene expression in a human A549 lung carcinoma cell line. They also found that by inhibiting some of these host restriction factor genes, baculovirus gene expression was greatly enhanced in mammalian cells.

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#### Summary and future outlook

Baculovirus surface-display technology is one of the most creative and valuable applications of BEVS. This technology benefits from all of the advantage of BEVS, in terms of high biosafety, large insertion capacity, high-level recombinant protein production, and appropriate eukaryotic post-translational modifications. Heterologous proteins can be displayed on the surfaces of viral particles, occlusion bodies, infected cells, and even transduced cells. Different cloning strategies have been developed for appropriate anchoring of proteins on these various surfaces. Based on the literature, baculovirus-display technologies have been applied in a wide range of fields. The baculovirus virion-display strategy has been used to study the functions of complex eukaryotic transmembrane proteins, to serve as vaccine antigens, and to substitute human-infectious viruses in diagnostic or antiviral assays. The cell surface-display technologies, either in insect cells or in mammalian cells, have shown great potential for establishing high-throughput assay platforms. GP64 may still be the most frequently applied fusion partner for

both virion and cell surface display strategies. By use of cryo-electron microscopy (cryo-EM), the baculovirus particle was shown to be an elongated ovoid shape with a lateral space between the envelope and nucleocapsid, rather than a rod-shaped particle (Wang *et al.*, 2016). More strikingly, the envelope proteins are located not only at one apical end, but are clustered at both polar ends (Fig. 11.6 and Wang *et al.*, 2016). These findings indicate that foreign proteins displayed by GP64 fusion may be more abundant and not restricted to one apical end on the virus particle. One of the major future applications of the baculovirus surface-display technology may be to generate vaccine antigens and diagnostic platforms for infectious pathogens, given the increasing incidence of emerging pathogens around the world. Another promising area for development is expansion of foreign protein display technologies in cells across different phyla in combination with the engineered baculovirus transgene



**Figure 11.6** Cryo-electron microscopy of baculovirus particle. Baculovirus particle (budded virus) with envelope proteins located on both polar ends. Bar: 100nm.

delivery system. Exploitation of strategies to enhance transgene expression will thereby be substantial. Likewise, the engineering of baculovirus vectors to allow for transgene expression in other organisms is an area ripe for investigation. Finally, as for practical applications in high-throughput assays, use in conjunction with automated systems is worth considering.

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