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GP64 of group I nucleopolyhedroviruses cannot readily rescue infectivity of group II *f*-null nucleopolyhedroviruses

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The genus *Nucleopolyhedrovirus* (NPV) of the family *Baculoviridae* can be subdivided phylogenetically into two groups. The same division can be made on the basis of their budded virus (BV) envelope fusion protein. Group I NPVs are characterized by the presence of a GP64-like major envelope fusion protein, which is involved in viral attachment and the fusion of virus and cell membrane, and is required for budding of progeny nucleocapsids. Group II NPVs have an envelope fusion protein unrelated to GP64, named F. In contrast to GP64, F proteins are found in all baculoviruses, but they are not functional as envelope fusion proteins in group I NPVs. *Autographa californica* multiple NPV (AcMNPV) lacking GP64 can be pseudotyped by the F protein of *Spodoptera exigua* multiple NPV (SeMNPV), suggesting that F proteins are functionally analogous to GP64. GP64 homologues are thought to have been acquired by group I NPVs during evolution, thereby giving these viruses a selective advantage and obviating the need for a functional F protein. To address this supposition experimentally, attempts were made to pseudotype a group II NPV, SeMNPV, with GP64. Transfection of an *f*-null SeMNPV bacmid into *Se*301 cells did not result in the production of infectious BVs. This defect was rescued by insertion of SeMNPV *f*, but not by insertion of AcMNPV *gp64*. This suggests that the functional analogy between GP64 and F is not readily reciprocal and that F proteins from group II NPVs may provide additional functions in BV formation that are lacking in the GP64 type of fusion protein.

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INTRODUCTION

The *Baculoviridae* are a family of large, enveloped, double-stranded DNA viruses that are pathogenic to arthropods, predominantly insects in the order Lepidoptera, but also to certain members of the orders Diptera, Hymenoptera and Decapoda (Adams & McClintock, 1991). The family encompasses two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Recently, a new baculovirus classification has been proposed, consisting of four genera: *Alphabaculovirus* (lepidopteran NPVs), *Betabaculovirus* (lepidopteran GVs), *Gammabaculovirus* (hymenopteran NPVs) and *Deltabaculovirus* (dipteran NPVs) (Jehle *et al.*, 2006). The proposed genus *Alphabaculovirus* can be subdivided further into group I and II NPVs, based on phylogenetic analysis of the viral DNA (Bulach *et al.*, 1999; Hayakawa *et al.*, 2000; Herniou *et al.*, 2001, 2003).

Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV) (Volkman & Summers, 1977). ODVs are present in occlusion bodies and are able to infect midgut epithelial cells by direct membrane fusion (Granados, 1978; Granados & Lawler, 1981; Horton & Burand, 1993). BVs infect insect cells via receptor-mediated endocytosis and are responsible for systemic spread of the virus in the insect (Hefferon *et al.*, 1999; Volkman & Goldsmith, 1985). The BVs of alphabaculovirus group I NPVs, e.g. *Autographa californica* multiple NPV (AcMNPV) and *Orgyia pseudotsugata* (Op)MNPV, contain a major envelope protein, GP64. This protein is involved in attachment of BVs to the cell, is required for low-pH-triggered membrane fusion during virus entry and is necessary later in the process of infection for efficient budding of progeny nucleocapsids (NCs) into the haemolymph or cell-culture supernatant (Blissard & Wenz, 1992; Hefferon *et al.*, 1999; Oomens & Blissard, 1999).

Recent data from complete genomic sequences of a growing number of baculoviruses suggest that all group I NPVs possess a *gp64*-like gene, whereas group II NPVs, beta-, gamma- and deltabaculoviruses lack this gene. For three group II NPVs, *Spodoptera exigua* (Se)MNPV, *Lymantria dispar* (Ld)MNPV and *Helicoverpa armigera* (Hear)NPV, it has been shown that low-pH-mediated membrane fusion is mediated by a novel type of envelope fusion protein, called F (IJkel *et al.*, 2000; Pearson *et al.*, 2000; Long *et al.*, 2006b). Like several mammalian viral

envelope fusion proteins, the baculovirus F protein must be cleaved post-translationally by a proprotein convertase (furin) to become fusiogenic (Lung *et al.*, 2002; Westenberg *et al.*, 2002). Homologues of the F gene have been identified in other group II NPVs, in beta- and deltabaculoviruses and in members of the insect retrovirus family *Errantiviridae*, but also exist in group I NPVs (Herniou *et al.*, 2003; Malik *et al.*, 2000; Rohrmann & Karplus, 2001; Terzian *et al.*, 2001). In the genome of group I NPVs, a truncated F homologue is present (Ac23 homologues). Its translation product is found on the envelope of BVs, but has been shown to be dispensable for viral replication and pathogenesis (Lung *et al.*, 2003; Pearson *et al.*, 2001).

Recently, it was shown that the F proteins of the group II NPVs SeMNPV, LdMNPV and HearNPV are capable of substituting functionally for GP64 in AcMNPV (Long *et al.*, 2006b; Lung *et al.*, 2002). An AcMNPV bacmid lacking the *gp64* gene was unable to produce BVs after transfection into insect cells (Monsma *et al.*, 1996), whereas this defect could be rescued by insertion of the SeMNPV, LdMNPV or HearNPV *f* gene (Long *et al.*, 2006b; Lung *et al.*, 2002). GP64 homologues are also found on Thogoto and Dhori viruses, which are tick-transmitted orthomyxoviruses that replicate in both ticks and mammals (Freedman-Faulstich & Fuller, 1990; Morse *et al.*, 1992). It has therefore been suggested that the group I NPVs have acquired the *gp64* gene later during evolution, either from the host or from another insect-infecting virus (Lung *et al.*, 2002; Morse *et al.*, 1992), thereby getting a selective advantage due to increased efficiency of either virus–receptor interaction and virus entry, or virus budding, obviating the need for a functional F protein. However, experimental evidence to support this view is lacking.

To address this hypothesis experimentally, a group II NPV (SeMNPV) lacking F was pseudotyped with GP64. The *f* gene of SeMNPV was deleted by using site-specific mutagenesis of an infectious SeMNPV bacmid (Pijlman *et al.*, 2002). Transfection of this *f*-null bacmid into insect cells showed that the virus replicates in the initially transfected cell, but is no longer able to propagate an infection. Reinsertion of the SeMNPV *f* gene rescued SeMNPV infectivity fully, but insertion of the *gp64* gene failed to rescue BV infectivity.

METHODS

Cells, insects and virus. The *Spodoptera exigua* cell line *Se*301 (Hara *et al.*, 1995) was cultured at 27 °C in plastic tissue-culture flasks (Nunc) in Grace's insect medium, pH 5.9–6.1 (Invitrogen), supplemented with 10 % fetal bovine serum (FBS). The SeMNPV isolate (Gelernter & Federici, 1986) was originally obtained from Dr B. A. Federici (Department of Entomology, University of California, CA, USA) and was called SeMNPV-US1 (Muñoz *et al.*, 1998).

Deletion of the SeMNPV *f* gene by ET recombination in *Escherichia coli*. For deletion mutagenesis of the *f* gene of the SeMNPV-US1 bacmid SeBAC10 (Pijlman *et al.*, 2002), 75- to 77-mer recombineering primers were designed with 50 nt comprising the left or right homology arm on the 5′ end. The forward primer was 5′-TTTGGTCGTCGTCGTCGTTGAAATGATACCCTTTGTCGTT-GAACTGGCCTTAGGTTTAAGGGCACCAATAACTG-3′, with viral flanking sequences [5′ untranslated region (UTR)] from nt 12248 to 12297 according to the SeMNPV complete genome sequence (IJkel *et al.*, 1999). The reverse primer was 5′-ATACATTATATATTGTT-TTATTTTACTCTACTACTATTACAATCAATCGGCCTAAGGTTC-CTGTGCGACGGTTAC-3′, with viral flanking sequences (3′ UTR) from nt 14545 to 14496. The 3′ ends of the primers anneal to the chloramphenicol-resistance gene (*cat*) of pBeloBac11 (Shizuya *et al.*, 1992; Wang *et al.*, 1997), and a *Bsu*36I site was designed between the viral and *cat* sequences (underlined).

PCR on pBeloBac11 was performed by using high-fidelity Expand Long Template PCR (Roche). The expected 1050 bp PCR fragment was gel-purified and digested with *Dpn*I to eliminate residual template plasmid DNA. After a second round of gel purification, about 500 ng PCR product was used for transformation of electrocompetent *E. coli* DH10*β* cells containing both SeBAC10 and the plasmid pBAD-*αβγ*, promoting homologous recombination (Fig. 1a), as described previously (Pijlman *et al.*, 2002). The altered sequence at the *f* locus of the recombinant bacmid, designated SeBACΔF, was confirmed by PCR using primers to the regions flanking the *f* gene.

Donor plasmid construction. The SeMNPV *polyhedrin* gene with its own promoter was cloned as an *Sma*I/*Hin*dIII fragment (Pijlman *et al.*, 2002) in the *Sma*I and *Kpn*I sites of pFastBac Dual (Invitrogen), generating pFBSePolh Dual. The SeMNPV *f* gene and the AcMNPV *gp64* gene, both under the control of the *gp64* promoter, were cloned as *Xba*I/*Eco*RI and *Xba*I/*Sst*I fragments from pΔFBgusSe8 and pΔFBgusGP64 (Lung *et al.*, 2002) into the *Sma*I and *Sst*I sites of pFBSePolh Dual. In this procedure, the AcMNPV p10 and polyhedrin promoters were removed, and plasmids pFBSepolh-SeF(*pGP64*) and pFBSepolh-GP64(*pGP64*) were obtained, respectively.

The 250 bp upstream of the SeMNPV *f* open reading frame (ORF) was amplified from pSe*Bgl*II-H (IJkel *et al.*, 1999) with primers 5′-AAACCCGGGTTTGGTCGTCGTCGTCGTTG-3′ and 5′-TAAGGATCCTATTTTGCTTGCGACTCGGTTCTC-3′ (underlined sequences generate *Sma*I and *Bam*HI restriction sites, respectively), using high-fidelity Expand Long Template PCR (Roche). The PCR fragment was cloned into the *Sma*I and *Bam*HI sites of pFBSePolh Dual, thereby removing the AcMNPV p10 and polyhedrin promoters and generating pFBSepolh-(*pSeF*). The SeMNPV *f* gene and the AcMNPV *gp64* gene were cloned as *Bam*HI/*Not*I and *Eco*RI/*Eco*RI fragments from pΔFBgusSe8 and pΔFBgusGP64 (Lung *et al.*, 2002) into the *Bam*HI and *Not*I sites of pFBSePolh-(*pSeF*), generating pFBSePolh-SeF(*pSeF*) and pFBSePolh-GP64(*pSeF*), respectively. For the generation of control bacmids, a vector was generated containing only the SeMNPV *polyhedrin* gene behind its own promoter. This was done by removing the SeMNPV *f* promoter as an *Sma*I/*Stu*I fragment from pFBSepolh-(*pSeF*), generating pFBSepolh.

Transfection of bacmids. The inserts of the donor plasmids were transposed into the *att*Tn*7* transposition sites of the SeMNPV bacmids SeBacΔF and SeBAC10 (Fig. 1b), or into the *gp64*-null AcMNPV bacmid (Lung *et al.*, 2002), according to the Bac-to-Bac manual (Invitrogen). Transposition was confirmed by PCR as described previously (Westenberg *et al.*, 2004).

*Se*301 cells (5.0×10^5) were seeded into 35 mm tissue-culture plates (Nunc). The cells were transfected with approximately 1 μg bacmid DNA, using 10 μl Cellfectin (Invitrogen). After 5 days, the cells were transferred to a T75 flask (Nunc) and, subsequently, one-third of the cells were transferred every 5 days to a new T75 flask until 90 % of the cells contained polyhedra. The insect-cell supernatants were clarified

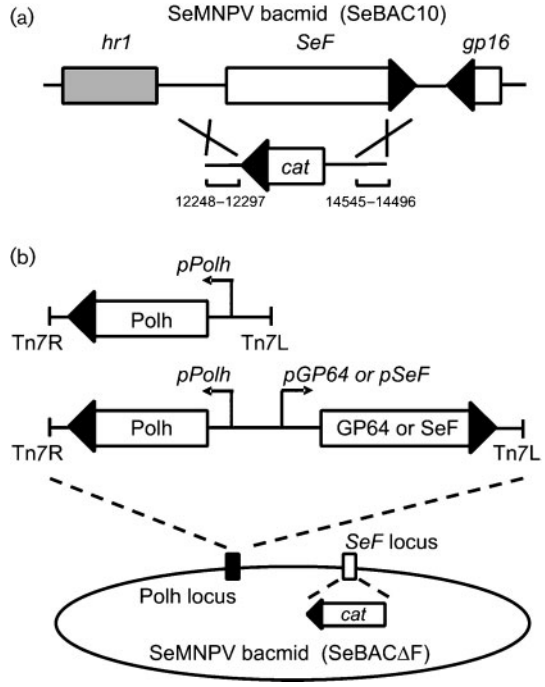

Fig. 1. (a) Strategy for the generation of an *f*-null SeMNPV bacmid by ET recombination in *E. coli*. The *SeF* locus in the SeMNPV bacmid SeBac10 is shown in the upper diagram. A PCR-amplified DNA fragment containing the chloramphenicol-resistance gene (*cat*) flanked by 50 bp SeMNPV sequences on both sides of the *f* ORF was used to substitute the *f* gene for the *cat* gene. Sequences included for homologous recombination (12248–12297 and 14545–14496) are indicated (IJkel *et al.*, 1999). (b) Strategy for insertion of gene-cassette constructs into the *polyhedrin* locus of the *f*-null SeMNPV bacmid. Inserts include the SeMNPV *polyhedrin* gene without the *f* gene (top), and the SeMNPV *polyhedrin* gene plus the AcMNPV *gp64* (GP64) or SeMNPV *f* (SeF) genes under the control of either the AcMNPV *gp64* promoter (*pGP64*) or the SeMNPV *f* promoter (*pSeF*) (centre). The cassettes were inserted into the *att*Tn*7* sites (indicated by the right and left insertion sites, Tn7R and Tn7L) in the *polyhedrin* locus by Tn*7*-based transposition (Pijlman *et al.*, 2002).

by centrifugation at 4000 ***g*** for 10 min and subsequently passed through a 0.45 μm filter. The presence of infectious BVs in the supernatant was investigated by infecting 1.0×10^6 *Se*301 cells in a T25 flask with 500 μl supernatant. Finally, the genotype of the BVs was verified by PCR.

RESULTS

Disruption of the *f* gene in an SeMNPV bacmid

Recently, it has been shown that a *gp64*-null AcMNPV bacmid, unable to propagate an infection upon transfection into *Sf*9 cells, could be rescued by the *f* gene of certain group II NPVs (Long *et al.*, 2006b; Lung *et al.*, 2002). To determine whether the reverse was also possible, a recently constructed SeMNPV bacmid (Pijlman *et al.*, 2002) was modified by deleting the *f* gene (Se8). The *f* ORF and its promoter were deleted from the SeMNPV bacmid SeBac10 by ET recombination (Muyrers *et al.*, 1999), using a PCR-amplified DNA fragment containing a *cat* cassette flanked by 50 bp of sequence 200 bp upstream of the translational start codon and 50 bp downstream of the stop codon (Fig. 1a). Bacterial colonies resistant for chloramphenicol (insert) and kanamycin (bacmid) were selected. The colonies were analysed for successful recombination by PCR using primers flanking the recombination sites and by sequencing the amplified DNA fragments (data not shown). The generated SeBacΔF DNA was transformed into *E. coli* DH10*β* cells, together with the plasmid pMON7124, encoding a Tn*7* transposase, which can facilitate transposition of gene cassettes into the bacmid (Luckow *et al.*, 1993).

To investigate whether the *f* gene is necessary for efficient budding and propagation of infection, the SeMNPV *polyhedrin* gene was inserted into SeBac10 and SeBacΔF bacmids at its original locus by Tn*7*-based transposition to mark successful infection (Fig. 1b) (Luckow *et al.*, 1993). *Se*301 cells were transfected with the generated bacmids SeBac10/Sepolh and SeBacΔF/Sepolh. As a positive control, wild-type SeMNPV DNA was also transfected in parallel. Polyhedra were observed 1 week post-transfection (p.t.) in cells initially transfected with SeMNPV wild-type DNA, whilst 2 weeks p.t., approximately 90 % of the cells contained polyhedra (Fig. 2a). A significant delay was observed for cells transfected with SeBac10^{Sepolh}, where 90 % was reached approximately 3 weeks p.t. (Fig. 2b). The presence of infectious BVs in the supernatants of the polyhedron-containing cell culture was demonstrated by infecting healthy *Se*301 cells with the supernatants (Fig. 2d, e). Cells initially transfected with SeBacΔF^{Sepolh} did contain polyhedra at 3 weeks p.t. (Fig. 2c), but subculturing of the cells showed that the virus did not spread to other cells (Fig. 2f). Therefore, it can be concluded that the SeMNPV *f* gene is essential for virus propagation in cell culture and probably in insects.

The *f*-null SeMNPV bacmid is not rescued by GP64

The inability to propagate an infection upon transfection with the *f*-null SeMNPV bacmid shows that the *f* gene is essential for BV production. To see whether the propagation defect could be rescued, the SeMNPV *f* gene was reinserted via a repair bacmid, SeBacΔF$^{Sepolh\text{-}SeF(pSeF)}$. This bacmid was generated by transposing the *f* gene under the control of its own promoter, together with the *polyhedrin* gene, into SeBacΔF (Fig. 1b). Transfection of the bacmid resulted in the formation of polyhedra in the majority of cells 3 weeks p.t. (Fig. 3a) and the production of infectious BVs was demonstrated upon passaging the supernatant (Fig. 3d). This shows that the *f*-null SeMNPV bacmid could be rescued by its own *f* gene and that a viable virus was generated. Further, the experiment shows that the homologous repeat region *hr1*, essential for viral replication (Broer *et al.*, 1998), and *gp16*, expressing a nuclear

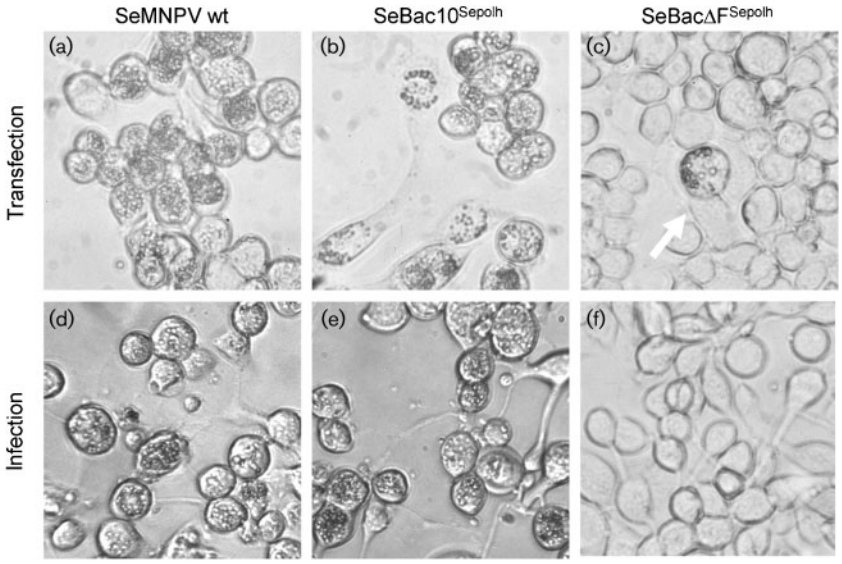

Fig. 2. Transfection–infection assay for wild-type SeMNPV or SeMNPV bacmids. *Se*301 cells were transfected with 1 μg SeMNPV wild-type or bacmid DNA and subcultured every 5 days for a maximum period of 4 weeks or until 90 % of the cells contained polyhedra. Polyhedron-containing cells were observed 2 weeks p.t. for wild-type SeMNPV DNA (a) and 3 weeks p.t. for SeBac10^{Sepolh} DNA (b), and polyhedra in the initially transfected cells (indicated by the arrow) were observed 3 weeks p.t. for SeBacΔF^{Sepolh} (c). Supernatant of polyhedron-containing cells was transferred to healthy *Se*301 cells and infected cells were observed at 4 days post-infection for SeMNPV wild-type (d) and SeBac10^{Sepolh} (e), but not for SeBacΔF^{Sepolh} (f).

membrane protein with unknown function (Gross *et al.*, 1993), both flanking the *f* gene in SeMNPV, were probably not affected by the deletion of *f*.

To investigate whether AcMNPV GP64 is also able to rescue *f*-null SeMNPV, the *gp64* gene under the control of the SeMNPV *f* promoter, together with the *polyhedrin* gene as a marker, was transposed into SeBacΔF, generating SeBacΔF$^{Sepolh\text{-}GP64(pSeF)}$ (Fig. 1b). Polyhedra were observed 3 weeks p.t. in *Se*301 cells that were initially transfected with this bacmid (Fig. 3b), but the infection did not spread to other cells. Transfer of the supernatant to fresh cells did not result in a secondary infection, suggesting that no infectious BVs were produced (Fig. 3e).

To exclude the possibility that the absence of spread was not due to low GP64 expression being governed by the SeMNPV *f* promoter or to less optimal replication of the bacmid, two 'control' bacmids, SeBacΔF$^{Sepolh\text{-}SeF(pGP64)}$ and SeBacΔF$^{Sepolh\text{-}GP64(pGP64)}$, were generated, from which either the F protein or the GP64 protein is expressed under the control of the AcMNPV *gp64* promoter. Polyhedron-containing cells were observed when cells were transfected with *f*-null bacmid expressing the F protein (Fig. 3c), and transfer of the supernatant to fresh cells resulted in a secondary infection (Fig. 3f), indicating that the *gp64* promoter is also able to drive *f* gene expression in SeMNPV. Despite this observation, rescue of *f*-null SeMNPV bacmid by *gp64* under the control of its own promoter was not detected, as polyhedra were only observed in the initially transfected cells (Fig. 3g) and not in cells incubated with the supernatant (Fig. 3j).

Expression of GP64 in insect cells

To verify whether functional GP64 could be expressed from the donor plasmids pFBSePolh-GP64(*pSeF*) and pFBSePolh-GP64(*pGP64*), the expression cassettes were transposed into a *gp64*-null AcMNPV bacmid. *Se*301 cells were transfected with the originating bacmids AcBacΔgp64$^{SePolh\text{-}GP64(pSeF)}$ and AcBacΔgp64$^{SePolh\text{-}GP64(pGP64)}$, respectively. Five days p.t., polyhedron-containing cells were observed (Fig. 3h, i). Cell supernatants were clarified and used to infect healthy *Se*301 cells. Three days post-infection, infected cells were observed, as determined by the presence of polyhedra (Fig. 3k, l), indicating that infectious viruses were made and, more importantly, that GP64 had been expressed. So, in principle, GP64 could be expressed from SeBacΔF$^{Sepolh\text{-}SeF(pGP64)}$ and SeBacΔF$^{Sepolh\text{-}GP64(pGP64)}$ as well in *Se*301 cells, but was not able to form budded SeMNPV virions.

DISCUSSION

Pseudotyping of viruses with heterologous envelope fusion proteins is a commonly used method to alter the host range of the virus (Landau *et al.*, 1991). Pseudotyping of baculoviruses was performed successfully for the first time with vesicular stomatitis virus G (VSV-G) protein in AcMNPV BVs (Barsoum *et al.*, 1997) and could even substitute for GP64 (Mangor *et al.*, 2001), although viral propagation in insect cells was greatly reduced. However, neither pseudotyping experiment seemed to alter the host range of AcMNPV. GP64 is involved in viral attachment to host cells, is required during virus entry and is necessary for efficient budding of progeny NCs (Blissard & Wenz, 1992; Hefferon *et al.*, 1999; Oomens & Blissard, 1999). GP64 is present in all group I NPVs. Group II NPVs lack the GP64 protein and use F proteins as envelope fusion protein (IJkel *et al.*, 2000; Pearson *et al.*, 2000). It has been postulated that, during evolution, the *gp64* gene was inserted into some baculoviruses (now group I NPVs), giving these a selective advantage due to increased efficiency either of virus–receptor interactions or of virus entry or budding, obviating the need for a functional F

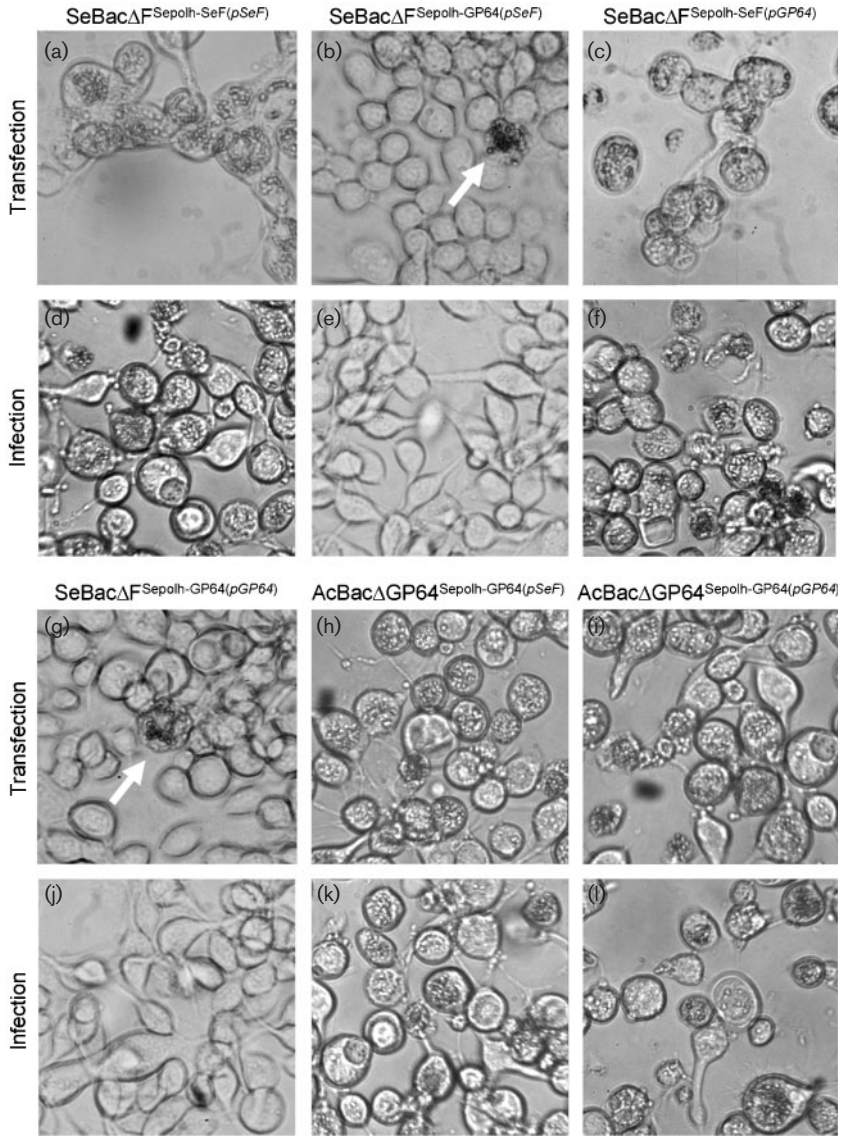

Fig. 3. Transfection–infection assay for *f*-null SeMNPV and *gp64*-null AcMNPV bacmids. *Se*301 cells were transfected with 1 μg SeMNPV bacmid DNA and subcultured every 5 days for a maximum period of 4 weeks or until 90 % of the cells contained polyhedra. Polyhedron-containing cells were observed 3 weeks p.t. for SeBacΔF$^{Sepolh\text{-}SeF(pSeF)}$ (a) and SeBacΔF$^{Sepolh\text{-}SeF(pGP64)}$ (c), and in the initially transfected cells (indicated by arrows) 3 weeks p.t. for SeBacΔF$^{Sepolh\text{-}GP64(pSeF)}$ (b) and SeBacΔF$^{Sepolh\text{-}GP64(pGP64)}$ (g). In the case of the *gp64*-null AcMNPV bacmids, cells were transfected with 1 μg DNA. Five days p.t., cells containing polyhedra were observed for AcBacΔgp64$^{SePolh\text{-}GP64(pSeF)}$ (h) and AcBacΔgp64$^{SePolh\text{-}GP64(pGP64)}$ (i). Supernatants were harvested and the presence of infectious virus was verified by infecting healthy *Se*301 cells. Three days post-infection, infected cells containing polyhedra were observed for SeBacΔF$^{Sepolh\text{-}SeF(pSeF)}$ (d), SeBacΔF$^{Sepolh\text{-}SeF(pGP64}$ (f), AcBacΔgp64$^{SePolh\text{-}GP64(pSeF)}$ (k) and AcBacΔgp64$^{SePolh\text{-}GP64(pGP64)}$ (l), but not for SeBacΔF$^{Sepolh\text{-}GP64(pSeF)}$ (e) or SeBacΔF$^{Sepolh\text{-}GP64(pGP64)}$ (j).

protein (Lung *et al.*, 2002; Morse *et al.*, 1992). The question was whether this assumption could be approached experimentally and validated.

It has previously been shown that AcMNPV could be pseudotyped with certain group II NPV F proteins, which substituted for the functions of GP64 (Long *et al.*, 2006b; Lung *et al.*, 2002). These experiments indicated that the group II NPV F proteins are functionally analogous to GP64 in an AcMNPV virion. In the current study, however, it was found that the AcMNPV GP64 protein cannot substitute readily for the F protein in SeMNPV (Fig. 3c, d) in functional terms. Viral propagation could be rescued by reinsertion of an SeMNPV *f* gene in an *f*-null SeMNPV bacmid (Fig. 3a, b), confirming that the *f* gene is essential for BV production and systemic spread of the virus of a group II NPV. This is in contrast to the F homologue (Ac23) in the group I NPV AcMNPV, where this protein can be deleted without affecting viral replication or pathogenesis in cell culture or infected animals (Lung *et al.*, 2003).

The inability of the *f*-null bacmid to propagate an infection could not be rescued by the introduction of AcMNPV *gp64* either downstream of the authentic SeMNPV *f* promoter or downstream of the AcMNPV *gp64* promoter. To exclude the possibility that propagation was affected by a deletion in the SeMNPV bacmid that could have been generated during transposition and transformation in *E. coli*, four different bacmid clones of SeBacΔF$^{Sepolh\text{-}GP64(pSeF)}$ and SeBacΔF$^{Sepolh\text{-}GP64(pGP64F)}$ were transfected into insect cells. However, none of the bacmids was able to propagate an infection, whereas the *f*-repair SeMNPV bacmid was able to do so each time. The ability to express GP64 from these bacmids was demonstrated by the insertion of the same expression cassettes into a *gp64*-null AcMNPV virus, restoring full BV infectivity (Fig. 3e, f). If GP64 is expressed in an SeMNPV background, the level of this expression can

only be very low. Western blot analysis using α-GP64 on *Se*301 cells transfected with the SeMNPV bacmids was not sensitive enough to detect GP64, also due to the low transfection rates (<0.1 %).

Despite the observation in this report that GP64 is not able to replace F functionally in SeMNPV, it has been demonstrated that GP64 can be inserted into the group II NPV HearNPV, but only when it carries an authentic *f* gene (Liang *et al.*, 2005). GP64 meets the requirements of an envelope glycoprotein that is independently able to insert into a membrane and form envelopes, as in pseudotyped lentiviruses (Kumar *et al.*, 2003). Human immunodeficiency virus type 1 (HIV-1) does not require its envelope protein (Env) for virion budding. However, the generated virions were not infectious (Shioda & Shibuta, 1990). Budding of retroviruses seems to occur at ordered lipid microdomains, called lipid rafts (Briggs *et al.*, 2003). It is possible that, when expressed in mammalian cells, GP64 may end up in these lipid rafts and thereby in the envelope of lentiviruses. Recent experiments, however, indicate that GP64 does not seem to be associated with lipid rafts in insect cells (Zhang *et al.*, 2003). In the context of group II NPVs, GP64 may not function on its own because it lacks essential interaction with other proteins to form BVs.

In contrast to HIV-1, the major envelope protein E2 of alphaviruses is absolutely required for efficient budding (Owen & Kuhn, 1997). One hypothesis to explain the synergistic roles of various proteins in the budding process is the push–pull model (Mebatsion *et al.*, 1996). The push represents the role of the matrix and perhaps other proteins on the inner surface of the plasma membrane, and the pull represents the role of the membrane proteins within and on the exterior of the membrane. The concerted or synergistic effects of the two components may accomplish budding. In this respect, it is possible that SeMNPV and perhaps all group II NPV NCs require a special interaction with the F protein to provoke budding, whereas this interaction is not needed for the incorporation of the SeMNPV F protein in AcMNPV BVs.

A possible candidate for this interaction may be the cytoplasmic tail domain (CTD) of the group II NPV F-like proteins. This CTD ranges in length from about 54 to 78 aa. In GP64-like proteins, this domain is much smaller, i.e. 3–8 aa, and probably inert (Oomens & Blissard, 1999). It has been shown for HearNPV (a group II NPV) that, except for the C-terminal 16 aa, the CTD of an F protein is important for virus spread from cell to cell (Long *et al.*, 2006a). Whilst the HearNPV F protein without its CTD is unable to do this in the context of HearNPV, it still rescues infectivity of *gp64*-null AcMNPV. The long CTDs of the F-like proteins could possess one or more specific protein motifs required for interaction with the viral NCs, whereas these motifs are absent in the rather short CTDs of GP64-like proteins.

The involvement of CTDs of viral envelope proteins in the budding process has indeed been supported for a number of other viruses, including Sendai virus (Ali & Nayak, 2000), influenza A virus (Bilsel *et al.*, 1993), VSV (Robison & Whitt, 2000), Mason–Pfizer monkey virus (Song *et al.*, 2003) and Semliki Forest virus (Zhao *et al.*, 1994). It is plausible that there is an interaction between the CTD of F and one or more viral NC proteins that are specific for group II NPVs. The putative protein Se107 (ORF 107 in SeMNPV), with a nuclear-localization signal and present only in group II NPVs (Herniou *et al.*, 2003), may be a candidate for such a protein. Group II NPVs share another unique gene, *Se30*. The putative translation product of this gene seems to have a signal peptide and transmembrane domain and it is therefore possible that this protein is translocated to the cell membrane and acts in conjunction with F in virion assembly and virus budding.

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REFERENCES

Adams, J. R. & McClintock, J. T. (1991). Nuclear polyhedrosis viruses of insects. In *Atlas of Invertebrate Viruses*, pp. 87–204. Edited by J. R. Adams & J. R. Bonami. Boca Raton, FL: CRC Press.

Ali, A. & Nayak, D. P. (2000). Assembly of Sendai virus: M protein interacts with F and HN proteins and with the cytoplasmic tail and transmembrane domain of F protein. *Virology* **276**, 289–303.

Barsoum, J., Brown, R., McKee, M. & Boyce, F. M. (1997). Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. *Hum Gene Ther* **8**, 2011–2018.

Bilsel, P., Castrucci, M. R. & Kawaoka, Y. (1993). Mutations in the cytoplasmic tail of influenza A virus neuraminidase affect incorporation into virions. *J Virol* **67**, 6762–6767.

Blissard, G. W. & Wenz, J. R. (1992). Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J Virol* **66**, 6829–6835.

Briggs, J. A., Wilk, T. & Fuller, S. D. (2003). Do lipid rafts mediate virus assembly and pseudotyping? *J Gen Virol* **84**, 757–768.

Broer, R., Heldens, J. G., van Strien, E. A., Zuidema, D. & Vlak, J. M. (1998). Specificity of multiple homologous genomic regions in *Spodoptera exigua* nucleopolyhedrovirus DNA replication. *J Gen Virol* **79**, 1563–1572.

Bulach, D. M., Kumar, C. A., Zaia, A., Liang, B. & Tribe, D. E. (1999). Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. *J Invertebr Pathol* **73**, 59–73.

Freedman-Faulstich, E. Z. & Fuller, F. J. (1990). Nucleotide sequence of the tick-borne, orthomyxo-like Dhori/Indian/1313/61 virus envelope gene. *Virology* **175**, 10–18.

Gelernter, W. D. & Federici, B. A. (1986). Isolation, identification, and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ Entomol* **15**, 240–245.

Granados, R. R. (1978). Early events in the infection of *Heliothis zea* midgut cells by a baculovirus. *Virology* **90**, 170–174.

Granados, R. R. & Lawler, K. A. (1981). *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology* **108**, 297–308.

Gross, C. H., Wolgamot, G. M., Russell, R. L., Pearson, M. N. & Rohrmann, G. F. (1993). A baculovirus encoded 16-kDa glycoprotein localizes near the nuclear membrane of infected cells. *Virology* **192**, 386–390.

Hara, K., Funakoshi, M. & Kawarabata, T. (1995). A cloned cell line of *Spodoptera exigua* has a highly increased susceptibility to the *Spodoptera exigua* nuclear polyhedrosis virus. *Can J Microbiol* **41**, 1111–1116.

Hayakawa, T., Rohrmann, G. F. & Hashimoto, Y. (2000). Patterns of genome organization and content in lepidopteran baculoviruses. *Virology* **278**, 1–12.

Hefferon, K. L., Oomens, A. G., Monsma, S. A., Finnerty, C. M. & Blissard, G. W. (1999). Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. *Virology* **258**, 455–468.

Herniou, E. A., Luque, T., Chen, X., Vlak, J. M., Winstanley, D., Cory, J. S. & O'Reilly, D. R. (2001). Use of whole genome sequence data to infer baculovirus phylogeny. *J Virol* **75**, 8117–8126.

Herniou, E. A., Olszewski, J. A., Cory, J. S. & O'Reilly, D. R. (2003). The genome sequence and evolution of baculoviruses. *Annu Rev Entomol* **48**, 211–234.

Horton, H. M. & Burand, J. P. (1993). Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. *J Virol* **67**, 1860–1868.

IJkel, W. F. J., van Strien, E. A., Heldens, J. G. M., Broer, R., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1999). Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *J Gen Virol* **80**, 3289–3304.

IJkel, W. F. J., Westenberg, M., Goldbach, R. W., Blissard, G. W., Vlak, J. M. & Zuidema, D. (2000). A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. *Virology* **275**, 30–41.

Jehle, J. A., Blissard, G. W., Bonning, B. C., Cory, J. S., Herniou, E. A., Rohrmann, G. F., Theilmann, D. A., Thiem, S. M. & Vlak, J. M. (2006). On the classification and nomenclature of baculoviruses: a proposal for revision. *Arch Virol* **151**, 1257–1266.

Kumar, M., Bradow, B. P. & Zimmerberg, J. (2003). Large-scale production of pseudotyped lentiviral vectors using baculovirus GP64. *Hum Gene Ther* **14**, 67–77.

Landau, N. R., Page, K. A. & Littman, D. R. (1991). Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *J Virol* **65**, 162–169.

Liang, C., Song, J. & Chen, X. (2005). The GP64 protein of *Autographa californica* multiple nucleopolyhedrovirus rescues *Helicoverpa armigera* nucleopolyhedrovirus transduction in mammalian cells. *J Gen Virol* **86**, 1629–1635.

Long, G., Pan, X., Westenberg, M. & Vlak, J. M. (2006a). Functional role of the cytoplasmic tail domain of the major envelope fusion protein of group II baculoviruses. *J Virol* **80**, 11226–11234.

Long, G., Westenberg, M., Wang, H., Vlak, J. M. & Hu, Z. (2006b). Function, oligomerization and *N*-linked glycosylation of the *Helicoverpa armigera* single nucleopolyhedrovirus envelope fusion protein. *J Gen Virol* **87**, 839–846.

Luckow, V. A., Lee, S. C., Barry, G. F. & Olins, P. O. (1993). Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol* **67**, 4566–4579.

Lung, O., Westenberg, M., Vlak, J. M., Zuidema, D. & Blissard, G. W. (2002). Pseudotyping *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. *J Virol* **76**, 5729–5736.

Lung, O. Y., Cruz-Alvarez, M. & Blissard, G. W. (2003). Ac23, an envelope fusion protein homolog in the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. *J Virol* **77**, 328–339.

Malik, H. S., Henikoff, S. & Eickbush, T. H. (2000). Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res* **10**, 1307–1318.

Mangor, J. T., Monsma, S. A., Johnson, M. C. & Blissard, G. W. (2001). A GP64-null baculovirus pseudotyped with vesicular stomatitis virus G protein. *J Virol* **75**, 2544–2556.

Mebatsion, T., Konig, M. & Conzelmann, K. K. (1996). Budding of rabies virus particles in the absence of the spike glycoprotein. *Cell* **84**, 941–951.

Monsma, S. A., Oomens, A. G. & Blissard, G. W. (1996). The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J Virol* **70**, 4607–4616.

Morse, M. A., Marriott, A. C. & Nuttall, P. A. (1992). The glycoprotein of Thogoto virus (a tick-borne orthomyxo-like virus) is related to the baculovirus glycoprotein GP64. *Virology* **186**, 640–646.

Muñoz, D., Castillejo, J. I. & Caballero, P. (1998). Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of *Spodoptera exigua*. *Appl Environ Microbiol* **64**, 4372–4377.

Muyrers, J. P., Zhang, Y., Testa, G. & Stewart, A. F. (1999). Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* **27**, 1555–1557.

Oomens, A. G. & Blissard, G. W. (1999). Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. *Virology* **254**, 297–314.

Owen, K. E. & Kuhn, R. J. (1997). Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. *Virology* **230**, 187–196.

Pearson, M. N., Groten, C. & Rohrmann, G. F. (2000). Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the *Baculoviridae*. *J Virol* **74**, 6126–6131.

Pearson, M. N., Russell, R. L. & Rohrmann, G. F. (2001). Characterization of a baculovirus-encoded protein that is associated with infected-cell membranes and budded virions. *Virology* **291**, 22–31.

Pijlman, G. P., Dortmans, J. C., Vermeesch, A. M., Yang, K., Martens, D. E., Goldbach, R. W. & Vlak, J. M. (2002). Pivotal role of the non-*hr* origin of DNA replication in the genesis of defective interfering baculoviruses. *J Virol* **76**, 5605–5611.

Robison, C. S. & Whitt, M. A. (2000). The membrane-proximal stem region of vesicular stomatitis virus G protein confers efficient virus assembly. *J Virol* **74**, 2239–2246.

Rohrmann, G. F. & Karplus, P. A. (2001). Relatedness of baculovirus and gypsy retrotransposon envelope proteins. *BMC Evol Biol* **1**, 1.

Shioda, T. & Shibuta, H. (1990). Production of human immunodeficiency virus (HIV)-like particles from cells infected with recombinant vaccinia viruses carrying the *gag* gene of HIV. *Virology* **175**, 139–148.

Shizuya, H., Birren, B., Kim, U. J., Mancino, V., Slepak, T., Tachiiri, Y. & Simon, M. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc Natl Acad Sci U S A* **89**, 8794–8797.

Song, C., Dubay, S. R. & Hunter, E. (2003). A tyrosine motif in the cytoplasmic domain of Mason-Pfizer monkey virus is essential

for the incorporation of glycoprotein into virions. *J Virol* **77**, 5192–5200.

Terzian, C., Pelisson, A. & Bucheton, A. (2001). Evolution and phylogeny of insect endogenous retroviruses. *BMC Evol Biol* **1**, 3.

Volkman, L. E. & Goldsmith, P. A. (1985). Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. *Virology* **143**, 185–195.

Volkman, L. E. & Summers, M. D. (1977). *Autographa californica* nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. *J Invertebr Pathol* **30**, 102–103.

Wang, K., Boysen, C., Shizuya, H., Simon, M. I. & Hood, L. (1997). Complete nucleotide sequence of two generations of a bacterial artificial chromosome cloning vector. *Biotechniques* **23**, 992–994.

Westenberg, M., Wang, H., IJkel, W. F., Goldbach, R. W., Vlak, J. M. & Zuidema, D. (2002). Furin is involved in baculovirus envelope fusion protein activation. *J Virol* **76**, 178–184.

Westenberg, M., Veenman, F., Roode, E. C., Goldbach, R. W., Vlak, J. M. & Zuidema, D. (2004). Functional analysis of the putative fusion domain of the baculovirus envelope fusion protein F. *J Virol* **78**, 6946–6954.

Zhang, S. X., Han, Y. & Blissard, G. W. (2003). Palmitoylation of the *Autographa californica* multicapsid nucleopolyhedrovirus envelope glycoprotein GP64: mapping, functional studies, and lipid rafts. *J Virol* **77**, 6265–6273.

Zhao, H., Lindqvist, B., Garoff, H., von Bonsdorff, C. H. & Liljestrom, P. (1994). A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding. *EMBO J* **13**, 4204–4211.