

Absence of *N*-linked glycans from the F₂ subunit of the major baculovirus envelope fusion protein F enhances fusogenicity

Gang Long,^{1,2} Xiaoyu Pan¹ and Just M. Vlak²

Correspondence

Just M. Vlak
just.vlak@wur.nl

¹State Key Laboratory of Virology, Key Laboratory of Molecular Virology and Joint Laboratory of Invertebrate Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China

²Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

The F protein is the major glycoprotein present in the envelopes of budded virus (BV) of members of the family *Baculoviridae*. The F protein mediates low-pH-activated fusion with insect cell membranes. Baculovirus F proteins are synthesized as a precursor (F₀) and cleaved post-translationally into two disulfide-bonded subunits, F₁ (C-terminal, large subunit) and F₂ (N-terminal, small subunit). Recently, *N*-linked glycosylation of the F₁ and F₂ subunits of *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV) was demonstrated [Long, G., Westenberg, M., Wang, H., Vlak, J. M. & Hu, Z. (2006). *J Gen Virol* **87**, 839–846]. Sequence analysis frequently predicts that one or more *N*-linked glycosylation sites are present in the F₂ subunit of baculovirus F proteins. *N*-glycans on envelope fusion proteins are usually required for proper conformational integrity and biological function, such as infectivity. This study examined the importance of *N*-linked glycosylation of the F₂ subunit of HearNPV by site-directed mutagenesis. The only putative *N*-linked glycosylation site in F₂ was eliminated by mutating asparagine (N¹⁰⁴) to glutamine (Q), resulting in the mutant HearNPV^{N104Q}. When inserted into an *f*-null HearNPV and a *gp64*-null bacmid of *Autographa californica multiple nucleopolyhedrovirus*, infectious BV could be retrieved that contained unglycosylated F₂. The virulence of HearNPV^{N104Q} was enhanced, as BV was produced earlier after infection and yielded larger plaques than *f*-null HearNPV repaired with the wild-type *f* gene. HearNPV^{N104Q} BV also induced much more efficient low-pH-activated syncytium formation. These results indicate that *N*-linked glycosylation of the HearNPV baculovirus F₂ subunit is not essential for viral infectivity and suggest that it is involved in BV production and fusogenicity.

Received 26 July 2006

Accepted 19 October 2006

INTRODUCTION

The *Baculoviridae* are a large family of enveloped DNA viruses that are exclusively pathogenic to arthropods, mainly insects (Theilmann *et al.*, 2005). The family is subdivided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Phylogenetic studies indicate that NPVs can be further subdivided into two subgroups: group I and group II (Bulach *et al.*, 1999; Herniou *et al.*, 2001). Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus and budded virus (BV) (Volkman & Summers, 1977). Occlusion-derived virus is present in occlusion bodies and is able to infect midgut epithelial cells by direct membrane fusion following its release from occlusion bodies. In contrast, BV is adapted to generate infection from cell to cell via receptor-mediated endocytosis and is responsible for the systemic spread of the virus in the infected insect larvae.

Two distinct envelope fusion proteins have been identified in BVs: GP64 in group I NPVs and F protein in group II

NPVs and in GVs (Blissard & Wenz, 1992; Ijkel *et al.*, 2000; Pearson *et al.*, 2000). The F protein is a functional analogue of GP64 (Lung *et al.*, 2002; Long *et al.*, 2006a). Unlike GP64, baculovirus F proteins show similar structural and functional characteristics to class I viral envelope fusion proteins from retroviruses, paramyxoviruses, coronaviruses and orthomyxoviruses with respect to the location of the signal peptide, heptad repeats, the fusion peptide and the transmembrane region (Fig. 1) and their respective functions (Eckert & Kim, 2001; Earp *et al.*, 2005). Like many mammalian viral envelope fusion proteins, the baculovirus F protein is synthesized as a precursor (F₀), which is subsequently cleaved by a cellular furin-like convertase into two disulfide-linked subunits, F₁ and F₂ (Westenberg *et al.*, 2002; Long *et al.*, 2006a). Cleavage of the F protein is necessary to allow low-pH-triggered membrane fusion activity to promote viral infectivity (Lung *et al.*, 2002; Westenberg *et al.*, 2002). F proteins reside in BV envelopes as non-covalently bound homotrimers, and both F₁ and

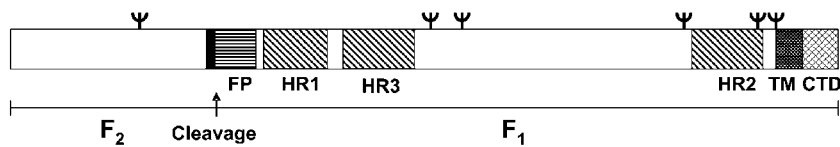


Fig. 1. Schematic diagram of the structure and organization of the HearNPV F protein. The locations of the cleavage site (arrow), fusion peptide (FP), heptad repeats (HRs), transmembrane domain (TM), cytoplasmic tail domain (CTD) and potential *N*-linked glycosylation sites (Ψ) are shown.

F₂ subunits show *N*-linked glycosylation (Long *et al.*, 2006a).

N-linked glycosylation is a common co-translational or post-translational modification for virus envelope fusion proteins (Doms *et al.*, 1993; Helenius & Aebi, 2001). *N*-linked glycans not only determine proper folding and intracellular trafficking of virus envelope fusion proteins (Gallagher *et al.*, 1992; Roberts *et al.*, 1993; Hu *et al.*, 1994, 1995; Braakman & van Anken, 2000; Daniels *et al.*, 2003), but are also critical for fusogenicity (Deng *et al.*, 1994; McGinnes *et al.*, 2001; Von Messling & Cattaneo, 2003; Panda *et al.*, 2004) and infectivity (Ohgimoto *et al.*, 1998). Despite the high degree of similarity among baculovirus F proteins with respect to the architecture of functional domains, the number and position of predicted *N*-linked glycosylation sites (NXS/T) vary among viruses. However, when comparing baculovirus F₂ subunit sequences, the putative *N*-linked glycosylation sites are frequently found at a conserved location. Recent studies on *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV) suggest that the only predicted *N*-linked glycosylation site (N¹⁰⁴) in the HearNPV F₂ subunit is probably used (Long *et al.*, 2006a). There is no information on the importance of *N*-linked glycosylation for the function and activity of F-like baculovirus envelope fusion proteins. In the present study, we eliminated the *N*-linked glycosylation site on the F₂ subunit of HearNPV by site-directed mutagenesis, resulting in a mutant with a conservative asparagine-to-glutamine change (N¹⁰⁴→Q) in F₂ (HearNPV^{N104Q}). We observed that this mutant was capable of rescuing the infectivity of an *f*-null HearNPV and of a *gp64*-null bacmid of *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV). Interestingly, the F^{N104Q} mutant produced more BV at earlier times post-infection and was more efficient in low-pH-activated syncytium formation than BV of wild-type HearNPV.

METHODS

Cells and bacmid. The *Heliothis zea* cell line HzAM1 (McIntosh & Ignoffo, 1983) and *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al.*, 1977) were 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). An *f*-null HearNPV bacmid (Long *et al.*, 2006c) and a *gp64*-null AcMNPV bacmid (Lung *et al.*, 2002) were used for reverse genetics studies on the functional role of *N*-linked glycans on the F₂ subunit of the HearNPV F protein.

Computational analysis. The amino acid sequences for the F₂ subunits of baculovirus F proteins were obtained from the following sequences deposited in GenBank: HearNPV (GenBank accession

no. AF271059), *Lymantria dispar* MNPV (GenBank accession no. AF081810), *Clanis bilineata* NPV (GenBank accession no. DQ504428), *Adoxophyes honmai* NPV (GenBank accession no. AP006270), *Agrotis segetum* NPV (GenBank accession no. DQ123841), *Spodoptera exigua* MNPV (GenBank accession no. AF169823), *Trichoplusia ni* SNPV (GenBank accession no. DQ017380), *Spodoptera litura* MNPV (GenBank accession no. AF325155), *Chrysodeixis chalcites* NPV (GenBank accession no. AY864330), *Mamestra configurata* NPV-B (GenBank accession no. AY126275) and *Leucania separata* NPV (GenBank accession no. AY394490). These sequences were aligned using MEGALIGN software with the CLUSTAL W method. Prediction of potential *N*-linked glycosylation sites was conducted using proteomics tools from the ExPASy proteomics server (<http://www.expasy.org>).

Mutagenesis and bacmids. Site-directed mutagenesis of N¹⁰⁴ of the *f* gene of HearNPV was carried out to replace asparagine (N) of the NXS/T sequon by glutamine (Q). The F protein preferred codon for Q (CAG, underlined in the mutagenesis reverse primer) replaced the AAT codon for N¹⁰⁴ by introducing this codon in the 5' end of the mutagenesis reverse primer (5'-CTGTTTATTCTTGATCC-ATTCAACTAGA-3'). Inverse PCR (Weiner *et al.*, 1994) was performed using a mutagenesis reverse primer and a forward primer (5'-TTAACCAGTTGCGAAGACAGCGA-3') with prior 5' phosphorylation of the primer pairs, *Pfu* polymerase (Promega) and the pFB-F&GFP vector containing the *f* gene cassette (nt 127811–130114) and a *p10*-promoter-controlled *egfp* gene (Long *et al.*, 2006c) as template. Following purification, the mutant PCR products were digested with *DpnI* to eliminate template plasmid DNA. Subsequently, the 5' ends of the purified PCR products were ligated to its own 3' ends generating a new vector containing mutated N¹⁰⁴ sequences. Clones containing the desired mutation were sequenced to confirm the mutation. The *f*^{N104Q} mutant gene cassette was subcloned into the pFB-F&GFP vector to replace the wild-type HearNPV *f* gene cassette by swapping the *Bst*1107I–*Hind*III fragments, resulting in donor plasmid pFB-*f*^{N104Q}&GFP carrying the *f*^{N104Q} mutant gene.

Competent cells containing either an *f*-null HearNPV bacmid (Long *et al.*, 2006c) or a *gp64*-null AcMNPV bacmid were prepared according to the Bac-to-Bac manual (Invitrogen). Successful transposition of inserts from donor plasmids pFB-F&GFP and pFB-*f*^{N104Q}&GFP to the *f*-null HearNPV bacmid and the *gp64*-null AcMNPV bacmid produced the recombinant HearNPV and pseudotyped AcMNPV with *f* and *f*^{N104Q}, respectively. The insertions were confirmed by diagnostic PCR using a gentamicin-resistance gene forward primer (5'-AGCCACCTA-CTCCCAACATC-3') in combination with the M13 forward primer (5'-CCCAGTCACGACGTGTGAAAACG-3') to check for successful transposition. Transfection and infection assays were conducted according to the methods of Long *et al.* (2006a).

Western blot analysis. Expression of the wild-type F protein and the F^{N104Q} mutant protein and their incorporation into BV were examined by Western blot analysis using polyclonal antibodies against F₁ and F₂ to probe sucrose-purified BV or cellular total protein samples throughout infection. Western blot analysis was performed as described previously (Long *et al.*, 2006a). Briefly, sucrose-purified BV was disrupted under reducing or non-reducing

conditions and denatured for 10 min at 95 °C. Proteins were separated by SDS-PAGE and subjected to Western blot analysis. Antisera were used at a 1:1000 dilution and proteins were detected by treatment with horseradish peroxidase-conjugated rabbit anti-chicken immunoglobulin (Sigma) diluted 1:10 000, followed by enhanced chemiluminescence, as described by the manufacturer (Amersham).

Plaque assays. To determine the infectivity of HearNPV BV carrying the F^{N104Q} mutation in the F₂ subunit (HearNPV^{fN104Q}) and the control HearNPV containing wild-type F₂ (HearNPV^f), plaque assays were conducted as described by Long *et al.* (2006c). Briefly, 1 × 10⁶ HzAM1 cells were transferred to 35 mm Petri dishes in Grace's medium (supplemented with 10% FBS). Cells were infected with HearNPV carrying wild-type F or mutant F^{N104Q} protein, at an m.o.i. of 0.01 TCID₅₀ per cell. After 4 days of incubation at 27 °C, the formation of plaques was examined by UV light microscopy. The relative diameters of ten separated plaques from each infection were measured and evaluated statistically.

One-step growth curve. Infectious BV production was measured using one-step virus growth curves. HzAM1 cells were infected with HearNPV^f or HearNPV^{fN104Q} at an m.o.i. of 5 TCID₅₀ per cell for 1 h. After infection, cells were washed with fresh medium once and incubated in fresh medium. Supernatants were collected at 12, 24, 36, 48, 60, 72, 84, 96 and 120 h post-infection (p.i.). Triplicate samples were collected for each mutant virus and at each time point. The amount of infectious BV in each sample was determined by end-point dilution assay on HzAM1 cells (King & Possee, 1992). Statistical analysis of the results was carried out using Microsoft EXCEL software. In addition, at each time point p.i., infected cells were collected and subjected to Western blot analysis to monitor temporal expression of the F protein following HearNPV^f and HearNPV^{fN104Q} infection.

Syncytium formation. Syncytium formation (Sf21–Sf21 or HzAM1–HzAM1 fusion) assays were performed by infection with pseudotyped AcMNPV or recombinant HearNPV BV (m.o.i. of 5 TCID₅₀ per cell), respectively. At 48 h p.i., cells were washed three times with 1 ml Grace's medium (pH 6.1) without FBS and treated for 5 min in 1 ml acidic Grace's medium at pH 5.0. The acidic medium was removed and replaced with 2 ml Grace's medium (pH 6.1) supplemented with 10% FBS. Syncytium formation was quantified by measuring the number of fused cells relative to the number of total cells and expressed as a percentage. Statistical analysis of the results was carried out using Microsoft EXCEL software.

RESULTS

Potential N-linked glycosylation sites in baculovirus F₂ subunits

The canonic recognition sequon for N-linked glycosylation is NXS/T, where X can be any amino acid residue except proline. Multiple N-linked glycosylation sites have been predicted in the F₁ and F₂ subunits of baculovirus F proteins. Comparison of the baculovirus F₂ subunits indicated that there are one or two potential N-linked glycosylation sites and that at least one is located at a conserved position around Cys¹⁰⁸ (not shown). A recent study showed that the F₁ and F₂ subunits of HearNPV are indeed N-glycosylated (Long *et al.*, 2006a). Five putative N-glycosylation sites are found in HearNPV F₁ and a single one in F₂ (Fig. 1). The single occurrence in HearNPV F₂ and its location at a conserved position suggest that N-linked glycosylation of F₂ plays an important role in baculovirus F protein function.

Effect of deletion of the N-linked glycosylation site from HearNPV F₂

To study the importance of N-linked glycosylation of F₂, an asparagine-to-glutamine mutant, f^{N104Q}, of HearNPV was constructed and the mutation was verified by nucleotide sequencing. Along with a p10-promoter-controlled *egfp* gene, the *f* or f^{N104Q} gene was transposed into an *f*-null HearNPV bacmid or into a *gp64*-null AcMNPV bacmid (Fig. 2a). Bacmid transfection and infection assays were conducted; expression of EGFP signalled successful transfection and virus infection. The transfection and infection experiments demonstrated that infectious BV of HearNPV^f and HearNPV^{fN104Q} was produced after transfection (Fig. 2b, left panels). This indicated that HearNPV F lacking a putative N-linked glycosylation site on the F₂ subunit, F₂^{N104Q}, was able to rescue infectivity of the *f*-null HearNPV bacmid, as infectious BV was produced, as evidenced by a secondary infection from the transfection supernatant (Fig. 2b, right panels). A similar result was obtained for AcMNPV pseudotyped with the wild-type HearNPV *f* or f^{N104Q} gene. The latter gene successfully rescued infectivity of the AcMNPV *gp64*-null mutant. Together, these results indicated that N-linked glycosylation of the HearNPV F₂ subunit is not essential for infectivity of HearNPV (Fig. 2b). N-linked glycans on the F₂ subunit are also not required for the production of infectious BV. Most likely, the F^{N104Q} proteins were properly processed and folded and were able to mediate successful envelope fusion processes.

To confirm correct incorporation of the F^{N104Q} protein into infectious BV, Western blot analysis was performed on purified BV from recombinant HearNPV (HearNPV^f and HearNPV^{fN104Q}) and pseudotyped AcMNPV (AcMNPV^f and AcMNPV^{fN104Q}). Under reducing conditions, F₁ (60 kDa) and F₂ (20 kDa) subunits migrated separately, suggesting that furin-like cleavage had occurred correctly during F protein synthesis in both HzAM1 and Sf21 cells. Unglycosylated F₂ subunits (19 kDa) from HearNPV^{fN104Q} and AcMNPV^{fN104Q} migrated faster than those from HearNPV^f and AcMNPV^f (Fig. 2c). This reflected the absence of N-linked glycosylation of F₂ and the successful construction of an N-glycosylation knockout (F^{N104Q}) mutant. Interestingly, unglycosylated F₂ subunits were also present in BV of both HearNPV^f and AcMNPV^f (Fig. 2c, left panel). This observation suggested that the single N-linked glycosylation site in the F₂ subunit is not always glycosylated in the wild-type protein. To ensure that the F₂^{N104Q} protein was free of N-linked glycans, the mobility of the deglycosylated F₂ subunit was compared with that of the F₂^{N104Q} protein by Western blot analysis (Fig. 2d). The mobility of deglycosylated F₂ was the same as that of the F₂^{N104Q} protein.

The N-linked glycosylation site of F₂ (N¹⁰⁴) is located slightly upstream of the only cysteine residue (Cys¹⁰⁸) in the F₂ subunit (Chen *et al.*, 2001). This is the only candidate amino acid of the F₂ subunit that can form a disulfide bond with the F₁ subunit. To study the effect of the absence of the

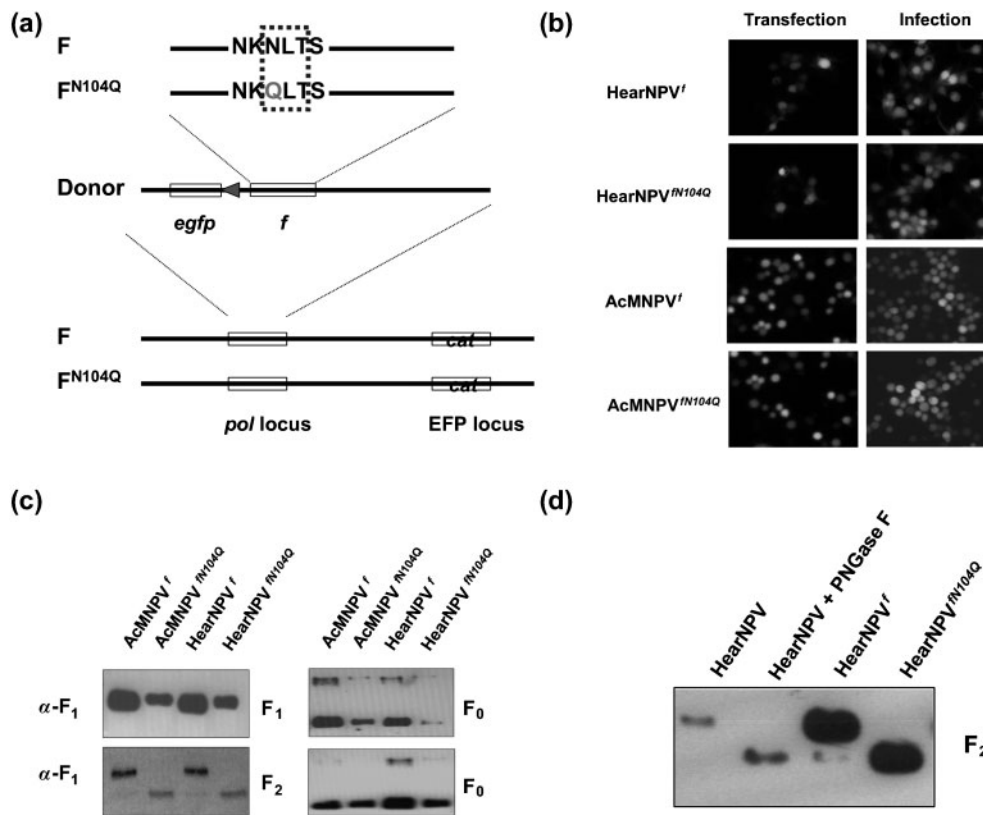


Fig. 2. Construction and functional analysis of the F_2 N -glycosylation knockout mutant (F^{N104Q}). (a) Mutation strategy of f^{N104Q} -rescued HearNPV and pseudotyped AcMNPV. (b) HzAM1 (upper four panels) or Sf21 (lower four panels) cells were transfected with f -rescued HearNPV bacmids (HearNPV^f and HearNPV^{fN104Q}) or pseudotyped AcMNPV bacmids (AcMNPV^f and AcMNPV^{fN104Q}), respectively (left panels). The results of infection of HzAM1 cells or Sf21 cells with the supernatants of transfections (right panels) are shown at 5 days p.i. GFP was used to monitor transfection and infection using epifluorescence microscopy. (c) Incorporation of F_2^{N104Q} and wild-type F protein in rescued HearNPV and pseudotyped AcMNPV BV. BV proteins were separated by SDS-PAGE under reducing (left) and non-reducing (right) conditions followed by Western blot analysis using antisera against F_1 (α - F_1) and F_2 (α - F_2). The positions of F_0 , F_1 and F_2 are indicated. (d) Verification of the absence of N -linked glycans from the F_2 subunit. Deglycosylation was carried out by treatment with N -glycosidase F (PNGase F). Western blot analysis was performed as in (c) (Long *et al.*, 2006a).

F_2 N -linked glycan on disulfide bridging, Western blot analysis was performed after denaturation of BV under non-reducing conditions (Fig. 2c). The F_1 and F_2 subunits comigrated with F_0 in all samples, with a molecular size of 80 kDa, demonstrating that disulfide bond formation between the F_1 and F_2 subunits was not affected by the absence of N -linked glycans from F_2 .

Infectivity and BV production of wild-type and mutant HearNPV

To analyse the effect of N -linked glycosylation of the F_2 subunit on BV production, one-step growth curves of BV of HearNPV^f and HearNPV^{fN104Q} were compared (Fig. 3). HzAM1 cells were infected with HearNPV^f or HearNPV^{fN104Q} at an m.o.i. of 5 TCID₅₀ per cell. BV production at various time points after infection was measured using an end-point dilution assay and the

experiment was carried out in triplicate. The results showed that HearNPV^f and HearNPV^{fN104Q} had comparable BV production kinetics, except that HearNPV^{fN104Q}-infected cells produced a greater number of BVs at an early stage of infection, whilst a smaller number of BVs of this mutant was found at a very late stage (Fig. 3a). At the end of the infection, the final BV yield was not significantly different between the two viruses. Expression of wild-type F protein and F^{N104Q} in cells infected with HearNPV^f or HearNPV^{fN104Q}, respectively, was monitored throughout infection (Fig. 3b). The cellular expression level of F^{N104Q} was much higher than that of wild-type F protein at 24 h p.i.

To evaluate further the effects of the absence of N -linked glycans on the F_2 subunit on viral infectivity, plaque size determination was performed on HearNPV^f- and HearNPV^{fN104Q}-infected HzAM1 cells (Fig. 4a, b). The results demonstrated that single plaques produced by

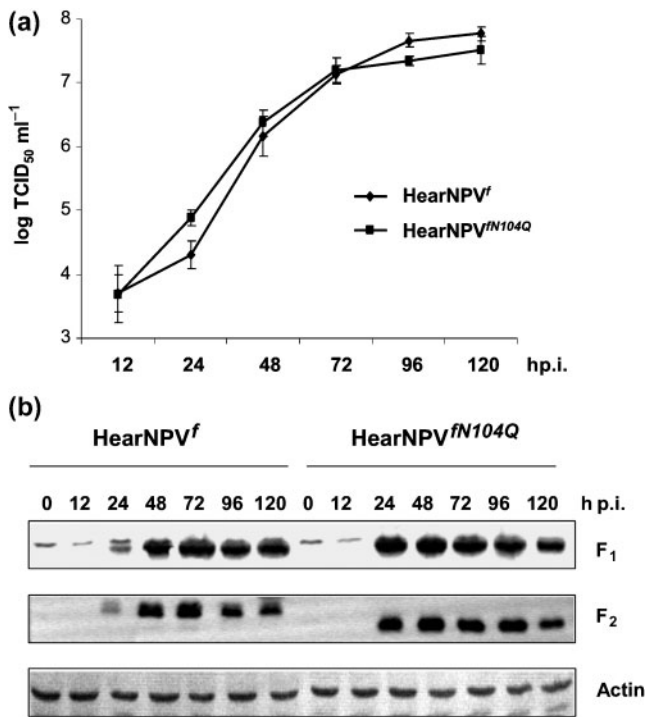


Fig. 3. BV production kinetics (a) and temporal expression (b) of wild-type *f*- and *f*^{N104Q}-rescued HearNPVs. (a) HzAM1 cells were infected at an m.o.i. of 5 TCID₅₀ per cell; supernatant samples were collected at the indicated times p.i. and titrated on HzAM1 cells. Each data point represents the mean ± SD from three separate experiments. (b) Temporal expression of wild-type F and F^{N104Q} in infected HzAM1 cells as described in (a). Total cellular protein at each time point was analysed by 12% SDS-PAGE. Western blot analysis was performed as described previously (Long *et al.*, 2006a).

HearNPV^{fN104Q} BV were significantly larger in size than those produced by HearNPV^f BV (Fig. 4c). This suggested that N-linked glycosylation of F₂ is not essential for plaque

formation of HearNPV per se, but that HearNPV^{fN104Q} spreads more quickly in cell culture than HearNPV containing wild-type F.

Low-pH-dependent fusion of wild-type and mutant HearNPV

BV of group II baculoviruses enters insect host cells via a clathrin-mediated and low-pH-dependent endocytic route (Long *et al.*, 2006b). The F protein is responsible for the low-pH-dependent cell fusion (Ijkel *et al.*, 2000). As the removal of N-linked glycans might change the conformation of viral fusion proteins and thus the fusogenicity of F, we examined syncytium formation at low pH following virus infection of HzAM1 cells (Fig. 5). Cells were infected at an m.o.i. of 5 TCID₅₀ per cell. At 48 h p.i., infected cells were subjected to low pH (pH 5.0) culture medium and syncytium formation was measured 24 h post-acidification. Syncytium formation of HearNPV^{fN104Q}-infected HzAM1 cells was significantly higher than for HearNPV^f-infected cells (Fig. 5a, lower panels, and b). In the case of syncytium formation mediated by wild-type F, the percentage of fused cells was less than 30% in HzAM1 cells, whereas the percentage of fused cells increased to about 70% for HearNPV carrying the F^{N104Q} mutation (Fig. 5b). Similar results were obtained with Sf21 cells infected with pseudotyped AcMNPV (AcMNPV^f and AcMNPV^{fN104Q}) (Fig. 5a, upper panels, and b). These observations indicated that the absence of N-linked glycans from the F₂ subunit of HearNPV enhanced low-pH-dependent syncytium formation by the baculovirus F protein.

DISCUSSION

N-linked glycosylation is important for proper protein processing during synthesis, and for structural integrity and functionality (Imperiali & O'Connor, 1999; Helenius & Aebi, 2001). For viral envelope fusion proteins, these functions include receptor binding (Ohuchi *et al.*, 1997; Nakayama *et al.*, 1998), envelope fusion (Deng *et al.*, 1994;

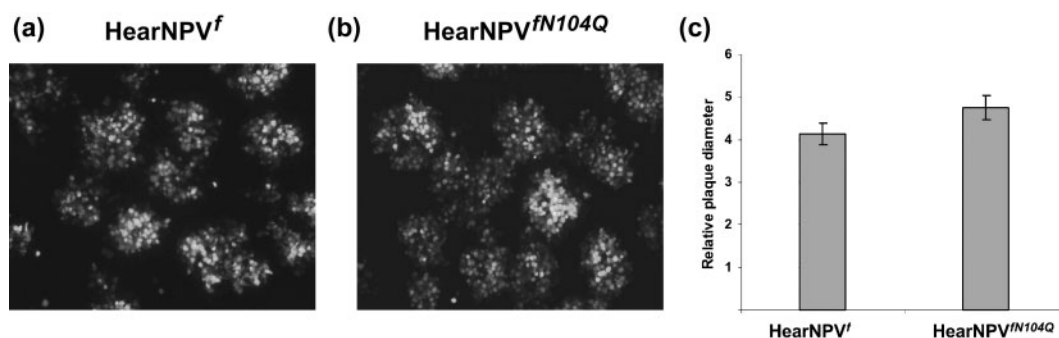


Fig. 4. Effect of the F^{N104Q} mutation on plaque formation. (a) HzAM1 cells (1×10^6) were infected with rescued HearNPVs (HearNPV^f and HearNPV^{fN104Q}) at a m.o.i. of 0.01 TCID₅₀ per cell. At 4 days p.i. at 27 °C, the formation of plaques was examined by UV light microscopy. (b) Plaque sizes of HearNPV^f- and HearNPV^{fN104Q}-infected HzAM1 cells. The relative diameters of ten separate plaques from each treatment were measured. Results are shown as mean ± SD.

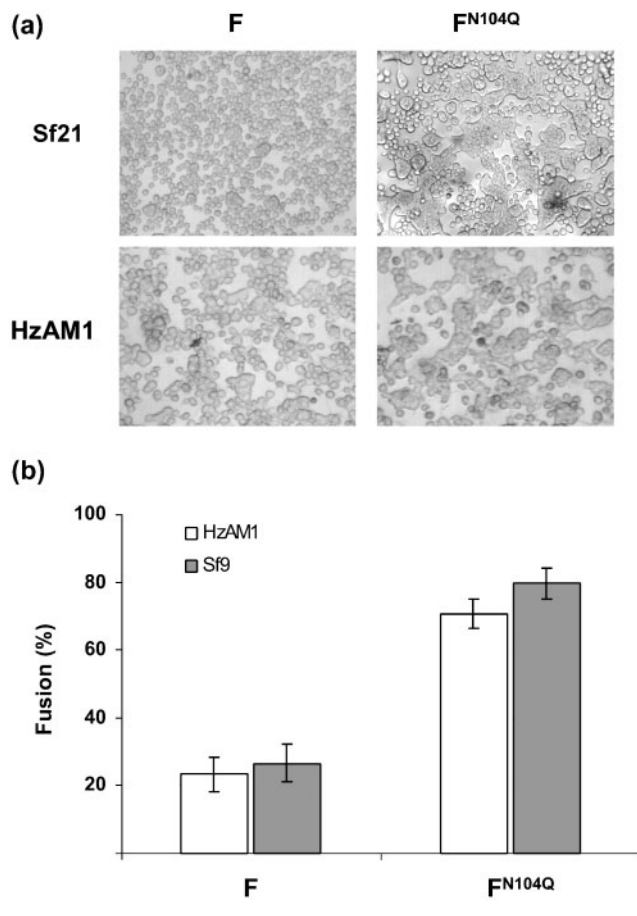


Fig. 5. Syncytium formation promoted by HearNPV BV infection. (a) HzAM1 cells (lower panels) and Sf21 cells (upper panels) were infected with rescued HearNPVs (HearNPV^f and HearNPV^{fN104Q}) and pseudotyped AcMNPVs (AcMNPV^f and AcMNPV^{fN104Q}), respectively, at an m.o.i. of 5 TCID₅₀ per cell. At 48 h p.i., the infected HzAM1 and Sf21 cells were incubated in Grace's insect medium (pH 5.0) for 5 min. After one time wash with fresh Grace's medium, the cells were incubated in Grace's medium plus 10% FBS. Syncytium formation was examined 12 h after low-pH treatment. (b) Comparison of syncytium formation mediated by HearNPV BV with wild-type F and with F^{N104Q}. Syncytium formation was measured by the percentage of fused cells relative to the number of total cells. Error bars represent SD.

McGinnes *et al.*, 2001; Von Messling & Cattaneo, 2003) and virulence (Li *et al.*, 1993; Ohgimoto *et al.*, 1998; Reitter *et al.*, 1998; Wagner *et al.*, 2000; Koch *et al.*, 2003; Panda *et al.*, 2004). In the case of baculovirus F proteins, multiple potential *N*-glycosylation sites are present in the F₁ and F₂ subunits at various positions, but their role in the above functions is unexplored. Both subunits of baculovirus F proteins are *N*-glycosylated (Long *et al.*, 2006a) and the question is which of the potential glycosylation sites indeed contain *N*-glycans and what the consequences of the presence of *N*-glycans are for baculovirus performance for each of these sites.

The F₁ subunit of HearNPV contains five predicted *N*-glycosylation sites, whereas the F₂ subunit has only one such site and contains *N*-glycans (Fig. 1). This suggests that *N*-linked glycosylation of this single site in the F₂ subunit may indeed occur and may be important for F protein function as a whole, perhaps in protein folding, intracellular trafficking, envelope fusion and virus infectivity. In the present study, we carried out a mutational analysis of this single putative *N*-glycosylation site of the F₂ subunit. Other group II baculoviruses have F₂ subunits with other putative glycosylation sites, but one is always in a more or less conserved position around Cys¹⁰⁸.

Using site-directed mutagenesis and functional rescue of bacmid-derived baculoviruses (Lung *et al.*, 2002) for F proteins, we have provided compelling evidence that the only *N*-linked glycosylation site (NLT) in the F₂ subunit of HearNPV F is indeed occupied by *N*-linked glycans (Fig. 2). An N→Q mutation at this sequon aborted *N*-glycosylation of F₂ but did not inhibit F protein synthesis, BV production or infectivity. However, this putative *N*-linked glycosylation site was not completely glycosylated, as unglycosylated F₂ was also found in the wild-type HearNPV F protein (Fig. 2c, lane 3). The oligosaccharyltransferase recognizes the consensus *N*-linked glycosylation sequon, NXS/T, but it has been shown that the amino acid residue at position X is an important determinant of glycosylation frequency at an individual site (Shakin-Eshleman *et al.*, 1996). Residues such as tryptophan, asparagine, glutamic acid and leucine at position X have been shown to be associated with less-efficient *N*-linked glycosylation. This incomplete *N*-linked glycosylation on the HearNPV F₂ subunit could be well explained by the presence of a leucine residue at the X position of the NXS/T sequon.

The fact that the *f*^{N104Q} gene was able to rescue the infectivity of an *f*-null HearNPV and also to rescue a *gp64*-null AcMNPV suggests that morphogenesis and maturation of BV could occur in the presence or absence of *N*-linked glycans on the F₂ subunit. These processes are apparently independent of the genetic background (HearNPV vs AcMNPV). However, the infectious BV production kinetics of the two rescued viruses, HearNPV^f and HearNPV^{fN104Q}, were significantly different (Fig. 3a). Compared with the wild-type rescued virus, HearNPV^f, more infectious HearNPV^{fN104Q} BV was produced at an early stage of infection but less at a later stage. This is in agreement with the larger plaque size for HearNPV^{fN104Q} (Fig. 4) and may be the consequence of increased BV production and quicker cell-to-cell spread of BV. We hypothesize that this quicker BV spread may enhance the speed of kill of insects of F₂ *N*-glycan mutants.

Intracellular expression of the F^{N104Q} protein at early times p.i. was much higher than that of wild-type F protein (Fig. 3b). This abundance of F most likely triggered increased BV production of the HearNPV^{fN104Q} mutant at earlier times. The mechanism resulting in higher expression levels of the F^{N104Q} protein and its relationship with

glycosylation and its pathway remain to be clarified. The absence of glycosylation might result in a slightly different folding of F or enhanced trafficking towards the cell envelope.

Like the GP64-like proteins, baculovirus F proteins are able to mediate low-pH-activated membrane fusion (Ijkel *et al.*, 2000; Pearson *et al.*, 2000). Syncytium formation mediated by the F protein is much less extensive than that mediated by GP64 protein (Ijkel *et al.*, 2000). In this study, we demonstrated that F^{N104Q} maintained mediation of low-pH-activated membrane fusion, suggesting that removal of N-linked glycans from F₂ did not change the overall mechanism of baculovirus F-mediated membrane fusion. However, the F^{N104Q} mutant was much more effective in low-pH-dependent fusion (Fig. 5). The explanation may be that N-linked glycans form a large hydrophilic side on the surface of F proteins. The absence of N-glycans forming this hydrophilic face on the F₂ subunit may enhance the interaction between F proteins in the virus envelope and the late endosomal cell membrane, thus assisting in the low-pH-induced conformational change to generate more effective membrane fusion. It is interesting to note that, in HearNPV F₂, the only N-glycosylation site (N¹⁰⁴) is located just upstream of the only cysteine (Cys¹⁰⁸). Cys¹⁰⁸ might be involved in disulfide bridging of F₂ with F₁ (Ijkel *et al.*, 2000) and it is conceivable that N-glycans so close to the disulfide bridge affect the conformation of the F protein and hence fusogenicity.

N-linked glycosylation is a prerequisite for proper folding, as the glycan addition occurs when the nascent protein folds into its native form. N-linked glycans are responsible for the binding of chaperones, which are essential for correct folding in the endoplasmic reticulum (Hebert *et al.*, 1997; Imperiali & O'Connor, 1999; Parodi, 2000; Helenius & Aebi, 2004). The fusogenicity of many viral envelope fusion proteins is dependent on correct folding and N-linked glycosylation. Removal of N-linked glycans from virus envelope fusion proteins often has a profound and cumulative effect, as has been shown for the vesicular stomatitis virus G, simian virus 5 F, respiratory syncytial virus F and bovine viral diarrhoea virus E2 proteins (Machamer *et al.*, 1985; Bagai & Lamb, 1995; Zimmer *et al.*, 2001; Pande *et al.*, 2005). In one case, elimination of multiple glycosylation sites in both F₁ and F₂ resulted in viruses with strongly enhanced fusogenicity (Aguilar *et al.*, 2006). Whether this is also the case for baculoviruses remains to be determined. Mutational analysis of the five putative N-glycosylation sites of F₁ should clarify this point.

It is tempting to speculate from an evolutionary perspective what the benefit would be for baculoviruses of having N-glycans on F₂ and reduced fusogenicity. As all baculovirus F₂ subunits have this conserved N-glycosylation site, there must be a strong evolutionary advantage in maintaining the N-glycans. It could be that enhanced fusogenicity and spread kills the insect before it can adequately produce complete polyhedra and that there is an ecological trade-off

between the production speed of BV in the insect and the spatio-temporal spread and survival of the baculovirus via polyhedra in the field or environment. This would imply that the HearNPV F^{N104Q} mutant has a shorter lethal time in insects than wild-type HearNPV.

In summary, our study demonstrates that N-linked glycosylation of the F₂ subunit of HearNPV BVs is not essential for BV formation and fusogenicity with host cells. Mutation of the N¹⁰⁴LT sequon and the absence of N-linked glycans on the F₂ subunit of HearNPV F resulted in enhanced fusogenicity of BV. The availability of the HearNPV F^{N104Q} mutant and the potential to generate F₁ N-glycosylation mutants will provide important tools to explore further the mechanisms of baculovirus F glycosylation and F-mediated fusion and to understand the pathobiology of this virus. Mutation of the single putative N-linked glycosylation site in baculovirus F₂ might also be a novel strategy to generate baculoviruses with increased speed of action for inundative insect control.

ACKNOWLEDGEMENTS

We thank Mark Zwart of the Laboratory of Virology (Wageningen University and Research Center) for technical assistance and helpful discussion. We are grateful to Marcel Westenberg for reading the manuscript. This work was funded by grants from the Royal Academy of Sciences of The Netherlands (KNAW) (project numbers 04-PSA-BD-02) and a PhD sandwich grant to G.L. from Wageningen University

REFERENCES

- Aguilar, H. C., Matreyek, K. A., Filone, C. M., Hashimi, S. T., Levrone, E. L., Negrete, O. A., Bertolotti-Ciarlet, A., Choi, D. Y., McHardy, I. & other authors (2006). N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. *J Virol* **80**, 4878–4889.
- Bagai, S. & Lamb, R. A. (1995). Individual roles of N-linked oligosaccharide chains in intracellular transport of the paramyxovirus SV5 fusion protein. *Virology* **209**, 250–256.
- Blissard, G. W. & Wenz, J. R. (1992). Baculovirus GP64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J Virol* **66**, 6829–6835.
- Braakman, I. & van Anken, E. (2000). Folding of viral envelope glycoproteins in the endoplasmic reticulum. *Traffic* **1**, 533–539.
- Bulach, D. M., Kumar, C. A., Zaia, A., Liang, B. & Tribe, D. E. (1999). Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedron and DNA polymerase gene sequences. *J Invertebr Pathol* **73**, 59–73.
- Chen, X., Ijkel, W. F. J., Tarchini, R., Sun, X., Sandbrink, H., Wang, H., Peters, S., Zuidema, D., Lankhorst, R. K. & other authors (2001). The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. *J Gen Virol* **82**, 241–257.
- Daniels, R., Kurowski, B., Johnson, A. E. & Hebert, D. N. (2003). N-Linked glycans direct the cotranslational folding pathway of influenza hemagglutinin. *Mol Cell* **11**, 79–90.
- Deng, R., Wang, Z., Glickman, R. L. & Iorio, R. M. (1994). Glycosylation within an antigenic site on the HN glycoprotein of

- Newcastle disease virus interferes with its role in the promotion of membrane fusion. *Virology* **204**, 17–26.
- Doms, R. W., Lamb, R. A., Rose, J. K. & Helenius, A. (1993).** Folding and assembly of viral membrane proteins. *Virology* **193**, 545–562.
- Earp, L. J., Delos, S. E., Park, H. E. & White, J. M. (2005).** The many mechanisms of viral membrane fusion proteins. *Curr Top Microbiol Immunol* **285**, 25–66.
- Eckert, D. M. & Kim, P. S. (2001).** Mechanisms of viral membrane fusion and its inhibition. *Annu Rev Biochem* **70**, 777–810.
- Gallagher, P. J., Henneberry, J. M., Sambrook, J. F. & Gething, M. J. (1992).** Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. *J Virol* **66**, 7136–7145.
- Hebert, D. N., Zhang, J.-X., Chen, W., Foellmer, B. & Helenius, A. (1997).** The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. *J Cell Biol* **139**, 613–623.
- Helenius, A. & Aebi, M. (2001).** Intracellular functions of *N*-linked glycans. *Science* **291**, 2364–2369.
- Helenius, A. & Aebi, M. (2004).** Roles of *N*-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* **73**, 1019–1049.
- 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.
- Hu, A., Cattaneo, R., Schwartz, S. & Norrby, E. (1994).** Role of *N*-linked oligosaccharide chains in the processing and antigenicity of measles virus haemagglutinin protein. *J Gen Virol* **75**, 1043–1052.
- Hu, A., Cathomen, T., Cattaneo, R. & Norrby, E. (1995).** Influence of *N*-linked oligosaccharide chains on the processing, cell surface expression and function of the measles virus fusion protein. *J Gen Virol* **76**, 705–710.
- 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 aproprotein convertase cleavage site. *Virology* **275**, 30–41.
- Imperiali, B. & O'Connor, S. E. (1999).** Effect of *N*-linked glycosylation on glycopeptide and glycoprotein structure. *Curr Opin Chem Biol* **3**, 643–649.
- King, L. A. & Possee, R. D. (1992).** *The Baculovirus Expression System: a Laboratory Guide*. London, UK: Chapman & Hall.
- Koch, M., Pancera, M., Kwong, P. D., Kolchinsky, P., Grundner, C., Wang, L., Hendrickson, W. A., Sodroski, J. & Wyatt, R. (2003).** Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. *Virology* **313**, 387–400.
- Li, S., Schulman, J., Itamura, S. & Palese, P. (1993).** Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. *J Virol* **67**, 6667–6673.
- Long, G., Westenberg, M., Wang, H., Vlak, J. M. & Hu, Z. (2006a).** Function, oligomerization and *N*-linked glycosylation of the *Helicoverpa armigera* single nucleopolyhedrovirus envelope fusion protein. *J Gen Virol* **87**, 839–846.
- Long, G., Pan, X., Kormerlink, R. J. & Vlak, J. M. (2006b).** Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. *J Virol* **80**, 8830–8833.
- Long, G., Pan, X., Westenberg, M., Hu, Z. & Vlak, J. M. (2006c).** Functional role of the cytoplasmic tail domain of the major envelope fusion protein of group II baculovirus. *J Virol* **80**, 11226–11234.
- 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.
- Machamer, C. E., Florkiewicz, R. Z. & Rose, J. K. (1985).** A single *N*-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. *Mol Cell Biol* **5**, 3074–3083.
- McGinnes, L., Sergel, T., Reitter, J. & Morrison, T. (2001).** Carbohydrate modification of the NDV fusion protein heptad repeat domains influence maturation and fusion activity. *Virology* **283**, 332–342.
- McIntosh, A. H. & Ignoffo, C. M. (1983).** Characterization of five cell lines established from species of *Heliothis*. *Appl Entomol Zool* **18**, 262–269.
- Nakayama, E. E., Shioda, T., Tatsumi, M., Xin, X., Yu, D., Ohgimoto, S., Kato, A., Sakai, Y., Ohnishi, Y. & Nagai, Y. (1998).** Importance of the *N*-glycan in the V3 loop of HIV-1 envelope protein for CXCR-4 but not CCR-5-dependent fusion. *FEBS Lett* **426**, 367–372.
- Ohgimoto, S., Shioda, T., Mori, K., Nakayama, E. E., Hu, H. & Nagai, Y. (1998).** Location-specific, unequal contribution of the *N*-glycans in simian immunodeficiency virus gp120 to viral infectivity and removal of multiple glycans without disturbing infectivity. *J Virol* **72**, 8365–8370.
- Ohuchi, M., Ohuchi, R., Feldmann, A. & Klenk, H. D. (1997).** Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. *J Virol* **71**, 8377–8384.
- Panda, A., Elankumaran, S., Krishnamurthy, S., Huang, Z. & Samal, S. K. (2004).** Loss of *N*-linked glycosylation from the hemagglutinin-neuraminidase protein alters virulence of Newcastle disease virus. *J Virol* **78**, 4965–4975.
- Pande, A., Carr, B. V., Wong, S. Y. C., Dalton, K., Jones, I. M., McCauley, J. W. & Charleston, B. (2005).** The glycosylation pattern of baculovirus expressed envelope protein E2 affects its ability to prevent infection with bovine viral diarrhoea virus. *Virus Res* **114**, 54–62.
- Parodi, A. J. (2000).** Protein glycosylation and its role in protein folding. *Annu Rev Biochem* **69**, 69–93.
- 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.
- Reitter, J. N., Means, R. E. & Desrosiers, R. C. (1998).** A role for carbohydrates in immune evasion in AIDS. *Nat Med* **4**, 679–684.
- Roberts, P. C., Garten, W. & Klenk, H.-D. (1993).** Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. *J Virol* **67**, 3048–3060.
- Shakin-Eshleman, S. H., Spitalnik, S. L. & Kasturi, L. (1996).** The amino acid at the X position of Asn-X-Ser sequon is an important determinant of *N*-linked core-glycosylation efficiency. *J Biol Chem* **271**, 6363–6366.
- Theilmann, D. A., Blissard, G. W., Bonning, B., Jehle, J. A., O'Reilly, D. R., Rohrmann, G. F., Thiem, S. & Vlak, J. M. (2005).** *Baculoviridae*. In *Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 177–185. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. San Diego, CA: Academic Press.
- Vaughn, J. L., Goodwin, R. H., Tompkins, G. J. & McCawley, P. (1977).** The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In Vitro* **13**, 213–217.
- 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.

Von Messling, V. & Cattaneo, R. (2003). N-linked glycans with similar location in the fusion protein head modulate paramyxovirus fusion. *J Virol* **77**, 10202–10212.

Wagner, R., Wolff, T., Herwig, A., Pleschka, S. & Klenk, H.-D. (2000). Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. *J Virol* **74**, 6316–6323.

Weiner, M. P., Costa, G. L., Schoettlin, W., Cline, J., Mathur, E. & Bauer, J. C. (1994). Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. *Gene* **151**, 119–123.

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.

Zimmer, G., Trotz, I. & Herrler, G. (2001). N-glycans of F protein differentially affect fusion activity of human respiratory syncytial virus. *J Virol* **75**, 4744–4751.