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

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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 HearNPVfN₁₀₄Q. 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 HearNPVfN₁₀₄Q was enhanced, as BV was produced earlier after infection and yielded larger plaques than f-null HearNPV repaired with the wild-type f gene. HearNPVfN₁₀₄Q 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.

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, Nucleopolyhedivirus (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

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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; McGinness 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 (HearNPVpN₁⁰⁴Q). 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 pN₁⁰⁴Q 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 (Vaugn 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. AFO81810), Clania bilineata NPV (GenBank accession no. DQ504428), Adoxophyes hondo 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. AY126273) 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’-CTGTATTATCCTGATCCATCAACTAGA-3’). Inverse PCR (Weiner et al., 1994) was performed using a mutagenesis reverse primer and a forward primer (5’-TAAACCAGTGCGAGACAGGGA-3’) with prior 5’ phosphorylation of the primer pair, Pfu polymerase (Promega) and the pFB-F&GFP vector containing the f gene cassette (nt 127811–130114) and a 10 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 fN₁⁰⁴Q mutant gene cassette was subcloned into the pFB-F&GFP vector to replace the wild-type HearNPV f gene cassette by swapping the BstNI–HindIII fragments, resulting in donor plasmid pFB-fN₁⁰⁴Q&GFP carrying the fN₁⁰⁴Q 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-fN₁⁰⁴Q&GFP to the f-null HearNPV bacmid and the gp64-null AcMNPV bacmid produced the recombinant HearNPV and pseudotyped AcMNPV with f and fN₁⁰⁴Q, respectively. The insertions were confirmed by diagnostic PCR using a gentamicin-resistance gene forward primer (5’-AGCCACCTCTCCCAACATC-3’) in combination with the M13 forward primer (5’-TCCCAGTCGACGTGTTAAAACG-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 pN₁⁰⁴Q 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 F\(_2\) subunit (HearNPV\(f^{N104Q}\)) and the control HearNPV containing wild-type F\(_2\) (HearNPV\(f\)), plaque assays were conducted as described by Long et al. (2006c). Briefly, 1 x 10\(^4\) 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 TCID\(_{50}\) 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\(f^{N104Q}\) at an m.o.i. of 5 TCID\(_{50}\) 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\(f^{N104Q}\) infection.

**Syncytium formation.** Syncytium formation (S21–S21 or HzAM1–HzAM1 fusion) assays were performed by infection with pseudotyped AcMNPV or recombinant HearNPV BV (m.o.i. of 5 TCID\(_{50}\) 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 F\(_2\) subunits**

The canonic recognition sequon for N-linked glycosylation is NXST, where X can be any amino acid residue except proline. Multiple N-linked glycosylation sites have been predicted in the F\(_1\) and F\(_2\) subunits of baculovirus F proteins. Comparison of the baculovirus F\(_2\) 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 Cys\(^{108}\) (not shown). A recent study showed that the F\(_1\) and F\(_2\) subunits of HearNPV are indeed N-glycosylated (Long et al., 2006a). Five putative N-glycosylation sites are found in HearNPV F\(_1\) and a single one in F\(_2\) (Fig. 1). The single occurrence in HearNPV F\(_2\) and its location at a conserved position suggest that N-linked glycosylation of F\(_2\) plays an important role in baculovirus F protein function.

**Effect of deletion of the N-linked glycosylation site from HearNPV F\(_2\)**

To study the importance of N-linked glycosylation of F\(_2\), 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\(f^{N104Q}\) was produced after transfection (Fig. 2b, left panels). This indicated that HearNPV F lacking a putative N-linked glycosylation site on the F\(_2\) subunit, F\(_2\)\(^{N104Q}\), 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\(f\) 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 F\(_2\) subunit is not essential for infectivity of HearNPV (Fig. 2b). N-linked glycans on the F\(_2\) 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\(f^{N104Q}\)) and pseudotyped AcMNPV (AcMNPV\(f\) and AcMNPV\(f^{N104Q}\)). Under reducing conditions, F\(_1\) (60 kDa) and F\(_2\) (20 kDa) subunits migrated separately, suggesting that furin-like cleavage had occurred correctly during F protein synthesis in both HzAM1 and S21 cells. Unglycosylated F\(_2\) subunits (19 kDa) from HearNPV\(f\) and AcMNPV\(f^{N104Q}\) and AcMNPV\(f^{N104Q}\) migrated faster than those from HearNPV\(f\) and AcMNPV\(f\) (Fig. 2c). This reflected the absence of N-linked glycosylation of F\(_2\) and the successful construction of an N-glycosylation knockout (F\(^{N104Q}\)) mutant. Interestingly, unglycosylated F\(_2\) 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 F\(_2\) subunit is not always glycosylated in the wild-type protein. To ensure that the F\(_2\)\(^{N104Q}\) protein was free of N-linked glycans, the mobility of the deglycosylated F\(_2\) subunit was compared with that of the F\(_2\)\(^{N104Q}\) protein by Western blot analysis (Fig. 2d). The mobility of deglycosylated F\(_2\) was the same as that of the F\(_2\)\(^{N104Q}\) protein.

The N-linked glycosylation site of F\(_2\) (N\(^{104}\)) is located slightly upstream of the only cysteine residue (Cys\(^{108}\)) in the F\(_2\) subunit (Chen et al., 2001). This is the only candidate amino acid of the F\(_2\) subunit that can form a disulfide bond with the F\(_1\) subunit. To study the effect of the absence of the
**Fig. 2.** Construction and functional analysis of the F2 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^{N104Q}F) or pseudotyped AcMNPV bacmids (AcMNPV^F and AcMNPV^{N104Q}F), 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 F2^{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 F1 (α-F1) and F2 (α-F2). The positions of F0, F1, and F2 are indicated. (d) Verification of the absence of N-linked glycans from the F2 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).

F2 N-linked glycan on disulfide bridging, Western blot analysis was performed after denaturation of BV under non-reducing conditions (Fig. 2c). The F1 and F2 subunits co-migrated with F0 in all samples, with a molecular size of 80 kDa, demonstrating that disulfide bond formation between the F1 and F2 subunits was not affected by the absence of N-linked glycans from F2.

**Infectivity and BV production of wild-type and mutant HearNPV**

To analyse the effect of N-linked glycosylation of the F2 subunit on BV production, one-step growth curves of BV of HearNPV^F and HearNPV^{N104Q} were compared (Fig. 3). HzAM1 cells were infected with HearNPV^F or HearNPV^{N104Q} at an m.o.i. of 5 TCID<sub>50</sub> 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^{N104Q} had comparable BV production kinetics, except that HearNPV^{N104Q} 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^{N104Q}, 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 F2 subunit on viral infectivity, plaque size determination was performed on HearNPV^F and HearNPV^{N104Q}-infected HzAM1 cells (Fig. 4a, b). The results demonstrated that single plaques produced by
HearNPV\textsuperscript{fN104Q} BV were significantly larger in size than those produced by HearNPV\textsuperscript{f} BV (Fig. 4c). This suggested that N-linked glycosylation of F\textsubscript{2} is not essential for plaque formation of HearNPV per se, but that HearNPV\textsuperscript{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 TCID\textsubscript{50} 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\textsuperscript{fN104Q}-infected HzAM1 cells was significantly higher than for HearNPV\textsuperscript{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 FN104Q mutation (Fig. 5b). Similar results were obtained with SF21 cells infected with pseudotyped AcMNPV (AcMNPV\textsuperscript{f} and AcMNPV\textsuperscript{fN104Q}) (Fig. 5a, upper panels, and b). These observations indicated that the absence of N-linked glycans from the F\textsubscript{2} 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;
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 Cys$^{108}$. 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 understood by the presence of a leucine residue at the X position of the NXS/T sequon.

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$^{fN104Q}$ and HearNPV$^{fN104Q\,gp64}$) and pseudotyped AcMNPVs (AcMNPV$^{f}$ and AcMNPV$^{f\,gp64}$), respectively, at an m.o.i. of 5 TCID$_{50}$ 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.

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$^{N104Q}$ 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 (N^{104}) is located just upstream of the only cysteine (Cys^{108}). Cys^{108} might be involved in disulphide bridging of F2 with F1 (Ijkel et al., 2000) and it is conceivable that N-glycans so close to the disulphide 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 diarrhea 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 N^{104}LT 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 inductive insect control.

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