Mutational and functional analysis of \( N \)-linked glycosylation of envelope fusion protein F of *Helicoverpa armigera* nucleopolyhedrovirus

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The envelope fusion (F) protein of baculoviruses is a heavily N-glycosylated protein that plays a significant role in the virus infection cycle. \( N \)-Linked glycosylation of virus envelope glycoprotein is important for virus envelope glycoprotein folding and its function in general. There are six predicted \( N \)-glycosylation sites in the F (HaF) protein of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV). The \( N \)-glycosylation site located in the F 2 subunit (N104) of HaF has been identified and functionally characterized previously (Long *et al.*., 2007). In this study, the other five potential \( N \)-glycosylation sites located in the HaF 1 subunit, namely, N293, N361, N526, N571 and N595, were analysed extensively to examine their \( N \)-glycosylation and relative importance to the function of HaF. The results showed that four of these five potential glycosylation sites in the F 1 subunit, N293, N361, N526, N571 and N595, were \( N \)-glycosylated in F proteins of mature HearNPV budded viruses (BV) but that N595 was not. In general, the conserved site N526 was critical to the functioning of HaF, as absence of \( N \)-glycosylation of N526 reduced the efficiency of HaF folding and trafficking, consequently decreased fusogenicity and modified the subcellular localization of HaF proteins, and thus impaired virus production and infectivity. The absence of \( N \)-glycosylation at other individual sites was found to have different effects on the fusogenicity and subcellular distribution of HaF proteins in HzAM1 cells. In summary, \( N \)-glycosylation plays comprehensive roles in HaF function and virus infectivity, which is further discussed.

INTRODUCTION

\( N \)-Linked glycans play a key role in directing proper folding, stability, intracellular trafficking and fusogenic activity of viral glycoproteins, which directly correlate with virus morphogenesis and infectivity. Many viral proteins are modified by the host cellular glycosylation machinery in order to ensure proper function and virus-host interactions. \( N \)-Glycosylations of various viral envelope proteins have been studied in much detail. For example, \( N \)-glycosylation of the haemagglutinin (HA) of influenza virus is important in modulating protein maturation and folding (Danielsson *et al.*, 2003), fusion activity (Ohuchi *et al.*, 1997b) and receptor affinity (Ohuchi *et al.*, 1997a), as well as antigenicity (Abe *et al.*, 2004).

The \( N \)-glycosylation site occupancies have an influence on the structure and function of glycoproteins (Jones *et al.*, 2005). In the \( N \)-glycosylation process, the \( N \)-glycosylation sequence (N-X-S/T) of glycoproteins, normally called the \( N \)-glycosylation sequon, is necessary for the recognition of the oligosaccharide transferase complex and the transfer of oligosaccharides onto the conserved asparagine (N) of the sequon. Once the sequon is disrupted, \( N \)-glycosylation would be abolished, which may affect the glycoprotein structure and function.

In members of the family *Baculoviridae*, the envelope fusion protein (EFP) is the major glycoprotein present in the envelopes of budded virus (BV). So far, two different types of EFP, GP64 and the F protein, have been identified. GP64s are class III EFPs and are identified only in members of group I of the genus *Alphabaculovirus* (Kadlec *et al.*, 2008). F proteins, assumed to be the ancestral EFPs of
baculoviruses, are widely found in members of the genera *Betabaculovirus* and *Deltabaculovirus* and group II of the genus *Alphabaculovirus* (Pearson et al., 2000). The F protein possesses the common features of class I EFPs. The precursor F₀ is synthesized in the endoplasmic reticulum (ER) and undergoes proteolytic cleavage by the trans-Golgi network protease furin (Ijkel et al., 2000). The two resulting subunits (F₁ and F₂) are linked by disulfide bonds (Long et al., 2006). Mature F protein is subsequently transported to the plasma membrane and finally incorporated into the viral envelope during BV budding. During the BV entry process, F proteins bind to an elusive cellular receptor and mediate fusion between the viral envelope and the endosome membrane to release viral nucleocapsids for replication (Tan et al., 2008; Westenberg et al., 2004). Therefore, efficient receptor binding, membrane fusion and virus egress depend largely on the proper functioning of the F protein.

The F protein (HaF) of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) contains six predicted N-glycosylation sequons (N-X-S/T), five in the F₁ subunit and one in the F₂ subunit. Deglycosylation assays have demonstrated that both the F₁ and F₂ subunits of HaF are N-glycosylated (Long et al., 2006). The unique N-glycosylation site in F₂ located at site N104 has been found to be important for virus infectivity (Long et al., 2007). The functions and significance of the remaining five potential sites, N293, N361, N526, N571 and N595, located in the F₁ subunit, have not been analysed so far. As N-glycosylation is a prerequisite for correct folding and functioning of the viral glycoprotein, a further in-depth study on the N-glycosylation of HaF is important to better understand its significance in the function of baculovirus F proteins. In this study, a series of single mutation at each putative site of HaF were introduced, and recombinant HearNPVs harbouring the various mutated HaFs were constructed. The mutant viruses were tested for N-glycosylation, fusogenicity and intracellular trafficking of the F proteins, as well as for virus replication and BV production.

**RESULTS**

Identification of N-glycosylation sites in the F₁ subunit of HaF by site-directed mutagenesis

HaF contains six predicted N-glycosylation site consensus sequons (N-X-S/T), comprising N104 in the F₂ subunit and N293, N361, N526, N571 and N595 in the F₁ subunit. N293, N526 and N571 are more conserved among the baculovirus F homologues than N104, N361 and N595 (Fig. 1a). N104 has been identified as the sole N-glycosylation site in the HaF₂ subunit (Long et al., 2007). In this research, six mutant viruses carrying single-site mutations – F₁N104Q, F₂N293S, F₁N361Q, F₂N526S, F₁N571S and F₁N595S – were generated to abolish each N-glycosylation site, respectively (Fig. 1b). Transfection and infection assays showed that all of these ‘single-site’ mutants produced foci of transfected cells indicating viable virus production and spread (Fig. 1c, upper panels) and that supernatants of these transfected cells were able to infect healthy cells (Fig. 1c, lower panels), although with different efficiencies of EGFP expression. This also indicated that absence of potential N-glycosylation at these positions individually did not abort HearNPV infectivity.

Western blot analysis was performed to identify a possible shift in molecular masses of the various HaF mutant proteins as a consequence of the mutations in both BVs (Fig. 2a) and infected cells (Fig. 2b). As a positive control, WT HaF was efficiently processed into F₁ and F₂ subunits, retaining only a small amount of uncleaved F₀ (Fig. 2, lanes a1, a6 and a11, and lane b5). Consistent with a previous report (Long et al., 2007), the results confirmed that N104 in the F₂ subunit is N-glycosylated (Fig. 2, lanes a8 and b9). A slightly faster-migrating F₁ band was detected in F₁N293S, F₁N361Q, F₁N526S and F₁N571S (Fig. 2, lanes a2–a5 and b1–b4), suggesting that the N-X-S/T sequons at these four sites were utilized for glycosylation. In contrast, both F₂ and F₁ bands of F₁N595S (Fig. 2, lanes a9 and b7) in BVs and infected cells appeared similar to the WT HaF (Fig. 2, lanes a11 and b5), suggesting N595 is not used for N-glycosylation in the F₁ subunit. The Western blot results further suggested that all the single mutations seemed to have little impact on the expression levels of HaF (Fig. 2b) and the incorporation of F proteins into BVs (Fig. 2a).

Rescue assay of N-glycosylation by nearby generated N-glycosylation sequons

To investigate whether the N-glycosylation site occupancy in HaF is a specific process, F₁N104S and F₁N361S mutants were also generated (Fig. 1b). These two mutants were different from F₁N104Q and F₁N361Q in that they not only abolished the native sites at N104 and N361 but at the same time created two new N-glycosylation sequons nearby, N-K-S at N102 and N-N-S at N359, respectively. The results of Western blotting showed that the molecular masses of the F₁N361S subunits (Fig. 2, lanes a10 and b6) appeared to be equivalent to the WT HaF (Fig. 2, lanes a11 and b5), suggesting that the N-glycosylation was probably rescued in F₁N361S. The newly created glycosylation site at N102 could not fully rescue N-glycosylation of F₂ subunit in the BV and cell samples. The F₂ subunits of a small portion of F₁N104S proteins were glycosylated, as most of the F₁N104S F₂ subunit sample ran faster, leaving a very weak signal of glycosylated F₂ with a similar molecular mass to the WT HaF₂ (Fig. 2, lanes a7 and b8, arrowheads). Therefore, N104 may be a specific N-glycosylation site in the F₂ subunit that plays an irreplaceable role in HaF functions.

Impact of elimination of individual N-glycosylation sites on infectious BV production and virus infectivity

The *in vitro* growth properties of the various mutated viruses were investigated (Fig. S1, available in the online
We found that mutant viruses carrying F N526S (2.2 ± 1.0 × 10^5 TCID50 ml^-1, mean ± SD) yielded much less infectious BVs than the control virus (4.0 ± 1.0 × 10^6 TCID50 ml^-1) (P < 0.01) (Fig. 3a). Infectious BVs of F N526S could barely be detected at 24 h post-infection (p.i.), and the BV titre of F N526S was more than 10 times lower than that of the control virus (P < 0.01) (Fig. S1 and Table 1). These indicated that F N526S was the most severely impaired among all the mutant viruses. BV production of F N104Q, F N293S and FN571S was about 5–10 times lower than that of the control virus (P < 0.05) (Fig. 3a). F N595S (2.0 ± 1.0 × 10^6 TCID50 ml^-1) also produced infectious BVs at 72 h p.i. of similar titres to the control virus, suggesting that N-glycosylation of N361 makes little contribution to virus production. However, one of the N-glycosylation-site-rescued HaF mutants, F N361S, yielded BV levels (1.0 ± 0.7 × 10^6 TCID50 ml^-1) about four times less than the control (P < 0.05), suggesting that it could not fully rescue the BV titres although N-glycosylation of F N361Q.
1 TCID50 was equivalent to approximately 10⁴ copies of viral genomic DNA. Most mutant viruses with single-site mutations in the F protein did not exhibit a significant difference in virus infectivity compared with the control virus (Table 1). However, for FNS265 mutant, the number of copies per TCID50 was 1.0 × 10⁶ ± 0.3 × 10⁵. Therefore, the FNS265 mutant was considered about 10 times less infectious than the control virus (P < 0.05). As the BV titre of FNS265 decreased by nearly 10-fold, as determined by an end-point dilution assay, the N-glycosylation site N526 of HaF appears to be important for virus production and infectivity.

**Removal of N-glycans at some identified N-glycosylation sites results in increased fusogenicity**

The baculovirus F protein is responsible for mediating virus–cell fusion in a low-pH-dependent manner (IJkel et al., 2000). The fusogenic activities of each mutant were evaluated by syncytium formation assays (Fig. 4a). The FNS265 mutant displayed a significantly lower fusion activity (66.1 ± 20.0 %, P < 0.05) (Fig. 4b), suggesting the importance of N526 for HaF fusogenicity. However, for most of the single-point mutants (FNI04S, FNI04Q, FN293S, FN361Q and FN571S), the fusion activity increased about twofold compared with that of the control virus. In addition, the site-rescued mutant FNS361S with the compensation of N-glycosylation was also much less efficient in inducing syncytium formation (63.5 ± 19.2 %, P < 0.05) (Fig. 4b). Thus, mutation of N-glycosylation sites of HaF was considered to have a profound effect on fusogenicity.

**Most N-glycans are involved in the intracellular trafficking of HaF protein**

N-Glycosylation contributes to proper glycoprotein folding and intracellular trafficking at different levels (Helenius, 1994). The cell-surface expression levels of the F mutants were quantified by an immunofluorescence assay and flow cytometric analyses (Fig. 5a). The cell-surface level of WT HaF was set as 100 %. When the cells were treated with tunicamycin, an inhibitor that blocks the N-glycosylation process, the expression level of the HaF protein was reduced to ~24.0 % (P < 0.01), demonstrating that N-glycosylation is important for the trafficking and distribution of F protein in insect cells (Fig. 5a). FNS361S showed a similar cell-surface expression level (94.5 ± 1.0 %) to that of the control virus. The level of cell-surface expression of FNS265 was reduced by ~70 % (P < 0.01), suggesting that N526 is an important N-glycosylation site for the efficient transport of F proteins to the cell surface. Most of the other mutants, FNI04S, FNI04Q, FN293S and FN571S, expressed slightly lower levels of ~80 % (P < 0.05) of cell-surface HaF in the infected cells compared with the WT control. However, N361 was different from the others as mutations at N361, either FNS361S (91.7 ± 1.3 %) or FNS361Q (108.0 ± 2.5 %), had a limited impact on the cell-surface expression level of HaF.

HaF might be rescued at N359 (Fig. 3b and S1). Therefore, the N-glycosylation of N361 site may not be critical for virus properties, and the compensation of N-glycans at neighbouring positions (N359) may be redundant and unnecessary. The titre of the other site-rescued mutant FNI04S (3.4 × 10⁶ ± 2.4 × 10⁵ TCID50 ml⁻¹) was about 10 times lower than that of the control (Fig. 3b) (P < 0.01). As FNI04S could not fully rescue N-glycosylation of HaF and the BVs titre of FNI04S was similar to FNI04Q in the absence of N-glycans in F₂ subunit, this indicated that N-glycosylation of the HaF₂ subunit is important for virus production.

The infectivity of the mutant baculoviruses at 96 h p.i. was also determined as genomic DNA copies per TCID50 (Table 1). For the control virus (vHaBacΔF-HaF), 1 TCID50 was equivalent to approximately 10⁴ copies of viral genomic DNA. Most mutant viruses with single-site
The cellular distribution of F protein mutants as the results of mutation of N-glycosylation sites was also visualized in Sf9 cells following fusion with the egfp gene (Fig. 5b). When fused with EGFP, WT F protein was located mainly on the cell membrane. For the other mutants, FN104S, FN104Q, FN293S and FN571S appeared in the cytoplasm and at the cell membrane, while FN526S clustered in the cytoplasm (Fig. 5b). These results confirmed that N-glycosylation of most sites (N104, N293, N526 and N571) is important for efficient transport of F proteins to the cell surface. FN361S and FN361Q were observed mainly at the cell membrane (Fig. 5b), suggesting a minor role of N-glycans on the rescued N359 or the authentic N361 in F protein transport.

**Glycosylation sites are important for F protein folding and transport to the Golgi apparatus**

The influence of N-glycosylation on HaF folding and trafficking was analysed by a pulse–chase folding assay. The newly synthesized WT F protein was detected as unfolded F0 at the beginning of the chase (Fig. 6). One other band running slightly faster than the WF F protein was observed under reducing conditions at a chase time 0 h p.i.

**Table 1.** Quantitative PCR analysis of BV genomic DNA production and comparison of infectivity of F protein-rescued virus and mutant viruses expressing F proteins with a single mutation

<table>
<thead>
<tr>
<th>Mutant virus</th>
<th>Genomic DNA (copies ml⁻¹)</th>
<th>Titre of mutant virus (TCID₅₀ ml⁻¹)</th>
<th>Virus infectivity (DNA copies per TCID₅₀)</th>
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<tbody>
<tr>
<td>vHaBacΔF-HaF</td>
<td>8.6 × 10¹⁰ ± 1.7 × 10¹⁰</td>
<td>3.5 × 10⁶ ± 1.5 × 10⁶</td>
<td>2.9 × 10⁴ ± 1.8 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴S</td>
<td>5.6 × 10⁹ ± 2.8 × 10⁹</td>
<td>4.3 × 10⁵ ± 1.8 × 10⁵</td>
<td>1.6 × 10⁴ ± 1.3 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴Q</td>
<td>6.0 × 10⁹ ± 0.8 × 10⁹</td>
<td>3.7 × 10⁵ ± 2.3 × 10⁵</td>
<td>2.5 × 10⁴ ± 2.2 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴S</td>
<td>5.5 × 10⁹ ± 0.2 × 10⁹</td>
<td>4.9 × 10⁵ ± 3.4 × 10⁵</td>
<td>1.6 × 10⁴ ± 1.1 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴S</td>
<td>1.4 × 10⁹ ± 0.7 × 10⁹</td>
<td>5.5 × 10⁵ ± 2.2 × 10⁵</td>
<td>2.7 × 10⁴ ± 1.1 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴Q</td>
<td>2.1 × 10⁹ ± 1.0 × 10⁹</td>
<td>2.0 × 10⁵ ± 0.0 × 10⁶</td>
<td>1.4 × 10⁴ ± 0.5 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴S</td>
<td>2.9 × 10⁹ ± 0.7 × 10⁹</td>
<td>2.5 × 10⁵ ± 0.9 × 10⁵</td>
<td>1.0 × 10⁴ ± 0.3 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴S</td>
<td>1.5 × 10⁹ ± 0.6 × 10⁹</td>
<td>6.0 × 10⁵ ± 2.9 × 10⁵</td>
<td>3.3 × 10⁴ ± 2.2 × 10⁴</td>
</tr>
<tr>
<td>vHaBacΔF-F¹⁰⁴S</td>
<td>2.8 × 10⁹ ± 0.2 × 10⁹</td>
<td>1.4 × 10⁶ ± 0.5 × 10⁶</td>
<td>2.2 × 10⁴ ± 0.8 × 10⁴</td>
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**Fig. 4.** Syncytium formation assays of HzAM1 cells infected with different mutant viruses. (a) Syncytium formation was observed and images were taken by fluorescence microscopy. Bars, 100 μm. (b) Quantitative characterization of the fusogenic ability of various mutant F proteins. The fusogenic ability of WT HaF protein was set as 100 %. Bars show means ± SD.

**Fig. 5.** Determination of the subcellular localization of the different mutant F proteins. (a) Cell-surface levels of mutant or WT F proteins were evaluated by an immunofluorescence assay and flow cytometry. Each sample was tested in triplicate, and bars represent means ± SD. (b) Subcellular localization of WT and mutant F proteins fused with EGFP was visualized in transfected Sf9 cells. Tuni, tunicamycin. Bars, 10 μm.
of 60 min (Fig. 6a, upper panel, arrowhead), suggesting that the oligosaccharides of F protein had been processed. The band of lower molecular mass was also detected under non-reducing conditions (Lázaro et al., 2007) at 10 min of chase time (Fig. 6a, bottom panel, arrowhead), indicating that the folding intermediates had already formed by this time. At 60 min of chase, more folding intermediates were detected (Fig. 6a, bottom panel, arrowhead).

As the intracellular transport of FN526S to the cell surface was severely impaired, the folding of FN526S was analysed. No other band of lower molecular mass was observed under reducing conditions until 60 min of chase time (Fig. 6a, upper panel, arrowhead), suggesting that the N-linked oligosaccharides of FN526S failed to be processed. In non-reducing conditions (Lázaro et al., 2007), the band of lower molecular mass could be detected after 10 min of chase but less efficiently compared with the WT F protein (Fig. 6a, bottom panel, arrowhead). This result suggested that elimination of the N526 glycosylation site impaired the initial truncation process of N-linked oligosaccharides of HaF and decreased the folding efficiency of this protein.

Transfer of the HaF protein from the ER to the Golgi apparatus was investigated using endoglycosidase H (Endo H). Under reducing conditions with Endo H treatment, N-glycans of the newly synthesized F protein would be removed to generate unglycosylated F (ugF0) (Fig. 6b). At 60 min of chase time, a band running slower than ugF0 was visualized (Fig. 6b, arrowhead), suggesting that a small portion of F protein molecules had been transferred to the Golgi apparatus and that N-glycans of the high-mannose type had been processed. However, FN526S was detected only in the ugF0 form (Fig. 6b), suggesting that FN526S failed to be transported to the Golgi apparatus for up to 60 min of chase time. These results showed that a mutation at N526 decreased F protein folding efficiency and strongly delayed HaF transport to the Golgi apparatus for further processing.

**DISCUSSION**

N-Glycosylation is a complex process that first requires the transfer of oligosaccharides onto the potential glycosylation sites in the ER via catalysis of oligosaccharide transferase complex (Burda & Aebi, 1999). Thus, N-glycosylation site occupancy is determined at the beginning of the N-glycosylation process. However, the site occupancy is not an independent event but relates to several events such as site availability, enzyme kinetics and substrate concentration required for N-glycosylation (Jones et al., 2005). Alternatively, not all potential N-glycosylation sequons (N-X-S/T) in one glycoprotein molecule will be occupied by N-linked oligosaccharides (Jones et al., 2005). In this study, the N-glycosylation sites used for HaF protein were mapped precisely. Consistent with the previous report on the N-glycoproteomic analysis of HearNPV BVs where N104, N526 and N571 were found to be glycosylated (Hou et al., 2013), we found that five of the six potential glycosylation sites (N104, N293, N361, N526 and N571) on the HaF protein were N-glycosylated, whereas the N595 site was not used. N595 is probably not a used N-glycosylation site because it is located in the pre-transmembrane region of F1 subunit where it may be difficult for the oligosaccharide transferase complex to get access (Fig. 7a). Moreover, the newly created site N102 (in the FN104S mutant) could not fully rescue N-glycosylation of the F2 subunit, suggesting that the unique N-glycosylation site N104 in the F2 subunit may be specific for site occupancy.
The next step of N-glycosylation is that the attached oligosaccharides interact with chaperones, glycosidases and glycosyltransferases in the ER to help protein folding. Only properly folded glycoproteins are transferred to the Golgi apparatus for extensive modification of N-glycans (trimming, branching and elongation) after which they are transported to the final destinations inside and outside cells (Burda & Aebi, 1999). Therefore, specific site occupancy of N-glycosylation will determine the destiny and property of glycoproteins and thus the protein functioning. Thus, the N-glycosylation sites used should be responsible for cellular distribution, trafficking and fusogenicity of HaF proteins, and consequently affect virus production and infectivity. In general, N526 is a very important N-glycosylation site for HaF functions. Other used N-glycosylation sites exhibited different degrees of effects on F protein function. As folding assays revealed that FN526S resulted in a decreased efficiency of F protein folding and trafficking, this indicated that mutation of N526 may dramatically change the F protein structure. This may also explain why the fusogenic activity of FN526S was impaired and the amount of infectious progeny viruses and the infectivity of vHaBacAF-FN526S were decreased significantly. In addition, N361 had limited effects on HaF protein

![Diagram](http://jgv.microbiologyresearch.org)
trafficking, because mutation of N361 did not significantly change the intracellular distribution of F protein. N-Linked oligosaccharides attached to conserved asparagine probably play more important roles in ER chaperone recognition and association, and thus are important for glycoprotein folding and trafficking (Jones et al., 2005). Alignment of baculovirus F proteins with putative N-glycosylation sites showed that N361 is a unique site of HaF present in a variable region compared with the others (Figs 1a and S2). Therefore, N-glycans on N361 may not be essential for HaF folding and trafficking. We also found that most mutant HaF proteins showed enhanced fusogenicity. The data suggested that N-glycans except for those on N526 may hinder the fusion process induced by F protein. Further evidence comes from the comparison of increased fusogenicity of F\textsuperscript{N361Q} in which the N-glycosylation at N361 is removed, and the decreased fusogenicity of F\textsuperscript{N361S}, which allowed rescue of the N-glycosylation of a newly formed sequon at N359.

The roles of N-glycosylation have been characterized in evolutionary processes (Gagneux & Varki, 1999; Varki, 2006; Zhang et al., 2004). N-Glycosylation events in both hosts and pathogens are determined by mutual selection pressure, and Darwinian selection has been introduced to illustrate the role of N-glycosylation sequons in eukaryotes and viruses (Cui et al., 2009). New N-glycosylation sites may be developed in virus envelope proteins at ‘better’ positions (Zhang et al., 2004). Some N-glycosylation sites may have been introduced at the expense of certain enhanced properties such as fusogenicity and in exchange for improvement of other function(s) such as virus spread in the environment (Aguilar et al., 2006). We found that, except for N526, N-glycans on most sites impaired HaF fusogenicity, an essential trait for virus infection. However, virus production and infectivity of mutant viruses with these mutations were lower than the control virus. This may indicate that the virus traded off higher fusogenic ability for virulence.

The three-dimensional (3D) structures of the F protein of both pre- and post-fusion forms were modelled using the paramyxovirus F proteins as the functional analogue (Chen et al., 2005) (Fig. 7b). Three domains of HearNPV F protein were defined and mapped in the modelled structures as well as the N-glycosylation sites (Fig. 7b). The structure of the pre-fusion form is composed of a head (N104, N293, N361 and N526) and a stalk region (N571) (Fig. 7b, left). The post-fusion form was defined with head (N361), neck (N104, N293 and N526) and stalk (N571) regions (Fig. 7b, right). The steric locations of N-glycosylation sites of the HaF protein present properties consistent with those of paramyxovirus F proteins (von Messling & Cattaneo, 2003). In general, firstly, in the post-fusion form, the neck region of HaF contains most of the used N-glycosylation sites (N104, N293 and N526); secondly, N-glycosylation sites involved in efficient intracellular trafficking and folding (N104, N293, N526 and N571) are located in the neck and stalk regions; and thirdly, at least one N-glycosylation site (N526) is important for F protein trafficking and folding.

In summary, the N-glycosylations in the HearNPV F protein were found to be specific and significant for intracellular transport and egress of the HaF protein and for the fusion activity with host cells. Among the four identified N-glycosylation sites on the F\textsubscript{1} subunit, N526 was found to play important roles in both F function and virus infectivity, and N361 is a special site that is not involved in HaF cellular trafficking. Other sites had impacts to various degrees on different properties of the F protein such as intracellular trafficking and fusogenicity. Detailed investigation of how N-glycosylation influences baculovirus F protein structure will be of great significance in understanding the functioning of this protein in baculovirus infection and pathogenicity.

METHODS

Insect cells and viruses. Heliothis zea HzAM1 cells were cultured at 27 °C in Grace’s insect medium (Gibco-BRL) at pH 6.0, supplemented with 10 % (v/v) FBS. The f-null bacmid (HaBac\textsubscript{ΔF}) used as a basis for generating glycosylation mutants and the HaF-Rescued f-null virus (vHaBac\textsubscript{F-HaF}) used as a positive control were generated as described previously (Wang et al., 2008).

Construction of mutant viruses carrying F proteins mutated at all canonical N-glycosylation sites. Site-directed mutagenesis was carried out at canonical N-glycosylation sites of the HearNPV F protein separately (Fig. 1b). Mutagenic primers (Table S1) were designed so that the asparagine (N)-encoding codon in each N-glycosylation sequon (N-X-S/T) was replaced with serine (S)- or glutamine (Q)-encoding codons. Mutant F genes were generated using overlap-extension PCR as described (Ho et al., 1989). Mutants carrying single substitutions were designated F\textsuperscript{N104S}, F\textsuperscript{N104Q}, F\textsuperscript{N293S}, F\textsuperscript{N361S}, F\textsuperscript{N361Q}, F\textsuperscript{N571S} and F\textsuperscript{N571Q}.

PCR products were cloned into pGEM-T Easy vector (Promega), verified by sequencing and then inserted into pFB-Op166 (Wang et al., 2008) using Eco RI and HindIII restriction sites to generate the transfer plasmids. Each individual plasmid was then transformed into competent DH10Bac cells containing a HaBac\textsubscript{F} bacmid and a helper plasmid expressing a transposase (Bac-to-Bac Baculovirus Expression System; Invitrogen). Mutant bacmids were selected by gentamycin resistance and blue/white screening and identified by PCR with M13 primers.

HzAM1 cells were seeded into 35 mm diameter tissue culture dishes at a density of 3 × 10\textsuperscript{5} cells per dish. After 24 h incubation at 27 °C, cells were transfected with 1 μg bacmid DNA using 12 μl Lipofectin (Invitrogen). At 6 days post-transfection (p.t.), 1 ml supernatant from each transfection was clarified by centrifugation (5 min at 2200 g) and used to infect fresh cells. EGFP expression and spread were determined by fluorescence microscopy.

Western blot analysis of F proteins in mutant BVs and infected cells. BVs were purified from supernatants as described previously (Wang et al., 2008). BVs and infected cells were disrupted by Laemmli sample buffer [125 mM Tris/HCl (pH 6.8), 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.001 % bromophenol blue] and separated by 12 % SDS-PAGE. The proteins in the gel were immobilized on a PVDF membrane and analysed using monoclonal antibodies against F\textsubscript{1} and F\textsubscript{2} (Long et al., 2006) and VP80 (Deng et al., 2007) as primary antibodies, and alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody (Novo-Gene Biosciences). Signals were detected with nitro-blue tetrazolium/BCIP (Sino-American Biotechnology Co.).
One-step virus growth curve analysis. One-step virus growth curve analysis was performed as described previously (Wang et al., 2008) with a slight modification. HzAM1 cells (5 × 10⁶ cells per well) were infected at an m.o.i. of 5. Supernatants were harvested at 0, 24, 48, 72 and 96 h.p.i. and titrated by an end-point dilution assay. Each virus infection was performed in triplicate. The mean log-transformed TCID₅₀ values and SD were determined using Microsoft Excel software (version 2003). Significant differences (P values) were analysed with one-way ANOVA (Hewson et al., 2004).

Quantitative PCR determination of mutant BV genome copies. Virus infectivity was measured by determining the number of genomic DNA copies compared with viral titres using quantitative PCR with Eva Green dye (Biotium). Viral genomic DNA was purified and used for quantitative PCR analysis as described previously (Wang et al., 2010).

Low-pH-induced envelope fusion assay. A syncytium formation assay was performed as described previously, with slight modifications (Bisslard & Wenz, 1992). HzAM1 cells were infected with mutant viruses at an m.o.i. of 5. At 48 h.p.i., the cells were rinsed three times with Grace’s insect medium (pH 6.0) and treated with 1 ml acidified Grace’s insect medium (pH 4.8) for 5 min. The cells were then further cultured with Grace’s insect medium containing 10 % FBS at a normal pH (pH 6.0) for 24 h followed by fluorescence microscopy.

The cells were fixed, washed and incubated with Hoescht 33258 (Bio-time) for 15 min at room temperature. After three washes, the cells were photographed. The fusion ability of BVs containing WT and mutant F proteins was measured as described elsewhere, with slight modifications (Long et al., 2007; Tan et al., 2008). Syncytial masses were defined as fused cells containing four or more nuclei. Five fields were chosen randomly for each analysis. The fusogenic ability was calculated as the number of fused cells compared with infected cells. The mean value of the fusogenic ability of WT F protein was set as 100 %.

Subcellular localization of mutant HaF proteins. To determine the localization of different mutant F proteins in insect cells, p166AcV5-fx-egfp plasmids (fx represents WT or mutant f genes) were constructed, allowing the expression of fused EGFP at the C-terminus of F proteins in insect cells. The stop codons were deleted from the parental and mutant f genes when they were PCR amplified with primers HaFdelTAAfor and HaFdelTAArev (Table S1) and verified by sequencing. Subsequently, the fragments were cloned into the p166AcV5-egfp plasmid (Ilkel et al., 2000) at the HindIII restriction site in frame with the egfp gene. SF9 cells (1 × 10⁶ cells per well) were transfected with the plasmids and examined in a Leica S2 confocal laser scanning fluorescence microscope at 48 h.p.i.

The cell-surface levels of mutant HaF proteins were analysed quantitatively by an immunofluorescence assay and flow cytometry analysis (Tong et al., 2001). HzAM1 cells were infected with viruses at an m.o.i. of 5. At 24 h.p.i. the cells were rinsed twice with PBS and blocked for 1 h at 27 °C with PBS containing 5 % BSA. The cells were then incubated with anti-F2 antiserum (1 : 500 dilution) overnight at 4 °C with and a secondary goat anti-rabbit antibody conjugated with R-phycocerythrin (1 : 300 dilution; Proteintech Group) for 1 h at 27 °C. The cells were washed and resuspended for flow cytometry analysis on a Beckman Coulter EPICS XL flow cytometer. Infected cells expressing EGFP and immunostained with R-phycocerythrin were scored. The relative levels of F proteins on the cell surface were measured by dividing the percentage of cells stained with R-phycocerythrin by the number expressing EGFP, and these levels were normalized to the data from the WT control which was set as 100 %.

Protein folding assays. F protein folding was investigated using pulse–chase experiments performed essentially as described previously (Braakman & Hebert, 2001; Braakman et al., 1991; Land et al., 2003).

HzAM1 cells were infected with viruses at an m.o.i. of 5. Cells were harvested at 48 h.p.i. and depleted of methionine for 15–30 min before they were pulse labelled with pro-mix [35S]cysteine and [35S]methionine (Easylight Express Protein Labelling Mix; PerkinElmer). Cells were chased for various intervals in medium containing an excess of unlabelled methionine. Chase samples were stopped by aspirating the medium and adding ice-cold Hanks’ balanced salt solution (Invitrogen-BRL) containing 20 mM N-ethylmaleimide to block free thiol groups. Cells were lysed in ice-cold 0.5 % v/v Triton X-100 in MNT [20 mM MES (pH 7.5), 100 mM NaCl, 30 mM Tris/HC1] and protease inhibitor cocktail (10 μg ml⁻¹ each chymostatin, leupeptin, antipain, and pepstatin, 1 mM PMSF and 1 mM EDTA). Cell lysates were spun for 10 min at 15 000 g at 4 °C to sediment the nuclei.

Immunoprecipitation was used to isolate the F proteins. Goat anti-rabbit IgG (1 μg per sample) was incubated for 1 h at 4 °C with protein A-Sepharose 4B fast-flow beads (50 μl 10 % suspension per sample; Amersham Pharmacia Biotech AB). Anti-F2 serum used as the primary antibody was added, and incubation was continued for 1 h. Cell lysates were added to the mixture and incubated at 4 °C for 1 h. The immunoprecipitates were washed twice for 10 min each with wash buffer [10 mM Tris/HC1 (pH 8.6), 0.05 % Triton X-100, 0.1 % SDS, 0.3 % NaCl] at room temperature; the washed pellets were resuspended in 20 μl 10 mM Tris/HC1 (pH 6.8) and sample buffer was added to a final concentration of 200 mM Tris/HC1 (pH 6.8), 3 % SDS, 10 % glycerol, 0.004 % bromophenol blue and 1 mM EDTA. Samples were incubated at 95 °C for 5 min and analysed by reducing or non-reducing 7.5 % SDS-PAGE (Braakman et al., 1991). The gels were then dried and signals were detected on Biomax MR films (Eastman Kodak).

For Endo H digestion, immunoprecipitates were resuspended in 0.2 % SDS in 100 mM sodium acetate (pH 5.5) and heated for 5 min at 95 °C. An equal volume of 100 mM sodium acetate (pH 5.5) was then added. Endo H (0.2 U) (Scalia et al., 1992) was added to each sample and incubated for 1 h at 37 °C.

Computational analysis of putative N-glycosylation sites and their superposition on the 3D structure of HaF. Sequences of F proteins of group II alphabaculoviruses were aligned using MEGALIGN in DNASTAR and edited with Genedoc software. Putative N-glycosylation sites of the F proteins were predicted with the NetNGlyc 1.0 Server (ExPaSy, Swiss Institute of Bioinformatics). The Phyre threading program (http://www.sbg.bio.ic.ac.uk/~phyre) was used to search appropriate templates for 3D computer animation. Simian virus 5 (SV5) F protein (PDB code: 2b9b) was used as the template for the pre-fusion state of HaF. Sequence alignment with HaF and SV5 F protein was carried out using CLUSTALW version 2.0 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the 3D structure was modelled in the alignment mode of SWISS-MODEL (Arnold et al., 2006; Kiever et al., 2009; Peitsch, 1995). The post-fusion structure was modelled by I-TASSER (Roy et al., 2010), and is a structure analogue compared with the human parainfluenza virus type 3 (HPV3) F protein (PDB code: 1ZTM). Domains of the HaF protein were defined according to those of SV5 F and HPV3 F proteins by using William Pearson’s LALIGN program as described elsewhere (Garry & Garry, 2008). The 3D structures were displayed using Pymol version 0.99.

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REFERENCES


