Function, oligomerization and N-linked glycosylation of the *Helicoverpa armigera* single nucleopolyhedrovirus envelope fusion protein

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In the family *Baculoviridae*, two distinct envelope fusion proteins are identified in budded virions (BVs). GP64 is the major envelope fusion protein of group I nucleopolyhedrovirus (NPV) BVs. An unrelated type of envelope fusion protein, named F, is encoded by group II NPVs. The genome of *Helicoverpa armigera* (Hear) NPV, a group II NPV of the single nucleocapsid or S type, also encodes an F-like protein: open reading frame 133 (*Ha133*). It was demonstrated by N-terminal sequencing of the major 59 kDa protein present in HearNPV BV that this protein is one of the two F subunits: F1 (transmembrane subunit of 59 kDa) and F2 (surface subunit of 20 kDa), both the result of cleavage by a proprotein convertase and disulfide-linked. The HearNPV F protein proved to be a functional analogue of GP64, as the infectivity of an AcMNPV *gp64*-deletion mutant was rescued by the introduction of the HearNPV F gene. It was also demonstrated by chemical cross-linking that HearNPV F is present in BVs as an oligomer whereby, unlike GP64, disulfide bonds are not involved. Deglycosylation assays indicated that both F1 and F2 possess N-linked glycans. However, when F was made in Hz2E5 cells, these glycans did not have an α-1-3 core fucose modification that usually occurs in insect cells. As α-1-3 core fucose is a major inducer of an allergic response in humans, the present observation makes the HearNPV–Hz2E5 system an attractive alternative for the production of recombinant glycoproteins for therapeutic use in humans.

INTRODUCTION

The *Baculoviridae* are a large family of enveloped DNA viruses that are almost exclusively pathogenic to arthropods, predominantly insects in the order Lepidoptera (Theilmann et al., 2005). Baculoviruses are divided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Phylogenetic studies indicate that NPVs can be subdivided into two subgroups: group I and group II (Bulach et al., 1999; Hayakawa et al., 2000; Herniou et al., 2003). Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV) (Volkman & Summers, 1977). ODVs are present in occlusion bodies and are able to infect midgut epithelial cells by direct membrane fusion (Horton & Burand, 1993). In contrast, BVs are adapted to propagate infection from cell to cell via receptor-mediated endocytosis and are responsible for the systemic spread of the virus in the infected insect (Hefferon et al., 1999; Volkman & Goldsmith, 1984).

Baculovirus, like other envelope viruses from a broad range of different families, overcomes the first barrier of host cells via membrane fusion, which is mediated by a specific envelope fusion protein (Eckert & Kim, 2001; Weissenhorn et al., 1999). The BV phenotype of group I NPVs contains the major envelope glycoprotein GP64, which mediates low pH-triggered membrane fusion (Blissard & Wenz, 1992; Kingsley et al., 1999; Plonsky et al., 1999) and is necessary for efficient budding of BVs from the surface of infected cells (Oomens & Blissard, 1999). Recent genomic research of group II NPVs and of GV suggests that they lack a GP64 homologue. A different BV envelope fusion protein has been identified from the group II NPV *Lymnantria dispar* (Ld)MNVP and *Spodoptera exigua* (Se)MNVP with structural similarity to vertebrate virus envelope fusion proteins (Ijkel et al., 2000; Pearson et al., 2000). Like the GP64-homologous protein, these proteins mediate low pH-dependent membrane fusion during BV entry and were named F proteins (Pearson & Rohrmann, 2002; Westenberg et al., 2002). Cleavage of the SeMNVP F protein into two disulfide-linked subunits by a cellular convertase (furin-like) is necessary for the low pH-triggered membrane-fusion activity and viral infectivity (Westenberg et al., 2002). This is
in contrast to LdMNPV F, which was originally found as an uncleaved BV protein (Pearson et al., 2000) despite the presence of a furin-like cleavage site, but this is probably due to the particular cell system (Westenberg et al., 2002). A recent study showed that both LdMNPV F and SeMNPV F can rescue virion production and infectivity of an AcMNPV virus with a deleted gp64 gene, which suggests that these F proteins of group II NPVs are functionally analogous to GP64 of group I NPVs (Lung et al., 2002). F-like proteins occur ubiquitously in envelopes of some vertebrate viruses, such as influenza and parainfluenza viruses (Eckert & Kim, 2001).

As in other group II NPVs, no gp64-like gene is present in the Helicoverpa armigera (Hear)NPV genome (Chen et al., 2001), but an f homologue has been found as open reading frame (ORF) 133 (Ha133). In this report, the HearNPV F protein is identified in BVs by protein-sequence analysis. To show the analogy between this HearNPV F protein and the SeMNPV or LdMNPV F proteins, the conformation of the HearNPV F protein in BVs and its ability to rescue BV production and infectivity of an AcMNPV virus with a deleted gp64 gene were studied. In addition, deglycosylation experiments revealed the presence of α-1-3 core-fucosylated N-glycans on the HearNPV F protein when produced in Trichoplusia ni cells, but the absence of such glycans in Hz2E5 cells. The oligomeric structure of the baculovirus F proteins was studied in more detail by chemical cross-linking of BV proteins.

**METHODS**

**Cells and virus.** Helicoverpa zea cell line Hz2E5 (McIntosh & Ignoffo, 1983) was cultured in plastic tissue-culture flasks (Nunc) in CCM3-HyQ medium (Hyclone) supplemented with 5% fetal bovine serum (FBS). Spodoptera frugiperda cell line IPLB-SF-21 (Vaugh et al., 1977) and T. ni BT-In-5B1-4 cells (High5) (Granados et al., 1994) were cultured at 27 °C in plastic tissue-culture flasks (Nunc) Grace’s insect medium, pH 5-9-6-1 ( Gibco-BRL), supplemented with 10% FBS. The HearSNPV G4 isolate was originally obtained from H. armigera (Lepidoptera, Noctuidae) in the People’s Republic of China (Sun et al., 1998). The gp64-null AcMNPV bacmid was described by Lung et al. (2002) and was obtained from Dr G. W. Blissard (Boyece Thompson Institute, Cornell University, Ithaca, NY, USA).

**Purification of HearNPV BVs.** Hz2E5 cells were infected with an m.o.i. of 5 TCID50 units HearSNPV per cell. After 3 days, the cell culture supernatant was collected and clarified at 3000 rpm for 10 min at 4 °C. The supernatant was passed through a 0-45 μm pore-size filter. BVs in the filtrate were sedimented through a 25% (w/w) sucrose cushion made up in 0.1× TE [10 mM Tris/HCl (pH 7-5), 1-0 mM EDTA] by centrifugation at 100,000 g for 90 min at 4 °C. The BVs were resuspended in 0.1× TE and used directly or stored at −20 °C.

**Protein sequencing.** Purified HearNPV BVs were disrupted in Laemmli buffer [125 mM Tris/HCl, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0-001% (w/v) bromophenol blue, pH 6-8] and incubated for 10 min at 95 °C. BV proteins were separated in a 12% SDS-PAGE gel and transferred by semi-dry blotting onto a PVDF membrane (Bio-Rad) using CAPS buffer [10 mM CAPS, 10% (v/v) methanol, pH 11]. Proteins were visualized on the PVDF membrane by using Cooamassie brilliant blue. The major protein band (59 kDa) from HearNPV BV was N-terminally sequenced (Protein Research Facility, Amsterdam, The Netherlands).

**Production of polyclonal antibodies.** The HearNPV ORF133 regions encoding aa 25 (M) to 173 (R) of F2 and aa 174 (N) to 599 (G) of F1 were amplified by high-fidelity Expand long-template PCR (Roche) with HearNPV G4 genomic DNA as template. Primers used to amplify F2 were 5′-CAAGGATATCCAAACTGGATGATTGAC-TCGGTTG-3′ (underlined nucleotides generate a BamHI restriction site) and 5′-AATAAAGTTATACCCGTACCTTAAATTTCCAACCG-3′ (underlined and bold nucleotides generate a HindIII restriction site and a translation stop codon, respectively). Primers used for amplifying F1 were 5′-GGATCTCTATGGTCTGGCAAGGCGCATGCGTGCTG-3′ (underlined nucleotides generate a BamHI restriction site) and 5′-TTAAGCTTATCGTGTTGCGACTGAAATTTTG-3′ (underlined and bold nucleotides generate a HindIII restriction site and a translation stop codon, respectively). The PCR products were cloned into the BamHI and HindIII cloning sites of expression vector pET28a (Novagen) to generate pET28-HaF1a600-678 and pET28-HaF2α1-24.

Proteins F1 and F2 were expressed in Escherichia coli BL21 cells. The insoluble fractions containing the HaF1 and HaF2 proteins were purified by continuous-elution electrophoresis using a model 491 Prep Cell (Bio-Rad) according to the manufacturer’s protocol. Elution fractions were collected and electrophoresed in 12-5% SDS-PAGE gels and the protein bands were visualized by silver staining. Fractions containing F1 or F2 protein were pooled and concentrated by using Centriprep filter devices (Amicon). Protein concentrations were determined with the Bio-Rad protein assay. Two chickens were each injected intramuscularly with 200 μg purified HaF1 or HaF2 protein by using a water-in-oil adjuvant. The chickens were boosted after 6 weeks with 100 μg purified protein. Two weeks after the booster, eggs were collected daily for 4 weeks. Antibodies were purified from the egg yolk as described previously (Westenberg et al., 2002).

**Western blot analysis.** HearNPV BVs were disrupted either under reducing conditions in Laemmli buffer as described above or under non-reducing conditions in 125 mM Tris/HCl, 8% (w/v) SDS, 37-5 mM iodoacetamide, 10% (v/v) glycerol, 0-001% (w/v) bromophenol blue, pH 6-8 by incubation for 10 min at 95 °C. Proteins were separated by SDS-PAGE and subjected to Western blot analysis as described by Ausubel et al. (1994) and Long et al. (2003). The antisera were used in a 1–1000 dilution and the proteins were detected by treatment with horseradish peroxidase-conjugated rabbit anti-chicken immunoglobulin (Sigma) diluted 1–10000 followed by enhanced chemiluminescence technology as described by the manufacturer (Amersham Biosciences).

**Chemical cross-linking.** Purified HearNPV BVs were suspended in cross-linking buffer (10 mM HEPES, 100 mM NaCl, pH 8-3). Ethylene glyco bis-(succinimidyl) succinate (EGS) (Sigma) was added as a cross-linking agent to a final concentration of 125–500 μM. After incubation on ice for 1 h, EGS was quenched with 50 mM Tris/HCl (pH 6-8) for 10 min. BV proteins were separated in a 6% non-reducing SDS-PAGE gel and transferred onto Immobilon-P membranes (Millipore) for Western blot analysis.

**Deglycosylation assay.** Deglycosylation experiments were performed on cell culture-derived and sucrose-purified HearNPV BVs. BVs were denatured by boiling for 10 min in the presence of 0-5% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Denatured BV proteins were incubated overnight either in Endo H G5 buffer [50 mM sodium citrate (pH 5-5), 5 mM PMSF] containing 1 U endoglycosidase H (Endo H; New England Biolabs) or in PNGase F incubation...
buffer [PBS (pH 7.4), 20 mM EDTA, 0–5% (v/v) NP40, 5 mM pMSF] containing 1 U peptide: N-glycosidase F (PNGase F; Roche). Deglycosylated BV proteins were separated by SDS-PAGE, immobilized onto an Immobilon-P membrane and probed with antibodies (IgY) against HaF1 and HaF2.

Pseudotyping gp64-null AcMNPV with HearNPV F. The whole coding region of the F gene was amplified by high-fidelity Expand long-template PCR (Roche) with primers Ha133FHindIII 5′-AAGGCTTATGCTTGGCGATATAAAAGTGAAGTAGTATG-3′ and Ha133RHindIII BamHI 5′-GGATCCTGATCGTGGATGATGGTTAGGCTGCAGCGCATCCCTAAAACGGCAGC-3′ (underlined and bold nucleotides generate HindIII and BamHI restriction sites, respectively) and HearNPV G4 genomic DNA as template. The PCR product was cloned as a HindIII fragment, of which the protruding ends were filled in by T4 DNA polymerase, into the also blunt-ended EcoRI and BamHI sites of the plasmid pFBgusAcgp64 (Lung et al., 2002) to obtain pAFBgsuHaF.

The expression cassette pAFBgsuHaF was transposed into the attTn7 transposition sites of the gp64-null AcMNPV bacmid (Lung et al., 2002) according to the Bac-to-Bac Baculovirus Expression Systems manual (Invitrogen) to obtain the gp64-null Ha133-AcMNPV bacmid. Transposition was confirmed by PCR using the M13/pUC forward primer (5′-CCGATCCTAGCGATTTGAAACG-3′) in combination with the M13/pUC reverse primer (5′-AGCGGATACCAATTTTCACAGACCGG-3′) and M13/pUC forward primer in combination with a primer corresponding to the sequence of the Gm gene, BAC-control (5′-GGGCCACCTACTCCCAACATC-3′).

S2I cells (9 × 10^5) were seeded into 35 mm tissue-culture plates (Nunc). Cells were transfected with approximately 1 µg bacmid DNA by using 10 µl Cefectin (Invitrogen). At 4 days post-transfection, cells were stained for β-d-glucuronidase (GUS) activity according to the Bac-to-Bac protocol (Invitrogen) to monitor transfection efficiency. The supernatant (passage 1) was clarified at 2000 × g for 10 min at 4 °C and subsequently filter-sterilized (0.22 µm pore size). To investigate the presence of infectious BVs, one-quarter (500 µL) of the supernatant was used to infect 9 × 10^5 S2I cells. Seventy-two hours post-infection (p.i.), the cells were stained for GUS activity. The gp64-positive AcMNPV bacmid and the gp64-null AcMNPV bacmid served as positive and negative controls for transfection and infection, respectively (Lung et al., 2002).

Computational analysis. The HearNPV F protein was analysed by using the EXPASY sever (Appel et al., 1994) to predict the transmembrane domain, signal peptide, heptad repeats (HRs) and potential glycosylation sites.

RESULTS

Identification of HearNPV F

To identify the HearNPV envelope fusion protein, BVs were purified from an infected Hz2E5-cell culture. Sucrose gradient-purified BVs proteins were separated in a 12.5% SDS-PAGE gel and the proteins were visualized by Coomassie brilliant blue staining. Four major protein bands were visible, with molecular sizes of 59, 45, 33 and 18 kDa (Fig. 1, lane 1). An N-terminal sequence NIGLNWF was obtained from the purified 59 kDa protein by protein sequencing. This obtained sequence is encoded by the ORF Ha133 (Chen et al., 2001), immediately downstream of the predicted furin-cleavage site R–N–K–R within the primary translational product. This suggests that Ha133 encodes

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![Fig. 1. SDS-PAGE and Western blot analysis of HearNPV BV proteins. Cell culture-derived BVs were incubated under reducing (lanes 1–3) or non-reducing (lanes 4–6) conditions and electrophoresed in a 12.5% gel (lanes 1–5) or 6% gel (lane 6) SDS-PAGE gel. Proteins were Coomassie brilliant blue-stained (lane 1) or subjected to Western blot analysis (lanes 2–6), probed with either x-HaF1 (lanes 2, 4 and 6) or x-HaF2 (lanes 3 and 5) and detected with a chemiluminescent substrate. Arrows indicate the four major protein bands with molecular sizes of 59, 45, 33 and 18 kDa. F0 (~80 kDa) = F1 (59 kDa) + F2 (20 kDa) disulfide-linked subunits of HearSNPV F protein. Size standards are indicated in kDa.](http://vir.sgmjournals.org)

Immunodetection of the HearNPV F protein

Post-translational cleavage of the fusion protein of enveloped viruses seems to be a general mechanism used to regulate viral membrane-fusion activity (Eckert & Kim, 2001; Klenk & Garten, 1994). The cleavage products could remain associated by covalent or non-covalent linkages. In case of the SeMNPV F protein, the cleavage products remain linked covalently by a disulfide bridge (Westenberg et al., 2002). To investigate which situation exists for the F protein, HearNPV BV proteins were disrupted, separated in a reducing SDS-PAGE gel and subjected to Western blot analysis using F1 (x-HaF1) or F2 (x-HaF2)-specific antibodies (Fig. 1, lanes 2–3). These results show that both the F1 (59 kDa) and F2 (20 kDa) cleavage products are present in the BV.
To examine whether the cleavage products remain associated covalently, HearNPV BVs were treated with iodoacetamide, which alkylates free thiol groups, preventing formation of artificial disulfide linkages during further treatment or electrophoresis. The proteins were denatured in the absence of reducing agents and electrophoresed in a 12.5 % SDS-PAGE gel. Western blot analysis with x-HaF1 and x-HaF2 detected in both cases a single protein with a molecular mass of 80 kDa (F0) (Fig. 1, lanes 4–5). The absence of F-protein subunits (59 and 20 kDa bands) indicates that F1 and F2 are associated covalently by a disulfide linkage.

Oligomerization of the HearNPV F protein

Viral fusion proteins generally form higher-order oligomers (Hernandez et al., 1996). In the case of the envelope fusion protein GP64 of the group I NPVs, this oligomer is formed by three stable, disulfide-linked GP64 proteins (Oomens et al., 1995). To determine whether HearNPV F proteins form disulfide-linked oligomers, non-reduced HearNPV BV proteins were separated in a 6 % SDS-PAGE gel, transferred onto Immobilon-P membranes and probed with x-HaF1 (Fig. 1, lane 6). A major monomeric band of 80 kDa (F0) was detected and no band was observed at about 160 or 240 kDa, indicating that, in contrast to GP64 (Oomens et al., 1995), possible oligomeric forms of the HearNPV F protein are not disulfide-linked.

BV proteins were also cross-linked chemically with different concentrations of EGS. Afterwards, proteins were separated in a 6 % SDS-PAGE gel, transferred onto Immobilon-P membranes and probed with x-HaF1 to detect F (Fig. 2). In addition to the monomeric form (~ 80 kDa), dimeric (~ 160 kDa) and trimeric (~ 240 kDa) forms of the F protein were clearly visible.

Pseudotyping of the AcMNPV gp64-null bacmid

It has been shown that the SeMNPV and LdMNPV F proteins are functional analogues of GP64, as they can rescue the infectivity of an AcMNPV gp64-null bacmid (Lung et al., 2002). To determine whether the HearSNPV F protein could also rescue the gp64-null bacmid, its coding region was placed under the control of the AcMNPV gp64 promoter and inserted, together with a gus reporter gene, into the gp64-null bacmid (vAcgp64-/HaF). The gp64-null bacmid with no envelope fusion protein gene (vAcgp64-) and the repair bacmid (vAcgp64+/gp64), in which the gp64 gene was reinserted, were used as negative and positive controls, respectively. The bacmids were transfected into Sf21 cells (Fig. 3a, panels 1–3). Cells transfected with vAcgp64- (panel 1) showed single infected cells with GUS, which did not produce infectious virus as expected (Monsma et al., 1996). When cells were transfected with vAcgp64-/HaF, a large number of infected cells was observed (panel 3), as was the case in the positive control using the repair bacmid vAcgp64+/gp64 (panel 2). Supernatant of both vAcgp64- and vAcgp64-/HaF-transfected cells produced infectious BVs when tested on uninfected Sf21 cells (panels 5 and 6).

Infectious viruses were produced from cells transfected with vAcgp64-/HaF (Fig. 3a, panels 3 and 6) and from the positive control Acgp64-/gp64 (Fig. 3a, panels 2 and 5). Infected cells, as well as BVs produced by those cells, were harvested and subjected to Western blot analysis using antibodies against GP64 (x-GP64) as a probe (Fig. 3b). GP64 was only detected in the vAcgp64-/gp64 BVs (Fig. 3b, lane 1) and vAcgp64-/gp64-infected cells (Fig. 3b, lane 3), but not in the vAcgp64-/HaF BVs (Fig. 3b, lane 2) or vAcgp64-/HaF-infected cells (Fig. 3b, lane 4).

The vAcgp64-/HaF BVs were further analysed for the presence of F and its post-translational cleavage products F1 and F2 in this pseudotyping system. BV proteins were separated in a 12.5 or 6 % SDS-PAGE gel, under either reducing (Fig. 3c) or non-reducing (Fig. 3d) conditions, and finally detected by Western blot analysis using antibodies against GP64 (x-GP64) or x-HaF2. All detected F protein in the BVs was cleaved entirely into F1 and F2 (Fig. 3d), which remains associated by disulfide bonds when analysed under non-reducing conditions (Fig. 3d). This result indicates that the HearNPV F protein is able to functionally pseudotype the gp64-null AcMNPV virus and that the F protein ends up in the BVs in a subunit configuration similar to that in the authentic HearNPV BVs.
The LdMNPV F protein appeared as an N-linked glycoprotein by tunicamycin treatment of infected Ld625Y insect cells (Pearson et al., 2000). To obtain more in-depth insight into the nature of the N-linked glycosylation status of the HearNPV F protein, deglycosylation assays were performed. Denatured HearNPV BV proteins were treated with Endo H and PNGase F, then separated by SDS-PAGE and subjected to Western blot analysis using either α-HaF1 (Fig. 4a, left panel) or α-HaF2 (Fig. 4a, right panel). Both F1 and F2 appeared to be N-glycosylated. The N-glycans on F1 are Endo H-sensitive (Fig. 4a, lane 2), implying that those N-glycans are of the high-mannose or hybrid type. Furthermore, the N-glycans on both F1 and F2 are PNGase F-sensitive (Fig. 4a, lanes 3 and 6), indicating that none of the glycans contains an α-1-3-linked core (C3) fucose, which is generally present in insect glycoproteins (Tomiya et al., 2004).

N-Linked glycosylation of HearNPV F protein expressed in T. ni High5 cells was analysed by PNGase F treatment of vAcgp64−/HaF BVs collected 5 days p.i. (Fig. 4b). Both F1 and F2 expressed in T. ni High5 cells were resistant to PNGase F (Fig. 4b, lanes 3 and 4). This result suggests that the HearNPV F protein expressed in T. ni High5 cells is C3-fucosylated and also that the C3 fucosylation on the F protein does not affect the infectivity of vAcgp64−/HaF in these two cell lines.

**DISCUSSION**

The *Baculoviridae* are a large family of enveloped viruses (Theilmann et al., 2005). The BV phenotype of these viruses enters cells by an endocytic mechanism facilitated by the presence of an envelope fusion protein. GP64 is the major BV envelope protein of group I NPVs (e.g. AcMNPV).
Recently, a novel type of baculovirus envelope fusion protein that is unrelated to GP64 was identified in SeMNPV (SE8) and LdMNPV (LD130) BVs (IJkel et al., 2000; Pearson et al., 2000). This envelope protein, named F protein (Westenberg et al., 2002), is similar to the envelope fusion protein of vertebrate viruses (IJkel et al., 2000; Pearson & Rohrmann, 2002). This report demonstrates that the Ha133 gene in the HearNPV G4 genome (Chen et al., 2001), being phenotypically a single-nucleocapsid NPV, encodes a protein homologous to the LdMNPV and SeMNPV F proteins. This result shows that both single (S) and multiple (M) nucleocapsid NPVs of group II NPVs have an F protein in the BV particle.

Four major proteins (59, 45, 33 and 18 kDa) were found in the HearNPV BV (Fig. 1, lane 1). The 59 kDa protein appeared to be the transmembrane-anchored F1 domain of the F protein. In HearNPV F, the furin-like cleavage occurs at the predicted cleavage site (SRNKR↓NIGLNF), resulting in an N-terminal sequence NIGLNF of F1. N-terminal protein sequencing of the 45 kDa BV protein showed that this protein is actin, which was confirmed by Western blot analysis using actin-specific antibodies (data not shown).

The origin of the major 18 kDa BV protein is not known, but it is not the F2 subunit of the HearNPV F protein because of the molecular mass difference between the 18 kDa BV protein and the protein recognized by antibody against F2. Using Western blot analysis, it appeared that F2 was about 20 kDa (Fig. 1, lane 3). This band is larger than the predicted molecular mass (18 kDa) of F2 with the signal peptide removed. Probably, this small difference is due to the N-linked glycosylation in F2 (see below).

Western analysis also showed that the HearNPV F protein is cleaved post-translationally and that the two cleavage fragments remain associated by a disulfide bridge (Fig. 1). The F2 fragment of the HearNPV F protein contains only a single cysteine (C108). In contrast, the F1 fragment contains nine conserved cysteines in its ectodomain. Mass-spectrometric analysis of peptides should reveal which of the cysteines in the F1 fragment forms the disulfide link with the F2 fragment. A similar situation is found for the SeMNPV F protein, for which it has been demonstrated that this post-translational cleavage is important for its fusogenic activity (Lung et al., 2002; Westenberg et al., 2002). In contrast, the LdMNPV F protein has been found as an uncleaved protein on BVs produced in Ld625Y cells (Pearson et al., 2000); however, when this protein was expressed singly in a different cell line, it appeared cleaved (Pearson et al., 2002). Probably, Ld625Y cells are unable to cleave the LdMNPV F protein properly, but maybe the protein is activated during viral uptake.

Both SeMNPV and LdMNPV F proteins have been used before to pseudotype a gp64-null AcMNPV virus (Lung et al., 2002). In a similar experiment, we demonstrated that the HearNPV F protein also has the ability to rescue infectious BV from this gp64-deletion mutant (Fig. 3). The protein was found on the pseudotyped AcMNPV BVs as two disulfide-linked F-protein fragments, similar to those on the HearNPV BV, which indicates that the post-translational modification and assembly of the F proteins in the BV envelope are similar in Hz2E5 and Sf21 cells. Pseudotyping of gp64-null AcMNPV virus has not been successful for all baculovirus F proteins. The F protein of the Plutella xylostella GV could be incorporated in the AcMNPV BVs,
but those BVs were not infectious (Lung et al., 2002). Maybe the Sf2 cells possess the F-protein receptor for NPVs, but not for GVs. Currently, these pseudotyped viruses are being used to investigate whether the F-protein receptors of HearNPV and SeMNPV on Sf21 cells are one and the same.

Viral fusion proteins generally form higher-order oligomers (Hernandez et al., 1996). Viral fusion protein from members of the families Orthomyxoviridae, Paramyxoviridae, Coronaviridae and Herpesviridae forms homo-oligomers and this formation is essential for its fusion activity and for the viral infectivity. The baculovirus fusion protein GP64 of the group I NPVs forms a stable, disulfide-linked homotrimer in infectivity. The baculovirus fusion protein GP64 of the formation is essential for its fusion activity and for the viral infection. The baculovirus fusion protein GP64 of the group I NPVs forms a stable, disulfide-linked homotrimer (Oomens et al., 1995). Disulfide linkage seems not to be involved in the oligomerization of the baculovirus F protein (Fig. 1; Westenberg et al., 2002), as the F protein is detected by Western blot analysis under non-reducing conditions predominantly as a monomer. Protein bands with a molecular mass greater than 80 kDa (f0) were found to react with the antibody against F1 (z-HaF1), but none of them had a multiple size of the monomer.

Chemical cross-linking has been used to identify, for instance, the envelope fusion protein interactions of paramyxoviruses (Russell et al., 1994; Sechoy et al., 1987). The HearNPV BVs were cross-linked with EGS and the F protein was detected both as non-cross-linked monomers and as cross-linked dimers and trimers (Fig. 2). Probably, this implies that the baculovirus F protein can be found as trimers on the BV particle. This situation also exists with F proteins of vertebrate enveloped viruses (Colman & Lawrence, 2003; Dutch et al., 2000). An HR domain on the F1 fragment with a putative leucine zipper (LAKNNALNEQVKELDDLIRL) has been identified in the HearNPV F protein, which might be involved in the oligomerization of the F protein (Eckert & Kim, 2001; IJkel et al., 2000). In-depth studies involving circular-dichroism techniques and mutational analysis are in progress to confirm whether this domain can indeed form a multimeric coiled-coil structure.

In general, viral envelope fusion proteins are heavily N-glycosylated, which is critical for proper folding, trafficking and fusogenicity of the fusion protein (Doms et al., 1993; Helenius & Aebi, 2001). N-Glycosylation and protein folding are closely interconnected processes that take place in the endoplasmic reticulum. The addition of N-linked glycans occurs cotranslationally before or whilst nascent polypeptide chains are translated (Braakman & van Anken, 2000). Six potential N-glycosylation sites are predicted in HearNPV F protein, one in F2 and five in F1. Deglycosylation of HearNPV BV proteins by Endo H and PNGase F showed that both F1 and F2 are N-glycosylated (Fig. 4).

Both F1 and F2 obtained from BVs produced in Hz2E5 cells were sensitive to PNGase F treatment (Fig. 4a), which implies that none of the N-glycans contains a C3 fucose residue. In contrast, HearNPV F protein from pseudotyped AcMNPV expressed in T. ni cells was C3-fucosylated (Fig. 4b). Glycobiology studies are in progress to elucidate the structure of these N-glycans on the F protein and on cellular glycoproteins of H. zea cell lines.

The baculovirus–insect cell expression system is often used to express large amounts of protein for pharmaceutical purposes (Jarvis, 2003; Kost et al., 2005). The major drawbacks of using the baculovirus (AcMNPV) expression system to produce glycoproteins as therapeutics are (i) the inability to produce complex types of N-linked glycans, as is the case with vertebrate systems, and (ii) the C3 fucosylation, which causes allergic reactions in humans (Tomiya et al., 2003, 2004). The observations that HearNPV F proteins do not contain C3-fucosylated N-linked glycans is thus of considerable interest and promotes the development of the HearNPV–Hz2E5 cell system as an alternative for the production of therapeutics for human use.

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