Identification and functional analysis of inter-subunit disulfide bonds of the F protein of *Helicoverpa armigera* nucleopolyhedrovirus

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The major envelope fusion protein F of the budded virus of baculoviruses consists of two disulfide-linked subunits: an N-terminal F2 subunit and a C-terminal, membrane-anchored F1 subunit. There is one cysteine in F2 and there are 15 cysteines in F1, but their role in disulfide linking is largely unknown. In this study, the inter- and intra-subunit disulfide bonds of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearNPV) F protein were analysed by site-directed mutagenesis. Results indicated that in a functional F protein, an inter-subunit disulfide bond exists between amino acids C108 (F2) and C241 (F1). When C241 was mutated, an alternative disulfide bond was formed between C108 and C232, rendering F non-functional. No inter-subunit bridge was observed in a double C232/C241 mutant of F1. C403 was not involved in the formation of inter-subunit disulfide bonding, but mutation of this amino acid decreased viral infectivity significantly, suggesting that it might be involved in intra-subunit disulfide bonds. The influence of reductant [tris(2-carboxyethyl) phosphine (TCEP)] and free-thiol inhibitors [4-acetamido-4-9-maleimidylstilbene 2,2'-disulfonic acid (AMS) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)] on the infectivity of HearNPV was tested. The results indicated that TCEP greatly decreased the infection of HzAm1 cells by HearNPV. In contrast, AMS and DTNB had no inhibitory effect on viral infectivity. The data suggested that free thiol/disulfide isomerization was not likely to play a role in viral entry and infectivity.

INTRODUCTION

The *Baculoviridae* is a family of large, enveloped, DNA viruses that are pathogenic to arthropods (Federici, 1997). The family is divided into four genera based on molecular phylogenetic analysis: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* (Jehle et al., 2006). The genus *Alphabaculovirus* is further subdivided into two groups (I and II) based on their host and major envelope fusion protein (EFP) (Herniou et al., 2001; Pearson et al., 2000). The major EFPs, GP64 and F protein, are significant for the infectivity of baculoviruses. Group I alphabaculoviruses utilize GP64 as their EFP, whereas group II alphabaculoviruses, and beta- and deltabaculoviruses, exploit the F protein as their EFP (Blissard & Wenz, 1992; IJkel et al., 2000; Long et al., 2006; Pearson et al., 2000; Yin et al., 2008). A functional degenerated F homologue (F-like protein) was identified in group I alphabaculoviruses (Lung et al., 2003; Wang et al., 2008a).

The baculovirus F proteins possess common features of class I viral fusion proteins. During the maturation of virions, the F proteins are cleaved post-translationally at their furin cleavage site, and the individual N-terminal F2 subunit and C-terminal subunit F1 are connected by a disulfide bond (IJkel et al., 2000; Long et al., 2006; Lung et al., 2002; Yin et al., 2008). It is generally believed that disulfide bonds and their dynamic rearrangement are critical for the accurate folding and transportation of proteins during synthesis and maturation (Day et al., 2006; Segal et al., 1992). In mature proteins, disulfide bonds play essential roles in stabilizing the structure required for their biological activity (Maar et al., 2012; Markovic et al., 1998; McCaffrey et al., 2012). Mutation of certain cysteine residues in the extracellular domain of the F protein of human respiratory syncytial virus (HRSV) abolished or dramatically reduced cell surface expression and fusion activity of the protein (Day et al., 2006). Cysteine residues in the...
ectodomain of influenza virus haemagglutinin (HA) are critical for efficient folding of the protein during synthesis and stabilize the structure of the matured protein (Segal et al., 1992). For baculovirus GP64 protein, the monomers are linked by a single intermolecular disulfide bond to form a trimer. Disruption of the intermolecular bridge resulted in inefficient transport of GP64 to the cell surface and in the loss of the ability to rescue a gp64-null Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV). In addition, the six conserved intramolecular disulfide bonds are all indispensable for GP64-mediated membrane fusion (Li & Blissard, 2010).

Membrane fusion mediated by baculovirus F proteins is triggered by acidification of the late endosome (IJkel et al., 2000; Pearson et al., 2000). Whether other factors play a role in the activation and conformational change during fusion of baculovirus F protein has not been explored. One potential mechanism to facilitate the conformational change required for membrane fusion is disulfide bond isomerization as suggested by studies of various virus membrane fusion proteins (Abou-Jaoude & Sureau, 2007; Fenouillet et al., 2007; Jain et al., 2007, 2009). One or multiple disulfide bonds in the human immunodeficiency virus (HIV) envelope protein are cleaved by cell-surface-associated protein disulfide isomerase (PDI) after binding to the CD4 receptor – a step which is a prerequisite for the subsequent virus–cell fusion event. Inhibition of the activity of PDI using membrane-impermeable inhibitors, such as free-thiol reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), can inhibit membrane fusion and viral infection (Barbouche et al., 2003; Fenouillet et al., 2007; Ryser et al., 1994). For Sindbis virus, where intact disulfide bonds of the envelope proteins are critical for the stability and function of the viral envelope, treatment with reducing reagents induces virus-mediated fusion and addition of DTNB during infection inhibits virus entry (Abell & Brown, 1993; Anthony et al., 1992). A further case of disulfide reduction during membrane fusion mediated by a viral envelope protein was observed in the case of Newcastle disease virus (NDV). Free thiols are produced in the NDV F protein during fusion and are critical for efficient virus entry into cells. Inhibitors of cell surface-associated thiol/disulfide isomerase, including DTNB, bacitracin and anti-PDI antibody, block F-protein-mediated cell–cell fusion and virus entry (Jain et al., 2007, 2009). The envelope glycoproteins of the pestiviruses bovine viral diarrhea virus also undergo disulfide reduction, which destabilizes the viral envelope during endocytosis to become fusogenic at endosomal acidic pH by an unknown mechanism (Krey et al., 2005).

Baculovirus F proteins have a low overall similarity (20–40% amino acid identity) (Wang et al., 2010). However, alignment of F proteins from group II alphabaculovirus (Ha133, Ld130 and Se8), betabaculovirus (Age25, Pxn26 and Cp31) as well as F-like proteins from group I alphabaculovirus (Ac23 and Op21) revealed that the positions of 11 cysteine residues were highly conserved and the position of one cysteine residue was relatively conserved in the protein (Fig. 1a) (Rohrmann & Karplus, 2001). An enlarged schematic diagram of the F protein of Heliothis armigera nucleopolyhedrovirus (HearNPV) (HaF), which is a group II alphabaculovirus, is shown in parallel in Fig. 1(a) to indicate the cysteine residues selected for mutagenesis in this study. Most of these cysteine residues are located in the membrane-anchored F1 subunit and only one of these is located in the F2 subunit (HaF C108). In F1, except for one highly conserved cysteine residue located at the N terminus (HaF C241) and another located in the cytoplasmic tail domain (CTD) (HaF C623), all the other eight cysteines cluster upstream of the transmembrane domain (TMD) (HaF C358, 365, 393, 403, 418, 436, 471 and 495) and are separated from the N-terminal conserved cysteine residue by an intervening polypeptide. A relatively conserved cysteine residue (HaF C232) can also be found in F1 located either upstream or downstream of the N-terminally conserved C241 (Fig. 1a).

To identify which cysteines form the inter-subunit disulfide bond of HaF, representative cysteine residues including C108, C232, C241 and one of the cysteines (C403) from the cysteine cluster upstream of the TMD were selected for site-directed mutagenesis, and the functions of mutated HaF proteins were investigated (Fig. 1a). Cysteines in the CTD were not selected because in general in F proteins they are fatty acid acylated and not involved in disulfide formation (Schultz et al., 1988). Furthermore, we investigated whether or not thiol/disulfide isomerization of HaF was important for viral entry by using membrane-impermeable thiol/disulfide exchange inhibitors. The results showed that an inter-subunit disulfide bond of HaF is formed between C108 in the F2 subunit and C241. When C241 is mutated, C232 then forms an inter-subunit disulfide bond with C108, but the infectivity is lost. Free thiol/disulfide isomerization of HaF is not likely to play a role in viral entry and infectivity. However, the surface-exposed disulfide bonds of HaF are important for the infectivity of HearNPV to HzAM1 cells.

### RESULTS

#### Functional analysis of cysteine residues of HaF

The cleavage of baculovirus F proteins by furin results in two disulfide-linked subunits (Long et al., 2006; Pearson et al., 2000; Westenberg et al., 2002; Yin et al., 2008). To identify the cysteine residues that participate in the formation of inter-subunit disulfide bonding in HaF, cysteine residues at positions 108, 232, 241 and 403 were mutated to a non-bulky glycine residue individually, and a double mutation at both C232 and C241 was also constructed (Fig. 1a). Mutated HaF genes as well as the WT HaF gene were transposed into the HaBacD bacmid, which resulted in recombinant bacmids HaBacΔF–HaF C108G, HaBacΔF–HaF C232G, HaBacΔF–HaF C241G, HaBacΔF–HaF C232/241G, HaBacΔF–HaF C403G and HaBacΔF–HaF (Fig. 1b, left). These mutants also expressed EGFP as a visible marker to follow infection.
To analyse the function of cysteine residues, recombinant bacmids were transfected into HzAM1 cells and supernatants were used to infect another batch of cells. Green fluorescence was detected to indicate the success of transfection and the spread of infectious budding virus (BV). Fluorescence was detected in all of the transfected cells (Fig. 2a–g), indicating successful transfer of recombinant bacmids. At 5 days post-infection (p.i.), infectious BVs were observed in cells infected with the supernatants of cells transfected with HaBacDF-HaF<sub>C108G</sub>, HaBacDF-HaF<sub>C232G</sub> and HaBacDF-HaF<sub>C241G</sub> (Fig. 2j, m, n). No secondary infection was detected in cells exposed to supernatants that were collected from cells that were transfected with HaBacDF-HaF<sub>C108G</sub> or HaBacDF-HaF<sub>C241G</sub> (Fig. 2i, k). For vHaBacDF-HaF<sub>C232/241G</sub>, although infection was observed, the spread of fluorescence was restricted to a limited number of adjacent cells (Fig. 2l). During the amplification process, BVs of vHaBacDF-HaF<sub>C232G</sub>, vHaBacDF-HaF<sub>C403G</sub> and vHaBacDF-HaF<sub>C232/241G</sub> could be propagated easily in HzAM1 cells; however, the amplification of vHaBacDF-HaF<sub>C232/241G</sub> failed in all the five attempts. The results demonstrated that C108 and C241 were crucial for the function of the HaF gene.

**Fig. 1.** (a) Schematic representation of baculovirus F proteins (adapted from Rohrmann & Karplus, 2001) and enlarged schematic diagram of HaF. The relative positions of the predicted signal peptide (SP), fusion peptide (FP), heptad repeats (HR1, HR2 and HR3), TMD, CTD and proteolytic cleavage site (arrow) are indicated. The positions of highly conserved and relatively conserved cysteine residues are indicated by bold dashed lines; non-conserved cysteine residues are indicated by normal dashed lines. Mutated cysteine residues are underlined. Ha133, Ld130, Se8, Agse25, Px26, Cp31, Ac23 and Op21 represent F proteins of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV; GenBank accession number AF271059), *Lymantria dispar* multiple nucleopolyhedrovirus (AF081810), Spodoptera exigua multiple nucleopolyhedrovirus (AF169823), Agrotis segetum nucleopolyhedrovirus (AY522332), Plutella xylostella granulovirus (AF270937), Cydia pomonella granulovirus (U53466), Autographa californica nucleopolyhedrovirus (L22858) and Orgyia pseudotsugata multiple nucleopolyhedrovirus (U75930), respectively. (b) The strategy to generate the recombinant f-null HearNPV bacmid (left) and the gp64-null AcMNPV bacmid (right). The WT HaF gene and mutated (MT) HaF genes listed were inserted into the polyhedrin locus by Tn7-mediated transposition. All the genes were under the control of the Op166 promoter (pOp166). Mutations in HaF included HaF<sub>C108G</sub>, HaF<sub>C232G</sub>, HaF<sub>C241G</sub>, HaF<sub>C403G</sub> and HaF<sub>C232/241G</sub>.
in rescuing the infectivity of the f-null HaBacmid. As C108 is the only cysteine residue in the F2 subunit of the mature form, it is reasonable to speculate that C108 participates in the inter-subunit disulfide linkage between HaF1 and HaF2.

**Inter-subunit disulfide bond formation in HaF mutants**

As some recombinant HearNPVs with cysteine-mutated HaF failed to produce infectious BVs for further analysis, all of the mutated HaF genes as well as the WT HaF gene were transposed into an AcBacmid carrying a GP64 gene to investigate inter-subunit disulfide bonds in mutated HaF proteins. The resultant recombinant bacmids were designated AcBac-HaF<sup>C108G</sup>, AcBac-HaF<sup>C232G</sup>, AcBac-HaF<sup>C241G</sup>, AcBac-HaF<sup>C232/241G</sup>, AcBac-HaF<sup>C403G</sup> and AcBac-HaF<sup>D</sup> (Fig. 1b, right). Recombinant bacmid DNAs were transfected into Sf9 cells, and BVs carrying both AcGP64 and HaF were purified from the supernatant of infected cells and subjected to Western blot analysis (Fig. 3). Under reducing conditions with antibodies specific for the membrane-anchored subunit HaF<sub>1</sub>, only a band of ~59 kDa was detected for all BVs (Fig. 3a, lanes 1–6). The molecular mass of the bands was consistent with the size of HaF<sub>1</sub>, indicating proper expression and cleavage of all HaF mutants (Long et al., 2006). Under non-reducing conditions, in which disulfide bonds were not disrupted, a band of 80 kDa was observed in vAcBac-HaF<sup>C232G</sup> (Fig. 3b, lane 2), vAcBac-HaF<sup>C241G</sup> (Fig. 3b, lane 3), vAcBac-HaF<sup>C403G</sup> (Fig. 3b, lane 5) and vAcBac-HaF<sup>D</sup> (Fig. 3b, lane 6), which corresponded to the size of F2 subunit linked with the F1 subunit (designated F1<sub>1</sub>+) (Long et al., 2006). For both vAcBac-HaF<sup>C108G</sup> (Fig. 3b, lane 1) and vAcBac-HaF<sup>C232/241G</sup> (Fig. 3b, lane 4), bands corresponding to the only size of F1 were observed, indicating the absence of any disulfide linkage between F1 and F2, but also incorporation into BVs as such. The result for vAcBac-HaF<sup>D</sup> was consistent with the supposition that C108 is the residue used in the linking the F2 subunit to the F1 subunit. Although the C241 residue is essential for HaF to rescue the infectivity of vHaBacAF (Fig. 2d, k), an inter-subunit disulfide linkage was detected in the HaF<sup>C241G</sup> protein (Fig. 3b, lane 3). The presence of the AcMNPV EFP GP64 and the major capsid protein VP39 in BVs of the recombinants was confirmed by SDS-PAGE under reducing conditions followed by Western blot analysis using antibodies against AcMNPV GP64 and VP39, respectively (Fig. 3c, d). It seemed that the expression of HaF or mutated HaF proteins disturbed the expression or insertion of GP64. When equal amounts of infectious BVs were analysed based on the capsid protein (Fig. 3d), higher levels of HaF or mutated HaF proteins correlated with a lower GP64 quantity in the same virus (Fig. 3a–c).

BV<sub>s</sub> of vHaBacAF-HaF<sup>C232G</sup>, vHaBacAF-HaF<sup>C403G</sup> and vHaBacAF-HaF<sup>D</sup> harvested from the supernatants of infected HzAM1 cells were also analysed in parallel (Fig. 3e, f, lanes 7–9), and the circumstances of the major inter-subunit disulfide bond formation were consistent with that of HaF expressed in WT AcMNPV BVs (Fig. 3b, lanes 2, 5 and 6). For vAcBac-HaF<sup>C108G</sup> under non-reducing conditions, besides the monomer of the F1 subunit, bands of ~120 and 180 kDa were observed, the apparent size of which was consistent with a possible dimer and trimer, although this would need to be confirmed (Fig. 3b, lane 1), suggesting intermolecular disulfide linkages of F1 subunits. It is possible that in the absence of C108 in the F2 subunit, most C241 residues form intermolecular disulfide linkages with cysteine residues of another F1 instead of forming intramolecular disulfide bonds with other cysteine residues within F1. Bands with higher molecular mass ~160 kDa, which might be a dimer of F1<sub>1</sub>+, were also observed in HaF<sup>C241G</sup> and HaF; for HaF<sup>C232G</sup> such a band was at 150 kDa (Fig. 3b, f). The original blots are shown in Fig. S1 (available in the online Supplementary Material).

**Production of infectious BVs and genomic DNA**

To evaluate the role of cysteine residues in HaF in BV production in more detail, one-step growth curve analysis was performed on vHaBacAF-HaF<sup>C232G</sup>, vHaBacAF-HaF<sup>C403G</sup> and vHaBacAF-HaF<sup>D</sup> (control). HzAM1 cells were infected with the respective BVs at m.o.i. 10 and supernatants were collected at the indicated time points. Titres of the progeny virus were determined by end-point dilution assays (EPDAs). Statistical analysis showed that at
48, 72 and 96 h p.i., infectious BV production of the three recombinant viruses was significantly different (P < 0.05) (Fig. 4). Initially, at 24 h p.i., the BV production of vHaBacΔF-HaF C232G was significantly higher than the control virus vHaBacΔF-HaF (P = 0.046), whilst no significant difference was found between vHaBacΔF-HaF C403G and the control virus (P = 0.96) (Fig. 4). These results indicated that the mutation at C232 increased the ability of HaF to rescue the infectivity of an f-null HaBacmid. In contrast, mutation at C403 impaired infectious viral production.

To further investigate whether the differences in viral titre were due to variation in genomic DNA production or in viral infectivity, quantitative real-time (qRT)-PCR analysis was performed. Primer pair Halef8-F/Halef8-R was designed to amplify a 64 bp fragment within the conserved LEF-8 gene of HearNPV. Samples collected at 72 h p.i. in the one-step growth curve assay were evaluated. The results showed that genomic DNA production of vHaBacΔF-HaF C232G and vHaBacΔF-HaF C403G was approximately equivalent, and ~1 log unit higher than that of the control vHaBacΔF-HaF (Table 1). For vHaBacΔF-HaF C403G, 1 TCID₅₀ unit was equivalent to 1.8 × 10⁶ copies of viral genomic DNA, and for vHaBacΔF-HaF C232G and vHaBacΔF-HaF 1 TCID₅₀ unit was equivalent to ~10³ copies of viral genomic DNA (Table 1). When corrected for the presence of viral genomes, there was no significant difference in productivity between vHaBacΔF-HaF C232G and vHaBacΔF-HaF (P = 0.057), but the productivity of vHaBacΔF-HaF C403G was 10-fold lower than vHaBacΔF-HaF C232G and vHaBacΔF-HaF (P < 0.001).

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**Fig. 3.** Western blot analysis of protein expression in recombinant virions collected from infected cells. (a, b) The expression and post-translational cleavage of HaF mutants HaF C108G (lane 1), HaF C232G (lane 2), HaF C241G (lane 3), HaF C232/241G (lane 4), HaF C403G (lane 5) and HaF (lane 6) in recombinant AcMNPV BVs were detected under reducing conditions (a) and non-reducing conditions (b) with anti-HaF₁ antiserum. (c, d) The expression levels of GP64 and nucleocapsid protein VP39 (internal control) in recombinant AcMNPV BVs were examined with anti-GP64 and anti-VP39 antiserum, respectively. (e, f) The vHaBacΔF-HaF C232G, vHaBacΔF-HaF C403G and vHaBacΔF-HaF BVs were analysed in parallel. The positions of F₁ and F₁+2 are indicated.

**Fig. 4.** One-step growth curve analysis of infectious BV production. HzAM1 cells were infected at m.o.i. 10 with either vHaΔF-HaF C232G, vHaΔF-HaF C403G or vHaΔF-HaF BVs. Supernatants were harvested at the indicated time points post-infection and infectious BV yield was determined by EPDAs in HzAM1 cells. Data represent the mean ± SD titres from triplicate infections.
Inhibitory effect of thiol/disulfide exchange inhibitors on the infectivity of HearNPV to HzAm1 cells

To investigate the role of cysteines and thiol/disulfide exchange during viral entry, infection assays were conducted in the presence of inhibitors of thiol/disulfide isomerization. Three membrane-impermeable inhibitors were chosen in our study: tris(2-carboxyethyl) phosphine (TCEP) is a reducer, and both 4-acetamido-4′-maleimidodisulfobenzene 2,2′-disulfonic acid (AMS) and DTNB are free-thiol reaction reagents. All three drugs were used at 1 and 2.5 mM, i.e. concentrations that were reported not to cause cytotoxicity (Abou-Jaoude & Sureau, 2007). To check the effect of selected drugs on cell metabolism and viral replication, control experiments were conducted in which the drugs were added to the cell supernatant after 2 h of virus exposure to the cells. Cells were then incubated with the drugs for 2 h, after which the medium was replaced with Grace’s medium containing 10% FBS. The proportion of infected cells was quantified 24 h p.i. using flow cytometry. The infection rates of groups did not show any dramatic difference compared with the group without any inhibitors (~95% for TCEP, 112% for DTNB and 115% for AMS), indicating that none of the drugs interfered with cell metabolism or HearNPV replication. To investigate the role of thiol/disulfide exchange at the stage of viral entry, HzAM1 cells were incubated with vHaBacAF-HaF in the presence of TCEP, DTNB or AMS and left for a 2 h virus–cell interaction period. The inocula were then removed and fresh Grace’s medium with 10% FBS was added. The infection rate of the control, which was not treated with any inhibitor, was set as 100%. As shown in Fig. 5(a), TCEP dramatically reduced viral entry in a dose-dependent manner, indicating a significant inhibitory effect, in contrast to the situation when free-thiol reaction reagents (DTNB and AMS) were used, where no obvious inhibitory effect was observed. These results indicated that disulfide bonds, but not free thiol groups, play critical roles in the entry of HearNPV into HzAM1 cells.

The roles of surface-exposed disulfide bonds and free-thiol groups on virion particles were investigated by treating virions with individual inhibitors for 2 h at room temperature. After treatments, viruses were diluted 100-fold in culture medium and inoculated to HzAm1 cells. At 2 h p.i., the supernatants were removed and replaced with fresh medium. The inhibition relationships were very similar to the above assays in that TCEP showed a significant inhibitory effect on viral infectivity, whilst neither DTNB nor AMS reduced viral infectivity compared with the control (Fig. 5b). These results implied that it was not the free thiol groups on the surface of vHaBacAF-HaF BVs, but the disulfide bonds within HaF that were important for viral infectivity. To investigate whether cell surface-associated PDI is involved in viral entry, HzAM1 cells were treated with individual drugs for 2 h at 27 °C. Inhibitors were then removed, and the cells were inoculated with vHaBacAF-HaF and left for a 2 h to allow cell–virus interaction. As shown in Fig. 5(c), no significant difference was observed between drug- and mock-treated groups, indicating that no disulfide bonds or free-thiol groups on the surface of host cells were necessary for viral entry.

DISCUSSION

As a member of the class I EFPs, the F protein of baculovirus is cleaved post-translationally into an N-terminal F2 subunit and a C-terminal, membrane-anchored F1 subunit by the cellular protease furin, and the two F subunits are linked by a disulfide bond (Westenberg et al., 2002). Cysteine residues are highly conserved among baculovirus F proteins, suggesting that they are important to the structure and function of the proteins. In this study, the identity and importance of disulfide bonds of HaF were investigated by mutagenesis of cysteine codons to glycine codons and the functions of the altered F proteins were analysed via rescue of the infectivity of an f-null HaBacmid. The role of thiol/disulfide exchange in viral entry and infectivity was also explored using membrane-impermeable thiol/disulfide exchange inhibitors.

The results demonstrated that a functional disulfide bond was formed between the F1 and F2 subunits of HaF between the only cysteine residue, C108, in the F2 subunit and C241, the most N-terminally conserved cysteine residue in the F1 subunit (Fig. 6a, solid line). Mutation of C108 in F1 completely abolished disulfide bond formation and the generation of infectious BVs. When C241 was mutated,
however, the inter-subunit disulfide bond between F1 and F2 was not destroyed, but the resulting protein failed to rescue the infectivity of the f-null HaBacmid. Inter-subunit disulfide bonds were no longer detected with the double mutation at both C241 and C232 (Fig. 6e). These results indicate a partial rescue effect of C241 by C232 in the formation of the inter-subunit disulfide bond.

Interestingly, a mutation at C232 increased infectious BV production by more than five times (Fig. 4, Table 1). Whether C232 plays a role in the synthesis of HaF and whether the linkage between C108 and C232 exists in the WT virus are currently unknown. Considering the existence of the residue in similar positions in almost all baculovirus F proteins, even in the F-like protein of group I nucleopolyhedroviruses (Fig. 1a), we propose that C232 plays an important role in F protein synthesis and/or function. Its potential importance needs to be addressed further.

The organization of cysteine residues and functional motifs of the baculovirus F protein shares large similarities with paramyxovirus F proteins when compared with other class I viral EFPs, such as influenza virus HA and HIV-1 envelope proteins (Fig. 6a, g) (Baker et al., 1999; Iwata et al., 1994; Lamb & Jardetzky, 2007). Assignment of disulfide bridges in the F protein of Sendai virus, a member of the family Paramyxoviridae, demonstrated that cysteine residues clustered upstream of the TMD form intra-subunit disulfide bonds and were supposed to contribute to the

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**Fig. 5.** Inhibition assay of HearNPV with thiol/disulfide exchange inhibitors. (a) Inhibitory effect of inhibitors on viral entry. HzAm1 cells were incubated with vHaBacΔF-HaF at m.o.i. 1 in Grace’s medium in the presence of TCEP, DTNB or AMS at concentrations of 1 or 2.5 mM for 2 h. The inocula were then removed and fresh Grace’s medium with 10% FBS was added. (b) Infection assay with vHaBacΔF-HaF virions pre-treated with inhibitors prior to inoculation. vHaBacΔF-HaF particles were treated with TCEP, DTNB or AMS at concentrations of 1 or 2.5 mM for 2 h at room temperature, and then 1:100 diluted in culture medium and inoculated to HzAm1 cells at m.o.i. 1. At 2 h p.i., the supernatants were removed and replaced with fresh medium. (c) Infection assay with HzAM1 cells pre-treated with inhibitors. HzAM1 cells were incubated with each inhibitor at concentrations of 1 or 2.5 mM for 2 h at 27°C. Supernatants with inhibitors were removed before inoculation with vHaBacΔF-HaF. After a 2 h cell–virus exposure period, the inocula were replaced with normal Grace’s culture medium. For all these assays, the infection rates were quantified at 24 h p.i. by flow cytometry. Each infection was performed in triplicate and the infection rate without inhibitors was set as 100%.
formation of a bunched structure (Iwata et al., 1994). Investigation of cysteine residues of the F protein of HRSV, a member of the subfamily Pneumovirinae within the family Paramyxoviridae, revealed that most of the cysteine residues in the cysteine cluster are critical for folding, transport and fusion activity of the protein (Day et al., 2006). For HaF, the mutation at C403, which is one of the cysteine residues in the cysteine cluster, decreased viral infectivity 10 times as compared with WT HaF (Figs 4 and 6f). It is reasonable to speculate that cysteine residues clustered upstream of the TMD of HaF form intra-subunit disulfide bonds, and play important roles in the synthesis, processing and functional structural stability of HaF.

For several viruses, such as NDV, which enter cells by fusion of the viral membrane to the plasma membrane, the activation of fusogenicity depends on the precise thiol/disulfide rearrangements (Abell & Brown, 1993; Jain et al., 2007, 2009). For other viruses, such as influenza virus, although the conformational changes needed to activate fusogenicity are triggered mainly by the acidic milieu, the exchange of thiol/disulfide groups still plays certain roles in the process of membrane fusion (Carr et al., 1997; Markosyan et al., 2001). No disulfide linkage was detected in the oligomerization of the baculovirus F protein and the protein was detected by chemical cross-linking to form homotrimers in which the monomers are associated by non-covalent interactions (Long et al., 2006).

In the current study, free-thiol groups in F proteins were not thought to play important roles in viral entry and infectivity, as evidenced by inhibition assays with free-thiol reaction reagents (Fig. 5a, b). These results reduce the likelihood that thiol/disulfide exchange plays a critical role.
in the conformational change of F proteins. The observation that inhibition of the activity of cell surface-associated PDI did not inhibit viral entry provides further support for this hypothesis (Fig. 5c). However, there is a possibility that functional free-thiol groups or disulfide bonds of PDI on the host cell plasma membrane are not accessible to the impermeable inhibitors. The observation that the reductant TCEP dramatically reduces the infectivity of HearNPV is possibly due to the breakage of disulfide bonds that are essential for the structural stability of HaF. Further detailed studies are needed to address whether thiol/disulfide exchange plays a role in baculoviral entry.

The high conservation of cysteine residues is a striking feature of baculovirus F proteins. In this study, we demonstrated that an inter-subunit disulfide bond of the F protein subunits of HearNPV is formed by C108 and C241, C232, the location of which is not highly conserved amongst baculovirus F proteins, appears to form a disulfide bond with C108 in the absence of C241, but this does not compensate functionally for the natural C108–C241 disulfide bond. We also detected a cysteine residue, C403, which may have the potential to form intra-subunit disulfide bonds in the F1 subunit on the basis of the structural similarity with paramyxovirus F proteins (Dutch et al., 2000). The observation that free-thiol groups are not involved in the F-protein-mediated entry of baculovirus in insect cells indicates that successful entry of BV is entirely dependent on the inter- and intra-subunit sulfur bridging. These results may also contribute to a further understanding of the structure–function relationship of the baculovirus F protein. Crystallization of a baculovirus F protein is required to substantiate the structure–function claims as discussed in this paper.

METHODS

Insect cells, virus and reagents. The HzAM1 and S9 cell lines were cultured at 27 °C in Grace’s medium (Gibco-BRL) supplemented with 10 % FBS, pH 6.0. The HearNPV bacmid (HaBacH28) and f-null bacmid HaBacAF used in the experiment were constructed previously in our laboratories (Wang et al., 2003; Wang et al., 2008b). TCEP and DTNB were purchased from Sigma. AMS was purchased from Invitrogen Molecular Probes.

Construction of recombinant bacmids. For the construction of donor plasmids, the HaF gene was PCR amplified from the HaBacH28 template with primers HaF-F and HaF-R using Pyrobest DNA polymerase (Takara). The PCR product was cloned into pGEM-T Easy vector (Promega) to generate pT-HaF. Following sequence confirmation, the HaF gene fragment was digested away from pT-HaF and then inserted into pFastBac1-Op166 (Wang et al., 2008b) to generate donor plasmid pFastBac1-Op166-HaF. Mutated HaF genes were constructed by overlapping extension PCR as reported by Ho et al. (1989). Briefly, mutated HaF genes were first PCR amplified as two fragments with pT-HaF as template. Each of the N-terminal domains was amplified with primer HaF-F and the N-terminal reverse mutagenic primer. For the C-terminal domain, PCR was performed with C-terminal forward mutagenic primer and HaF-R. For example, the primer pair used to generate the N-terminal domain of the mutated HaF<sup>C108G</sup> gene was HaF-F/HaF<sub>C108G</sub>-NR (Table S1) and the primer pair used to generate the C-terminal domain was HaF<sup>C108G</sup>-CF/HaF-R (Table S1). The other HaF gene mutants, HaF<sup>C232G</sup>, HaF<sup>C241G</sup> and HaF<sup>C403G</sup>, were amplified similarly with the respective primers listed in Table S1. The two PCR products were then annealed together and primer pair HaF-F/HaF-R was used to generate complete mutated HaF genes. Mutated HaF gene fragments were then cloned into pGEM-T Easy vector, and designated pT-HaF<sup>C108G</sup>, pT-HaF<sup>C232S</sup>, pT-HaF<sup>C241G</sup> and pT-HaF<sup>C403G</sup>. The double-cysteine mutated HaF gene pHaF<sup>C232C241G</sup> was constructed according to the method described for single-residue mutation, but using pT-HaF<sup>C232G</sup> as template, and the primers were the same as used for generating the HaF<sup>C241G</sup> gene mutant. All of the HaF gene mutants were verified by sequencing and then inserted into pFastBac1-Op166 to generate donor plasmids.

Mutated HaF genes and the WT HaF gene under the control of the Orgyia pseudotsugata multiple nucleopolyhedrovirus GP64 gene promoter (Op166) were transposed into both HaBacAF (Fig. 1b) and AcBacmid (Fig. 1c) by Tin<sup>7</sup>-mediated transposition according to the Bac-to-Bac Baculovirus Expression System manual (Gibco-BRL). Colonies resistant to both tetracycline and kanamycin were further confirmed by PCR with M13/pUC-F and M13/pUC-R primers.

Transfection and infection assay. Samples of 3 μg of each recombinant HearNPV bacmid DNA were transfected into 5 × 10<sup>5</sup> HzAM1 cells using 12 μL Lipofectin reagent (Invitrogen). Supernatants were harvested at 5 days post-transfection. After centrifugation at 1000 g for 5 min, 0.5 ml supernatant was used to infect another batch of HzAM1 cells. The percentage of transfected and infected cells was monitored using fluorescence microscopy. For BV amplification, 5.0 × 10<sup>5</sup> HzAM1 cells were infected at m.o.i. 0.1 TCID<sub>50</sub> units per cell and the supernatant was collected at 5 days p.i. Each of the transfections was performed in triplicate. The transfection and infection assays of recombinant AcBacmids were performed on S9 cells using a similar procedure.

Western blot analysis. BVs used for Western blot analysis were collected from infected cell culture supernatants and purified by ultracentrifugation through a 25 % sucrose cushion. Samples were disrupted in Laemmli buffer for electrophoresis under reducing conditions or in buffer containing 10 mM Tris/HCl (pH 6.8), 0.5 % SDS, 10 % glycerol, 50 mM iodoacetamide and 0.001 % bromophenol blue for electrophoresis under non-reducing conditions. Proteins were separated by 10 % SDS-PAGE and then electroblotted onto Immobilon-P membranes (Millipore). Western blot analysis was performed with various polyclonal antibodies: anti-HaF<sub>D</sub>, anti-HaF<sub>C403G</sub>, anti-GP64 (diluted 1 : 2000; Wang et al., 2008a) and anti-VP39 (diluted 1 : 1000; Wang et al., 2008a). Detection of antibody binding was visualized by alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies (NovoGene Biosciences). The final signals were detected with nitro-blue tetrazolium and BCIP.

One-step growth curve analysis. To perform one-step growth curve analysis, 5 × 10<sup>5</sup> HzAM1 cells were incubated with vHaA<sub>F-HaF<sup>C232G</sup></sub>, vHaA<sub>F-HaF<sup>C403G</sup></sub> or vHaA<sub>F-HaF</sub> at m.o.i. 10 TCID<sub>50</sub> units per cell for 1.5 h at 28 °C. Cells were then washed for three times with Grace’s medium before addition of 2 ml fresh Grace’s medium (supplemented with 10 % FBS). Supernatants were harvested at 0, 12, 24, 48 and 72 h p.i., and, after clarification by centrifugation, infectious BV yields were determined by EPDAs using HzAM1 cells. The infection experiments were performed in triplicate, and the mean BV titres were analysed using one-way ANOVA (SPSS) with virus type and time as factors.

qRT-PCR. To determine the differences of genomic DNA production among recombinant viruses by qRT-PCR, primers Hale8-F and Hale8 were designed to amplify a 64 bp fragment within the LEP-8
gene. Aliquots of 100 µl supernatant were collected in each sample of the one-step growth curve infections at 72 h p.i. and genomic DNA was extracted as described by Wang et al. (2010). qRT-PCR was carried out with EvaGreen fluorescent dye (Biotium) using 40 cycles of 95 °C for denaturation and 53 °C and 72 °C for annealing/extension.

Infection of HzAm1 cells with thiol/disulfide exchange inhibitors. To test the effect of selected drugs on cell metabolism and viral replication, cells were inoculated with vHaBacΔF-HaF at m.o.i. 1 TCID₅₀ unit per cell for 2 h, and drugs were then added to the supernatant and incubated for another 2 h. The supernatant was then replaced with fresh Grace’s medium and left for 24 h before quantification by flow cytometry. The inhibitory effects of TCEP, DTNB and AMS on viral infectivity of vHaBacΔF-HaF were tested during different stages of infection. (i) Inhibitory effect of inhibitors on viral entry. HzAm1 cells were inoculated with vHaBacΔF-HaF at m.o.i. 1 TCID₅₀ unit per cell in the presence of each inhibitor at a final concentration of 1 or 2.5 mM and left for 2 h. (ii) Pre-treatment of virions with inhibitors. vHaBacΔF-HaF BVs were pre-treated with each inhibitor for 2 h at room temperature, and then diluted 100-fold in culture medium and inoculated to HzAm1 cells at m.o.i. 1 TCID₅₀ unit per cell. (iii) Pre-treatment of cells with inhibitors. HzAm1 cells were pre-incubated with each inhibitor for 2 h at 27 °C; supernatants with inhibitors were then removed and the cells were inoculated with vHaBacΔF-HaF at m.o.i. 1 TCID₅₀ unit per cell. Infections without any inhibitor were set up as control. For all the infections, after a 2 h cell–virus exposure period at 27 °C, the inocula were removed and normal Grace’s medium with 10% FBS was added. The infection rates were quantified at 24 h p.i. by flow cytometry.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (31215003, 31100120, 31300191, 3130058 and 31200124), National Basic Research Program of China (2010CB530300) and Programme Strategic Scientific Alliances between China and The Netherlands (2008DBFB3020). The authors would like to thank Dr Basil M. Arif for the scientific editing of the manuscript. We thank Professor Kai Yang for providing anti-VP39 antiserum, Dr Xiulian Sun for statistical analysis and Ms Yanfang Zhang for cell culture.

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