INTRODUCTION

The family Baculoviridae is a large family of pathogens that infect arthropods, particularly insects of the Lepidoptera. Nucleopolyhedroviruses (NPVs), a genus of family Baculoviridae, have a large circular, supercoiled and double-stranded DNA genome packaged into rod-shaped virions. NPVs produce two types of virions during the infection cycle to bring about efficient viral replication within infected insect larvae and to spread virus from insect to insect. Occlusion-derived virus (ODV) transmits infection to neighbouring cells (Federici, 1997). The end of infection is marked by a dramatic degradation (or melting) of the host cadaver. By this process, the dispersal of occlusion bodies (OBs) containing several hundred ODVs is facilitated, resulting in horizontal transmission in nature.

Baculoviruses possess a number of auxiliary genes that presumably provide an evolutionary advantage to baculoviruses. It has been reported that baculovirus-encoded auxiliary genes are utilized to control host physiology, development, and behaviour, at both the cellular and organismal levels (O’Reilly, 1997). In Autographa californica NPV (AcMNPV) and Bombyx mori NPV (BmNPV), two auxiliary genes, cathepsin (v-cath) and chitinase (v-chiA), have been shown to be required for post-mortem host degradation (Slack et al., 1995; Ohkawa et al., 1994; Hawtin et al., 1997; Katsuma et al., 2004b). V-CATH resembles the lysosomal protease, cathepsin L, in sequence (Slack et al., 1995). The substrate specificity of V-CATH is closer to that of cathepsin B than cathepsin L even though the sequence of V-CATH is more closely similar to that of cathepsin L (Brömmel & Okamoto, 1995). Also, it has been reported that V-CHIA possesses both exo- and endochitinolytic activities (Hawtin et al., 1995; Daimon et al., 2007), and the deletion of v-chiA from the virus results in blocking host degradation after death (Hawtin et al., 1995; Katsuma et al., 2004b).

Recent studies also showed that inactivation of v-chiA resulted in the accumulation of insoluble V-CATH within virus-infected cells (Hom & Volkman, 2000; Daimon et al., 2007), proposing an additional role for V-CHIA as a molecular chaperone during pro-V-CATH processing. Also, V-CATH activity was not detected in v-chiA-inactivated NPV-infected cells (Hom & Volkman, 2000; Katsuma et al., 2004b; Daimon et al., 2007), suggesting that reduced host degradation by infection with a v-chiA-inactivated NPV is mainly due to the loss of V-CATH activity, but not to the loss of chitinase activity of V-CHIA.

N-linked glycans located in the pro-region of Bombyx mori nucleopolyhedrovirus V-CATH are essential for the proper folding of V-CATH and V-CHIA

Susumu Katsuma, Tadashi Nakanishi, Takaaki Daimon and Toru Shimada

Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

Post-mortem host degradation by infection of Bombyx mori nucleopolyhedrovirus (BmNPV) requires the synergistic activation of two virus-encoded genes, cathepsin (v-cath) and chitinase (v-chiA). Recent studies also showed that inactivation of v-chiA results in the accumulation of insoluble V-CATH within virus-infected cells (Hom & Volkman, 2000; Daimon et al., 2007), proposing an additional role for V-CHIA as a molecular chaperone during pro-V-CATH processing. Also, V-CATH activity was not detected in v-chiA-inactivated NPV-infected cells (Hom & Volkman, 2000; Katsuma et al., 2004b; Daimon et al., 2007), suggesting that reduced host degradation by infection with a v-chiA-inactivated NPV is mainly due to the loss of V-CATH activity, but not to the loss of chitinase activity of V-CHIA.

Post-mortem host degradation by infection of Bombyx mori nucleopolyhedrovirus (BmNPV) requires the synergistic activation of two virus-encoded genes, cathepsin (v-cath) and chitinase (v-chiA). Previous studies have suggested that V-CHIA is essential for the proper folding of the nascent V-CATH polypeptide in the endoplasmic reticulum, and that the putative V-CHIA–V-CATH interaction might be mediated by N-linked glycans of V-CATH. Sequence analysis shows that BmNPV V-CATH includes three consensus N-linked glycosylation sites (asparagine 38, 65 and 158). To clarify the role of N-linked glycans of V-CATH in its biological activity, we generated three recombinant BmNPVs expressing mutant V-CATHs, and found that the two residues, asparagine 38 and 65, which are localized in the pro-region of V-CATH, are the glycosylation sites of BmNPV V-CATH. Western blot analysis also showed that removal of N-linked glycans from BmNPV V-CATH resulted in production of the insoluble forms of V-CATH and V-CHIA. These results demonstrate that N-linked glycans located in the pro-region of BmNPV V-CATH are essential for the proper folding of V-CATH and V-CHIA.

Post-mortem host degradation by infection of Bombyx mori nucleopolyhedrovirus (BmNPV) requires the synergistic activation of two virus-encoded genes, cathepsin (v-cath) and chitinase (v-chiA). Previous studies have suggested that V-CHIA is essential for the proper folding of the nascent V-CATH polypeptide in the endoplasmic reticulum, and that the putative V-CHIA–V-CATH interaction might be mediated by N-linked glycans of V-CATH. Sequence analysis shows that BmNPV V-CATH includes three consensus N-linked glycosylation sites (asparagine 38, 65 and 158). To clarify the role of N-linked glycans of V-CATH in its biological activity, we generated three recombinant BmNPVs expressing mutant V-CATHs, and found that the two residues, asparagine 38 and 65, which are localized in the pro-region of V-CATH, are the glycosylation sites of BmNPV V-CATH. Western blot analysis also showed that removal of N-linked glycans from BmNPV V-CATH resulted in production of the insoluble forms of V-CATH and V-CHIA. These results demonstrate that N-linked glycans located in the pro-region of BmNPV V-CATH are essential for the proper folding of V-CATH and V-CHIA.
AcMNPV V-CATH has been shown to be modified with N-linked glycans (Slack et al., 1995; Hom & Volkman, 2000). Hom & Volkman (2000) reported that, when Sf-9 cells infected with AcMNPV were treated with tunicamycin to block N-linked glycosylation, no proteolytic processing of pro-V-CATH was observed. This non-glycosylated form of pro-V-CATH was detected in the insoluble fraction of infected cells, as was the case with pro-V-CATH produced in v-chiA-inactivated AcMNPV-infected cells. Together with these results, it was proposed that the putative V-CHIA–V-CATH interaction might be mediated by N-linked glycans of V-CATH, although the positions of N-linked glycans on V-CATH have not been determined.

Amino acid sequence analysis of V-CATHs from Group I NPVs shows that V-CATH includes two consensus N-linked glycosylation sites, asparagine 65 and 158, whereas BmNPV has two sites in the pro-region, and one in the mature region (Fig. 1b). Here we report the role of N-linked glycans of BmNPV V-CATH. We generated three recombinant BmNPVs expressing mutant V-CATHs, and identified two residues, asparagine 38 and 65, as the glycosylation sites of BmNPV V-CATH. Biochemical experiments showed that N-linked glycans are required for the proper production and activation of BmNPV V-CATH. We also observed that mutation of either of two of the glycosylation sites, but not the deletion of v-cath, resulted in the formation of insoluble V-CHIA. This suggests that non-glycosylated V-CATH inhibits the function of V-CHIA, probably due to co-aggregation of V-CATH and V-CHIA.

**METHODS**

Insect, cell lines and viruses. B. mori larvae were reared as described previously (Katsuma et al., 2006a). The BmN-4 (BmN) and SF-9 cells were cultured at 27°C in TC-100 medium supplemented with 10% fetal bovine serum. BmNPV T3 and AcMNPV C6 were used as wild-type (wt) viruses. A v-cath deletion mutant BmCysPD was reported previously (Ohkawa et al., 1994). Virus titres of BmNPV and AcMNPV were determined by plaque assay on BmN and SF-9 cells, respectively (Katsuma et al., 2006a).

Construction of plasmids containing mutant v-caths. The 3.76 kb fragment of the BmNPV T3 genome containing v-cath and v-chiA (nt 96909–100668; GenBank accession no. L33180) was amplified by PCR with the primers vcatf1 and vchat2 and cloned into pcDNA3.1(−) (Invitrogen), and designated wt/pDNA. To construct plasmids containing mutant v-caths, two-step PCR-based mutagenesis was performed using BmNPV T3 genomic DNA as a template (Katsuma et al., 2006b). Primers used in the mutagenesis experiments are shown in Supplementary Table S1. Three mutant plasmids (N38D/pDNA, N65D/pDNA and N158D/pDNA) containing mutant v-caths were constructed. The nucleotide sequence was confirmed using an ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI Prism 3100 DNA sequencer (Applied Biosystems).

Generation of recombinant BmNPVs expressing mutant V-CATHs. The pcDNA3.1-based plasmids (wt/pDNA, N38D/pDNA, N65D/pDNA and N158D/pDNA) were cotransfected with the Bsu36I-digested BmCysPD genomic DNA into BmN cells as described previously (Katsuma et al., 2006a). Five days after transfection, the medium was collected and stored at 4°C until use. Recombinants identified as white plaques were isolated by plaque assay with agarose overlays containing 400 μg X-Gal per 60 mm dish. In order to confirm whether the replacement of lacZ with the mutated v-caths was correctly performed, the v-cath gene regions of isolates with white plaque phenotype were amplified by PCR using the cathORF1 and cathORF2 primers (Supplementary Table S1, Fig. 1b, and Supplementary Fig. S2), cloned into the pGEM-T Easy vector (Promega) and sequenced by commercial primers.

Western blot analysis of V-CATH and V-CHIA. BmN cells were infected with T3 or recombinant BmNPVs at an m.o.i. of 5. At 3, 4 and 5 days post-infection (p.i.), the cells and medium were collected and subjected to Western blot analysis using antibodies against BmNPV V-CATH and B. mori BmCHI-h as described previously (Daimon et al., 2007).

**Cysteine protease assay.** Cysteine protease activity was examined as described previously (Katsuma et al., 2004b). Haemolymph was collected from virus-infected B. mori larvae at 5 days p.i., centrifuged at 20,000 g for 10 min at 4°C, and the supernatants were used immediately for cysteine protease assays (30 μl/assay). Protease activity was measured in an azocasein assay. BmNPV-infected BmN cells were collected at 5 days p.i., resuspended in 100 μl PBS, and...
sonicated with Sonifier 250 (Branson). Each 83 μl aliquot was used immediately to measure proteolytic activity in an azocasein assay.

Assays for BV production. For the virus growth curves, BmN cells were infected with wt or mutant BmNPVs at an m.o.i. of 5. After 1 h incubation, virus-containing culture medium was removed and fresh medium was added after two washes with serum-free TC-100 medium (0 h p.i.). A small amount of culture medium was harvested at 48 h p.i. BV production was determined by plaque assay.

OB production in B. mori larvae and BmN cells. Fifth instar B. mori larvae were starved for several hours, injected with 50 μl of a viral suspension containing 1 × 10^8 p.f.u. and returned to the artificial diet at 27 °C. At 4 days p.i., haemolymph of infected larvae was collected and released OBs were counted using a haemocytometer as described previously (Daimon et al., 2007). BmN cells infected with T3 or recombinant BmNPVs at 6 days p.i. were gently scraped with a rubber policeman, and released OBs were counted using a haemocytometer. Total OB production was measured as described elsewhere (Hong et al., 2000).

Statistical analysis. One-way analysis of variance (ANOVA) was used to evaluate treatment effects. If the ANOVA value was significant, comparisons between the control and treatment group were performed using ANOVA followed by Dunnett’s test to localize the significant difference. A P value of less than 0.05 was considered significant. All statistics were run with InStat 2.00 (GraphPad Software).

RESULTS

Positions of N-linked glycans on BmNPV V-CATH

The deduced amino acid sequence of BmNPV V-CATH includes three consensus N-linked glycosylation sites [asparagine 38 (N38), N65 and N158], two of which are localized in the pro-region and the other in the mature region of V-CATH (Fig. 1a). Sequence analysis of V-CATHs from group I NPVs showed that N65 and N158 are completely conserved, but N38 is not conserved among group I NPVs (Supplementary Fig. S1). Previous studies using tunicamycin, which blocks N-linked glycosylation, have shown that BmNPV V-CATH might possess N-linked glycans in BmNPV-infected BmN cells (Daimon et al., 2007). To determine the positions of N-linked glycans of BmNPV V-CATH, we constructed three recombinant BmNPVs (BmCysN38D, BmCysN65D and BmCysN158D) expressing V-CATH derivatives, N38D (asparagine 38 mutated to aspartic acid), N65D and N158D, by site-directed mutagenesis (Fig. 1b). A v-cath repair virus, BmCysPDR, was also constructed as a control virus (Fig. 1b). We confirmed the correct replacement of lacZ with the mutated v-caths by PCR (Supplementary Fig. S2) and DNA sequencing (data not shown).

We examined V-CATH expression in wild-type (T3 strain) or recombinant BmNPV-infected BmN cells in either NP-40-soluble or insoluble fractions since V-CATH was detected predominantly in the insoluble fraction of cells infected with a BmNPV lacking functional v-chiA (Daimon et al., 2007). As shown in Fig. 2 and Supplementary Fig. S3, Western blot analysis using anti-V-CATH antiserum showed that BmNPV T3 expressed three forms of pro-V-CATH (35, 33 and 30 kDa) and two forms of putative mature V-CATH (22 and 22 kDa) in the soluble fraction of BmN cells. As reported previously (Daimon et al., 2007), V-CATH was detected predominantly in the soluble fraction of BmN cells. We observed that the band migrating slowest, which seemed a fully glycosylated form of pro-V-CATH (35 kDa), was missing in the cells infected with BmCysN38D or BmCysN65D. Also, tunicamycin treatment revealed that a non-glycosylated form of pro-V-CATH is 30 kDa (Supplementary Fig. S5 and Supplementary Fig. S6), indicating that two residues, N38 and N65, are used as N-linked glycosylation sites during biosynthesis of BmNPV pro-V-CATH. In contrast, the pattern of V-CATH expression in BmCysN158D-infected BmN cells was quite similar to that of T3-infected cells, suggesting that N158 is not glycosylated in BmNPV-infected cells.

Solubility of V-CATH and V-CHIA by removal of N-linked glycans from BmNPV V-CATH

We observed that V-CATH was detected mainly in the insoluble fraction when cells were infected with BmCysN38D or BmCysN65D (Fig. 2 and Supplementary Fig. S3). This strongly suggests that N-linked glycans of these two residues (N38 and N65) are required for production of the soluble V-CATH. A 15 kDa band, a putative degradation product, was heavily accumulated in the insoluble fraction of BmN cells infected with BmCysN38D or BmCysN65D (Supplementary Fig. S3). Also, Western blot analysis showed that BmNPV V-CHIA predominantly formed insoluble aggregates in BmCysN38D- or BmCysN65D-infected BmN cells, although BmCysPD, a v-cath-deleted BmNPV, produced V-CHIA mainly in the soluble fraction (Fig. 3 and Supplementary Fig. S4). This shows that non-glycosylated V-CATH blocks the production of the soluble form of V-CHIA.

To know whether non-glycosylated V-CATH specifically inhibits the production of soluble V-CHIA, we examined
V-CHIA expression in BmCysPD-infected cells treated with tunicamycin. As shown in Supplementary Fig. S5, the insoluble form of V-CHIA was detected in BmCysPD-infected cells treated with tunicamycin, suggesting that other non-glycosylated proteins expressed in BmNPV-infected BmN cells can block the production of the soluble form of V-CHIA.

A single N-linked glycosylation site in the pro-region of AcMNPV V-CATH

Slack et al. (1995) reported that AcMNPV V-CATH is modified with N-linked glycans and the putative site of glycosylation is N158, whereas Hom & Volkman (2000) showed that N-glycosylation occurs in the pro-region of AcMNPV V-CATH. To verify position(s) at which AcMNPV V-CATH is glycosylated, we examined the expression pattern of V-CATH during AcMNPV infection. As shown in Supplementary Fig. S6, Western blot analysis using anti-V-CATH antiserum showed that two forms of pro-V-CATH (33 and 30 kDa) and a single form of putative mature V-CATH (23 kDa) were present in the soluble fraction of Sf-9 cells, suggesting that AcMNPV V-CATH possesses a single N-linked glycosylation site. Also, tunicamycin treatment revealed that the pro-V-CATH, but not the mature form of V-CATH, was glycosylated in AcMNPV-infected Sf-9 cells (Supplementary Fig. S6). Taken together with the existence of a single N-glycosylation site (N65) in the pro-region (Supplementary Fig. S1), we conclude that AcMNPV V-CATH is glycosylated in the pro-region during AcMNPV infection, which is consistent with our results that BmNPV V-CATH is glycosylated only in the pro-region (Fig. 2 and Supplementary Fig. S3).

Release of BmNPV V-CATH by removal of N-linked glycans

We next examined release of V-CATH from BmN cells infected with T3 or mutant BmNPVs expressing V-CATH derivatives. Western blot analysis using culture medium of BmNPV-infected cells did not detect V-CATH secretion at 3 or 4 days p.i. (data not shown). At 5 days p.i., we observed three bands in the medium of BmN cells infected with T3, but not in that of BmCysPD-infected BmN (Fig. 4). The 22 kDa band appears to be a mature form of V-CATH. In the medium of BmCysN38D-infected BmN cells, each form of V-CATH was released, but its amount was markedly reduced. In addition, two forms of pro-V-CATH, but not the mature form, were expressed as smaller proteins than those observed in T3-infected BmN cells. Also, release of V-CATH into the medium was not detected in BmCysN65D-infected BmN cells, whereas we detected three bands in the medium of BmN cells infected with BmCysN158D (Fig. 4). These results suggest that N-linked glycans of BmNPV V-CATH are required for its release, and N65 rather than N38 is an essential residue for V-CATH release from BmNPV-infected BmN cells.

Activity of BmNPV V-CATH in BmN cells by removal of N-linked glycans

We next examined V-CATH activities in BmN cells infected with T3 or mutant BmNPVs expressing V-CATH derivatives. Assays were performed with or without addition of E-64, a cysteine protease-specific inhibitor, to determine if detected activity was derived from cysteine proteases. We detected significant activities in T3-, BmCysPDR-, and BmCysN158D-infected BmN cells, but extracts from BmCysPD, BmCysN38D, and BmCysN65D showed a complete loss of V-CATH activities (data not shown). Also, we observed a marked reduction of V-CATH activities in the medium of BmCysN38D- and BmCysN65D-infected BmN cells, although the activity in BmCysN38D-infected cells was significantly higher than that observed in BmCysPD- or BmCysN65D-infected cells (Fig. 5a). These results suggest that the proper folding of V-CATH in BmN cells is required for its activation.

![Fig. 3. Solubility of BmNPV V-CHIA. BmN cells were infected with T3 or mutant BmNPVs and harvested at 3 days p.i. in cell lysis buffer containing 0.5% NP-40 and 30 μg cysteine protease inhibitor E-64 ml⁻¹. The soluble and insoluble fractions of the lysates were then analysed by Western blotting using anti-BmCHI-h antibody. The molecular masses of protein standards are indicated on the left.](http://vir.sgmjournals.org) 173

![Fig. 4. Release of BmNPV V-CATH from infected cells. BmN cells were infected with wt or mutant BmNPVs and the culture medium was harvested at 5 days p.i. Western blotting of the medium samples was performed using anti-V-CATH antibody. The molecular masses of protein standards are indicated on the left. Arrowheads indicate pro V-CATHs and asterisk shows a non-specific band seen in each lane. P, ProV-CATH; M, mature form of V-CATH.](http://vir.sgmjournals.org)
Effects of removal of \(N\)-linked glycans from BmNPV V-CATH in \(B.\ mori\) larvae

We examined OB release into the haemolymph of infected larvae. As shown in Fig. 6(a), we found a significant reduction of OB release into the haemolymph of BmCysN38D- and BmCysN65D-infected larvae. Released OBs were slightly, but significantly, more abundant in BmCysN38D-infected larvae than in BmCysPD- or BmCysN65D-infected larvae (Fig. 6a). In addition, we found that haemolymph from BmCysPD-, BmCysN38D- and BmCysN65D-infected larvae showed a marked reduction in V-CATH activities, with a low, but significant, activity in the haemolymph of BmCysN38D-infected larvae (Fig. 6b). This is consistent with the \textit{in vitro} results from Fig. 5(a). Collectively, these results suggest that \(N\)-linked glycans of BmNPV V-CATH are required for OB release and V-CATH activation in BmNPV-infected \(B.\ mori\) larvae.

---

**OB release from BmNPV-infected cells by removal of \(N\)-linked glycans**

We next investigated the effects of loss of V-CATH \(N\)-linked glycans on BmNPV infection of BmN cells. BV production was not significantly different among T3 and mutant viruses at 48 h p.i. (T3, \(4.7 \times 10^7\) p.f.u. ml\(^{-1}\); BmCysPD, \(4.5 \times 10^7\) p.f.u. ml\(^{-1}\); BmCysPDR, \(4.7 \times 10^7\) p.f.u. ml\(^{-1}\); N38D, \(3.3 \times 10^7\) p.f.u. ml\(^{-1}\); N65D, \(4.0 \times 10^7\) p.f.u. ml\(^{-1}\); N158D, \(3.8 \times 10^7\) p.f.u. ml\(^{-1}\)). In contrast, we found a significant reduction of OB release in BmCysN38D- and BmCysN65D-infected BmN cells (Fig. 5b), although OB production was not statistically different among viruses (data not shown). OB release was slightly more abundant in BmCysN38D-infected BmN cells than that in BmCysPD- or BmCysN65D-infected cells (Fig. 5b). These results suggest that \(N\)-linked glycans of BmNPV V-CATH are involved in OB release from infected cells.
**DISCUSSION**

In AcMNPV and BmNPV, two viral genes, v-cath and v-chiA, have been shown to be required for the process of host larval degradation after death (Slack et al., 1995; Ohkawa et al., 1994; Hawtin et al., 1997; Katsuma et al., 2004b). Further studies also showed that inactivation of v-chiA or tunicamycin treatment resulted in the formation of insoluble V-CATH aggregates in virus-infected cells (Hom & Volkman, 2000; Daimon et al., 2007). From the results showing that V-CATH is a glycosylated protein with N-linked glycans (Slack et al., 1995; Hom & Volkman, 2000), it was proposed that V-CHIA might work as a molecular chaperone during pro-V-CATH processing and that the putative V-CHIA–V-CATH interaction might be mediated by N-linked glycans of V-CATH. To date, however, the role of N-linked glycans of V-CATH in its solubility remains unclear, since tunicamycin treatment can block N-linked glycosylation of all proteins synthesized in virus-infected cells, and it is possible that other glycoproteins are involved in the solubility of V-CATH. Here we generated three recombinant BmNPVs expressing mutant V-CATHs to determine the positions and roles of N-linked glycans of V-CATH. Biochemical studies clearly showed that the two residues N38 and N65, which are localized in the pro-region of V-CATH, are the glycosylation sites of BmNPV V-CATH (Fig. 2, Supplementary Fig. S5 and Supplementary Fig. S6). Our present study also revealed that N-linked glycans are required for the functions of V-CATH.

There are two inconsistent reports on the positions of N-linked glycans of AcMNPV V-CATH: Slack et al. (1995) described that AcMNPV V-CATH is modified with N-linked glycans at N158, whereas Hom & Volkman (2000) showed that N-glycosylation occurs in the pro-region. Thus, we examined the expression pattern of V-CATH in AcMNPV-infected Sf-9 cells treated with or without tunicamycin. Western blot analysis using anti-V-CATH antiserum showed that AcMNPV V-CATH possesses a single N-linked glycosylation site, probably N65, in the pro-region (Supplementary Fig. S6). These results suggest that the existence of N-linked glycosylation site(s) in the pro-region is a common characteristic of V-CATHs among group I NPVs. We found that asparagine 38 is also used as the N-linked glycosylation site during BmNPV infection, although this residue is not conserved among group I NPVs (Supplementary Fig. S1, Fig. 2 and Supplementary Fig. S3). Further experiments using recombinant BmNPVs showed that N-linked glycosylation at N38 is involved in the V-CATH functions, but the mutation of this residue did not inhibit the activity of V-CATH completely (Fig. 5 and Fig. 6). Infection with BmCysN38D resulted in reduced OB release observed in BmN cells and B. mori larvae, whereas OB release was completely inhibited in BmCysN65D infection. These results suggest that N38 is an important residue, but not essential for activation of BmNPV V-CATH. By sequence analysis, V-CATH of *Antheraea pernyi* NPV (AnpeNPV) is likely glycosylated at this residue (Supplementary Fig. S1), implicating that V-CATHs of these two NPVs might share a unique property which is different from those of other group I NPVs.

Surprisingly, we found that BmNPV V-CHIA as well as V-CATH predominantly formed insoluble aggregates in BmCysN38D- or BmCysN65D-infected BmN cells (Fig. 3 and Supplementary Fig. S4). In BmN cells infected with BmCysPD, a v-cath-deleted BmNPV, V-CATH was mainly detected in the soluble fraction (Fig. 3 and Supplementary Fig. S4). These results indicate that the expression of non-glycosylated V-CATH inhibits the production of the soluble V-CHIA, whereas a defect in V-CATH expression does not affect the solubility of V-CHIA. To examine whether non-glycosylated V-CATH is the sole determinant to inhibit the production of the soluble V-CHIA, we examined V-CHIA expression in BmCysPD-infected cells treated with tunicamycin. Surprisingly, we observed that the insoluble form of V-CHIA was also detected in BmCysPD-infected cells treated with tunicamycin (Supplementary Fig. S5). This suggests that other non-glycosylated viral and/or host proteins in BmNPV-infected BmN cells can cause the production of the insoluble form of V-CHIA. SDS-PAGE analysis of cell extracts from BmNPV-infected BmN cells showed that V-CHIA, but not V-CATH, could be easily detected by Coomassie brilliant blue staining (Wang et al., 2005), indicating that V-CHIA accumulates to high levels during BmNPV infection. This strongly suggests that V-CHIA may also assist folding processes of a wide variety of host or viral proteins other than V-CATH.

Several studies on the role of N-linked glycosylation in baculoviral proteins have been reported. Jarvis et al. (1998) reported mutational analysis of N-linked glycans on AcMNPV GP64, the major envelope glycoprotein in the budded form of group I NPVs. They found that four of the five consensus N-glycosylation sites are used during AcMNPV infection, and that the infectious BVs produced by AcMNPV mutants lacking one, two or three N-linked glycosylation sites were 10- to 100-fold lower than wt levels. Together with the results from the binding experiments, N-linked glycans of GP64 were shown to play an important role in binding of BVs to the cell. Recently, the role of N-linked glycans from the F2 subunit of *Helicoverpa armigera* NPV F protein, the major envelope fusion protein of group II NPVs, was described (Long et al., 2007). Mutational analysis showed that N-linked glycans of the F2 subunit are involved in BV production and fusogenicity. Also, our recent studies have revealed that BmNPV fibroblast growth factor is modified with N-linked glycans at N44 and N171, and N-linked glycans are involved in the infectious BVs produced by BmNPV mutants lacking one, two or three N-linked glycosylation sites were 10- to 100-fold lower than wt levels. Together with the results from the binding experiments, N-linked glycans of GP64 were shown to play an important role in binding of BVs to the cell. Recently, the role of N-linked glycans from the F2 subunit of *Helicoverpa armigera* NPV F protein, the major envelope fusion protein of group II NPVs, was described (Long et al., 2007). Mutational analysis showed that N-linked glycans of the F2 subunit are involved in BV production and fusogenicity. Also, our recent studies have revealed that BmNPV fibroblast growth factor is modified with N-linked glycans at N44 and N171, and N-linked glycans are involved in the infectious BVs produced by BmNPV mutants lacking one, two or three N-linked glycosylation sites were 10- to 100-fold lower than wt levels. Together with the results from the binding experiments, N-linked glycans of GP64 were shown to play an important role in binding of BVs to the cell. Recently, the role of N-linked glycans from the F2 subunit of *Helicoverpa armigera* NPV F protein, the major envelope fusion protein of group II NPVs, was described (Long et al., 2007).
In this study, we identified two residues, N38 and N65, as the glycosylation sites of BmNPV V-CATH. Also, we found that N-linked glycans are essential for the functions of BmNPV V-CATH. Surprisingly, we observed that the proper folding of V-CHIA is dependent on N-linked glycans of V-CATH, but not the V-CATH activity. Further studies are required to identify additional viral or host proteins that require the assistance of V-CHIA during BmNPV infection.

ACKNOWLEDGEMENTS

We would like to thank Taro Ohkawa for critical reading of the manuscript, Naoko Omuro for the DNA sequencing, and Masashi Iwanaga for providing AcMNPV. This work was supported by grants from MEXT (no. 17018007 to T. S. and no. 19688004 to S. K.), MAFF-NIAS (Agrigenome Research Program) and JST (Professional Program for Agricultural Bioinformatics), Japan.

REFERENCES


