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A single amino acid substitution modulates low-pH-triggered membrane fusion of GP64 protein in *Autographa californica* and *Bombyx mori* nucleopolyhedroviruses

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ABSTRACT

We have previously shown that budded viruses of *Bombyx mori* nucleopolyhedrovirus (BmNPV) enter the cell cytoplasm but do not migrate into the nuclei of non-permissive Sf9 cells that support a high titer of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) multiplication. Here we show, using the syncytium formation assay, that low-pH-triggered membrane fusion of BmNPV GP64 protein (Bm-GP64) is significantly lower than that of AcMNPV GP64 protein (Ac-GP64). Mutational analyses of GP64 proteins revealed that a single amino acid substitution between Ac-GP64 H155 and Bm-GP64 Y153 can have significant positive or negative effects on membrane fusion activity. Studies using bacmid-based GP64 recombinant AcMNPV harboring point-mutated *ac-gp64* and *bm-gp64* genes showed that Ac-GP64 H155Y and Bm-GP64 Y153H substitutions decreased and increased, respectively, the multiplication and cell-to-cell spread of progeny viruses. These results indicate that Ac-GP64 H155 facilitates the low-pH-triggered membrane fusion reaction between virus envelopes and endosomal membranes.

Introduction

Nucleopolyhedroviruses (NPVs) belonging to the family Baculoviridae are large, enveloped, double-stranded DNA viruses that are pathogenic to insects (Theilmann et al., 2005). In their infection cycle, NPVs yield two types of virions, occlusion body-derived virions (ODVs) and budded virions (BVs), with distinct envelope origins and compositions (Funk et al., 1997). ODVs are enveloped within the nucleus by *de novo* synthesized membranes and are subsequently occluded into protein matrices called occlusion bodies or polyhedra. BVs, in contrast, are enveloped during budding from infected cells by plasma membranes that have been modified by the GP64 envelope fusion protein. ODVs play important roles in the primary infection of insects and in the insect-to-insect transmission of occlusion body-embedded progeny viruses through oral infection, while BVs have been shown to be essential for cell-to-cell transmission within infected insects (Blissard, 1996; Blissard and Rohrmann, 1990; Volkman, 1997).

Group I NPVs encode GP64 protein, which is located at one end of the BV envelope. GP64 protein is essential for virus multiplication and plays important roles in viral attachment to cells, membrane fusion during viral entry into the host cell through endocytosis, and budding of progeny BVs from the surface of infected cells (Blissard and Wenz, 1992; Hefferon et al., 1999; Monsma et al., 1996; Oomens and Blissard, 1999). During the initial stages of infection, BVs are internalized by host cells through clathrin-mediated endocytosis (Blissard and Wenz, 1992; Long et al., 2006; Volkman and Goldsmith, 1985), and GP64 protein mediates BV binding to host cell receptors and regulates low-pH-triggered membrane fusion between BV envelopes and endosomal membranes to release nucleocapsids from the endosomes (Blissard and Wenz, 1992; Hefferon et al., 1999). During the final stages of progeny BV production, nucleocapsids bud from infected cells and form mature BVs, acquiring an envelope containing GP64 protein that is incorporated into the plasma membrane of infected cells. The efficient nucleocapsid budding resides in optimal GP64 protein production (Oomens and Blissard, 1999).

GP64 protein is synthesized during both the early and late phases of viral infection and is transported to the plasma membrane of infected cells. During the transportation process, GP64 protein undergoes oligomerization to a homotrimer and glycosylation to ensure efficient cell surface localization and membrane fusion activity (Oomens et al., 1995). Two functional domains, the membrane fusion and oligomerization domains, have been identified as key elements in the low-pH-triggered membrane fusion activity of GP64 protein (Jarvis et al., 1998; Monsma and Blissard, 1995).

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Autographa californica multicapsid NPV (AcMNPV) has been shown to migrate into the nuclei of a wide variety of cell lines derived from both insects and mammals (Boyce and Bucher, 1996; Condreay et al., 1999; Hofmann et al., 1995; Miller and Lu, 1997; Shoji et al., 1997; van Loo et al., 2001), suggesting that AcMNPV GP64 protein (Ac-GP64) effectively functions as a mediator of membrane fusion between BV envelopes and endosomal membranes in a wide variety of cell types. In contrast, *Bombyx mori* NPV (BmNPV), which is closely related to AcMNPV, exhibits defective nuclear transport of nucleocapsids in non-permissive insect cell lines, including Sf9 and High Five, both of which are permissive for AcMNPV (Katou et al., 2006). In addition, recombinant BmNPV carrying Ac-GP64 in place of its own BmNPV GP64 protein (Bm-GP64) has been shown to be able to enter the nuclei of Sf9 and High Five cells, suggesting that the low-pH-triggered membrane fusion activity of Ac-GP64 differs from that of Bm-GP64.

In this study, we demonstrate that the low-pH-triggered membrane fusion activity of Bm-GP64 is significantly lower than that of Ac-GP64, and that the observed difference in membrane fusion activity between these two proteins is the result of a single amino acid substitution in the GP64 protein. Using bacmid-based recombinant AcMNPV carrying point mutations in the GP64 protein, we also show that a single amino acid substitution in the Ac-GP64 histidine residue at position 155 (H155) and the corresponding Bm-GP64 Y153 can have significant negative and positive effects, respectively, on low-pH-triggered membrane fusion activity and the multiplication and cell-to-cell spread of progeny viruses.

Results

Membrane fusion activity of Bm-GP64 and Ac-GP64

In a previous study, we showed that BmNPV BVs enter non-permissive Sf9 cells and are transported to the vicinity of the nucleus without actually being imported into the nucleus (Katou et al., 2006). To determine whether the observed defects in the nuclear import of BmNPV BVs in Sf9 cells are related to the reduced low-pH-triggered membrane fusion activity of Bm-GP64, BmN-4 cells were infected with AcMNPV and BmNPV and subjected to syncytium formation assay according to methods described previously (Blissard and Wenz, 1992). BmN-4 cells at 24 and 48 h post-infection were exposed to TC100 medium (pH 5.0) for 5 min and syncytium formation was examined under a microscope 4 h after low pH exposure (Fig. 1A). Both AcMNPV- and BmNPV-infected BmN-4 cells underwent cell fusion and formed syncytia containing multiple nuclei. BmNPV-infected cells formed fewer syncytia with fewer nuclei than AcMNPV-infected cells. Immunoblot analysis revealed that BmNPV-infected cells contained increased levels of GP64 protein compared to AcMNPV-infected cells (Fig. 1B).

To determine whether the membrane fusion activity of Ac-GP64 was higher than that of Bm-GP64, the syncytium formation assay was carried out in uninfected Sf9 cells transiently expressing hemagglutin (HA)-tagged GP64 protein (GP64HA). First, Sf9 cells were transfected with pHyHr6IE1/AcGP64HA or pHyHr6IE1/BmGP64HA that expressed Ac-GP64HA or Bm-GP64HA, respectively (Supplementary Figs. 1A, B). The transfected cells were then exposed to TC100 medium (pH 5.0) for 5 min at 24 h post-transfection and incubated for 4 h in TC100 medium (pH 6.2). Microscopic observations revealed that Sf9 cells expressing Ac-GP64HA formed a number of syncytia containing several nuclei, while relatively few syncytia were produced in cells expressing Bm-GP64HA (Supplementary Fig. 1C). These findings indicate that, at pH 5.0, Bm-GP64HA exhibits reduced membrane fusion activity compared to Ac-GP64HA.

To determine whether the threshold pH triggering membrane fusion differs between Ac-GP64 and Bm-GP64, Sf9 cells expressing Ac-GP64HA or Bm-GP64HA were exposed to TC100 medium at various pH (3.75, 4.0, 4.5, 5.0, 5.5 and 6.0) for 5 min, and syncytium formation was examined. Sf9 cells expressing Ac-GP64HA formed syncytia at pH ≤ 5.0, while cells expressing Bm-GP64HA generated comparatively fewer syncytia, and only at pH 4.0 and 3.75 (Fig. 2). These findings indicate that both the pH threshold for low-pH-triggered membrane fusion and the membrane fusion activity at specific low pH are lower for Bm-GP64 than Ac-GP64.

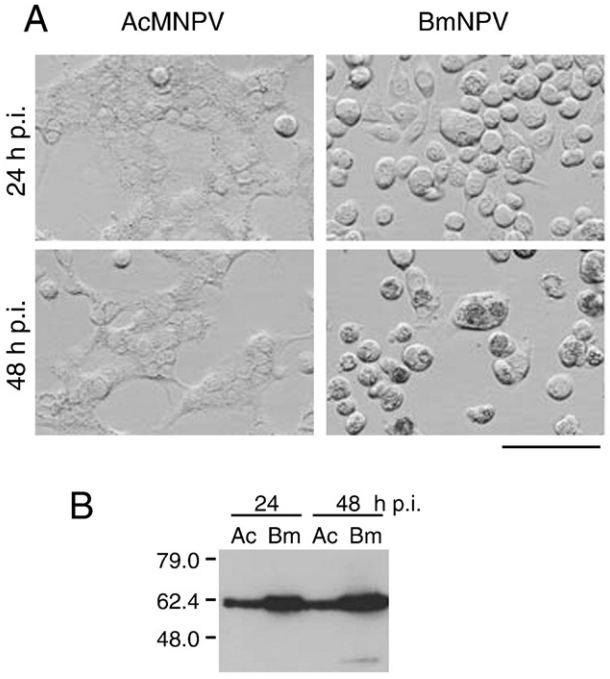

Fig. 1. Low-pH-mediated membrane fusion of BmN-4 cells infected with AcMNPV and BmNPV. (A) Syncytium formation of infected BmN-4 cells. BmN-4 cells were infected with AcMNPV and BmNPV at a multiplicity of infection of 10. At 24 and 48 h post-infection, infected cells were incubated in TC100 medium at pH 5.0 for 5 min. After washing with TC100 medium, infected cells were incubated in TC100 medium at pH 6.2 for 4 h and observed under a microscope. Scale bar = 100 μm. (B) GP64 protein expression in BmN-4 cells infected with AcMNPV and BmNPV. BmN-4 cells infected with AcMNPV (Ac) and BmNPV (Bm) were harvested at 24 and 48 h post-infection and the polypeptides isolated from the infected cells were analyzed by immunoblotting using an anti-GP64 monoclonal antibody. Polypeptides from the same number of infected cells at infection were analyzed in each lane. The numbers on the left of the panel indicate the molecular mass (kDa) of the marker proteins.

Oligomerization and cell surface localization of Bm-GP64 in Sf9 cells

Membrane fusion activity observed by syncytium formation assay has been shown to be dependent on the oligomerization and cell surface localization of GP64 protein (Monsma and Blissard, 1995). To exclude the possibility that the reduced membrane fusion activity of Bm-GP64 in Sf9 cells is caused by a defect in oligomerization or cell surface localization of Bm-GP64, Sf9 cells expressing Ac-GP64HA and Bm-GP64HA were subjected to non-reducing SDS-PAGE followed by immunoblot analysis with anti-GP64 monoclonal antibody (Fig. 3A). Both Ac-GP64HA and Bm-GP64HA expressed in Sf9 cells migrated predominantly as two bands with molecular masses of >180 kDa, representing the two forms of trimeric GP64 protein (Monsma and Blissard, 1995). Immunocytochemistry with anti-GP64 antibody further demonstrated that, like Ac-GP64HA, Bm-GP64HA was localized on the surface of Sf9 cells (Fig. 3B), and cELISA showed that there was no significant defference in the cell surface levels between Ac-GP64HA and Bm-GP64HA (Fig. 3C). These results indicate that the reduced low-pH-triggered membrane fusion activity of Bm-GP64 is not caused by defects in oligomerization or cell surface localization.

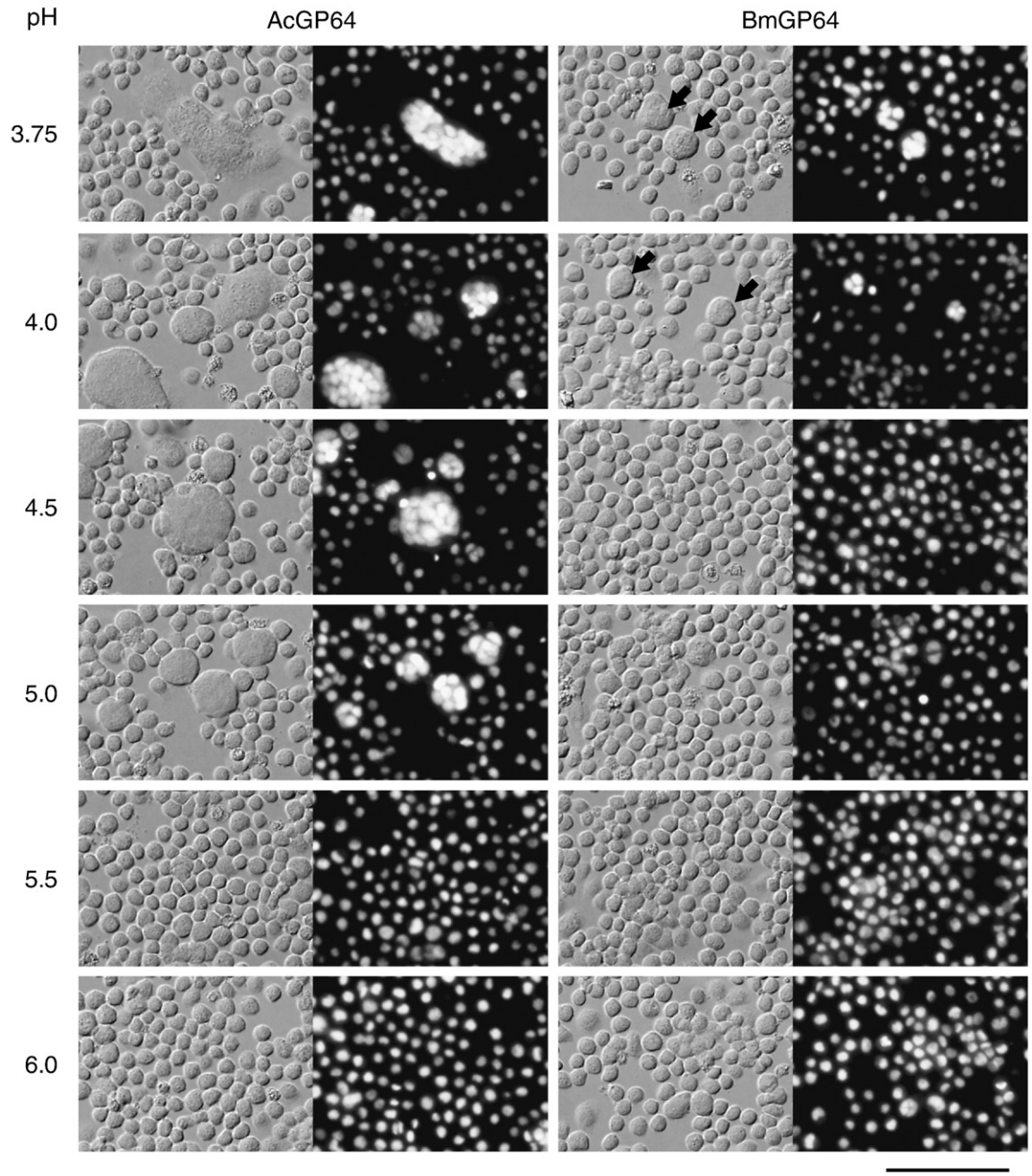

Fig. 2. pH sensitivity of membrane fusion in Sf9 cells expressing Ac-GP64 and Bm-GP64. Sf9 cells were transfected with pHyHr6IE1/AcGP64HA (AcGP64) or pHyHr6IE1/BmGP64HA (BmGP64) and exposed to TC100 medium at pH ranging from 3.75 to 6.0 for 5 min at 24 h post-transfection. After 4 h incubation in TC100 medium at pH 6.2, the cells were fixed with 2% paraformaldehyde and stained with PI to identify the nuclei. Bright field (left) and PI fluorescence (right) are shown. Arrows indicate syncytium formation in Bm-GP64-expressing cells. Scale bar = 100 μm.

Identification of amino acid residues responsible for fusion activity differences between Ac-GP64 and Bm-GP64

Previous studies have shown that the amino acid sequence identity between Bm-GP64 and Ac-GP64 are over 95%, in which the fusion and the oligomerization domains were completely or almost completely conserved between the two proteins (Ayres et al., 1994; Gomi et al., 1999). These two GP64 proteins are also conserved in terms of cystein residue distribution, potential *N*-glycosylation sites and overall hydropathy profile. In order to identify the regions responsible for the differences in membrane fusion activity between Ac-GP64 and Bm-GP64, 10 different chimeric mutant *gp64* genes containing amino acid sequence substitutions between Bm-GP64 and Ac-GP64 were constructed using four conserved restriction endonuclease sites (Fig. 4A). These chimeric mutants were then transfected into Sf9 cells and subjected to syncytium formation assay at pH 5.0 to examine membrane fusion activity (Fig. 4B). Ac-GP64 containing Bm-GP64 region II in place of its own region II exhibited reduced membrane fusion activity, while Ac-GP64 with Bm-GP64 regions I, III, IV and V in place of its analogous regions exhibited membrane fusion activity similar to that observed in Sf9 cells transfected with wild-type (wt) Ac-GP64. In contrast, Bm-GP64 containing Ac-GP64 region II in place of its own region II demonstrated increased membrane fusion activity, while Bm-GP64 substituted with Ac-GP64 regions I, III, IV and V, like Sf9 cells transfected with wt Bm-GP64, exhibited no membrane fusion activity. These results indicate that Ac-GP64 requires region II

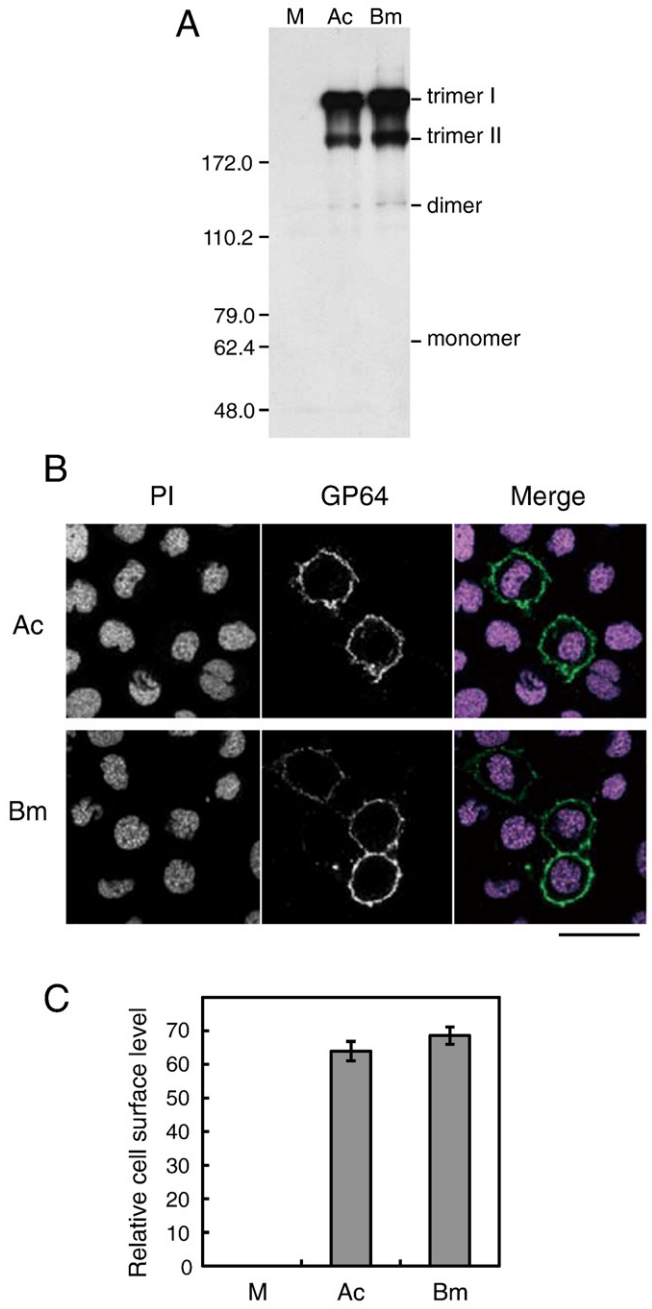

Fig. 3. Oligomerization and cell surface localization of Ac-GP64 and Bm-GP64 transiently expressed in Sf9 cells. (A) Oligomerization of GP64 proteins expressed in Sf9 cells. Sf9 cells were transfected with pHyHr6IE1/AcGP64HA (Ac) and pHyHr6IE1/BmGP64HA (Bm). Cells were harvested at 24 h post-transfection and the polypeptides were separated by non-reducing SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis with an anti-GP64 monoclonal antibody. The numbers on the left to the panel indicate the molecular mass (kDa) of the marker proteins. M indicates mock-transfected cells (control). (B) Cell surface localization of GP64 proteins expressed in Sf9 cells. Sf9 cells transfected with pHyHr6IE1/AcGP64HA (Ac) or pHyHr6IE1/BmGP64HA (Bm) were fixed with 2% paraformaldehyde at 24 h post-transfection. GP64 proteins were visualized with anti-GP64 monoclonal antibody and Alexa488-conjugated goat anti-mouse IgG antibody (GP64, green in merged image). These cells were also stained with PI to identify the nuclei of the transfected cells (PI, magenta in merged image). Scale bar = 20 μm. (C) Relative cell surface levels of GP64 proteins measured by cELISA. Sf9 cells in a 24-well plate were transfected with pHyHr6IE1/AcGP64HA (Ac) or pHyHr6IE1/BmGP64HA (Bm), fixed with 0.5% glutaraldehyde, and reacted with anti-GP64 monoclonal antibody, followed by reaction with HRP-conjugated goat anti-mouse IgG. The peroxidase activity was determined by QuantaBlu™ fluorogenic peroxidase substrate kit (PIERCE) and accumulation of fluorescent product was monitored using Fluoroskan Ascent (Thermo Scientific), with excitation and emission wavelengths of 325 and 420 nm, respectively. Vertical bars represent the standard deviations from six determinations. M, mock-transfected cells.

(amino acids 135 to 176) for low-pH-triggered membrane fusion activity at pH 5.0, and that substitution of region II between Ac-GP64 and Bm-GP64 can negatively or positively influence membrane fusion activity.

Alignment of amino acid sequences revealed that only two amino acid residues in region II (amino acids 135 to 176 for Ac-GP64 and amino acids 133 to 174 for Bm-GP64) differed between Ac-GP64 and Bm-GP64 (Fig. 5A; Ac-GP64 H155 and I166 vs. Bm-GP64 Y153 and V164, respectively). In order to determine which amino acid residues were responsible for the observed differences in membrane fusion activity between the two proteins, we generated six *gp64* expression vectors containing point mutations (Fig. 5A) that efficiently expressed their respective GP64 proteins (Fig. 5B). Syncytium formation assay revealed that Ac-GP64 containing H155Y and H155Y/I166V substitutions (Ac-GP64^{H155Y} and Ac-GP64$^{H155Y/I166V}$) demonstrated no membrane fusion activity, whereas Ac-GP64^{I166V} exhibited membrane fusion activity in a manner similar to wt Ac-GP64 (Fig. 5C). In contrast, Bm-GP64^{Y153H} and Bm-GP64$^{Y153H/V164I}$ demonstrated membrane fusion activity similar to wt Ac-GP64, while Bm-GP64^{V164I} exhibited no membrane fusion activity. Therefore, the observed discrepancy in membrane fusion activity between Ac-GP64 and Bm-GP64 can be attributed to a single amino acid difference located at position 155 of Ac-GP64 and position 153 of Bm-GP64. These findings suggest that the histidine residue located at position 155 (H155) of Ac-GP64 is essential for the higher membrane fusion activity of Ac-GP64 in comparison to that of Bm-GP64.

Generation and infectivity of recombinant AcMNPVs expressing point-mutated GP64 proteins

It has been demonstrated previously that GP64 protein plays a critical role in BV infection and yield (Blissard and Wenz, 1992; Hefferon et al., 1999; Monsma et al., 1996; Oomens and Blissard, 1999; Volkman and Goldsmith, 1985). To determine whether the single amino acid difference observed between Ac-GP64 and Bm-GP64 affects BV infection and multiplication in Sf9 cells, we generated recombinant AcMNPV (rAcMNPV) expressing point-mutated GP64 proteins from the bacmid. The endogenous *gp64* gene was knocked out by homologous recombination in Sf9 cells as described previously (Kitagawa et al., 2005; Lung et al., 2003) (Supplementary Figs. 2A, B), and disruption of the *gp64* gene was confirmed by Southern blot hybridization (data not shown). The virus based on this *gp64* knockout bacmid completely lost infectivity in Sf9 cells (Supplementary Fig. 2C, right panel). The wt and mutated *gp64* genes were then introduced into the *polyhedrin* locus, together with the *ie1* promoter-driven *egfp* gene, by the Tn7 transposon (Supplementary Fig. 2A). Four *gp64*-introduced bacmids were transfected into Sf9 cells, and the culture media were collected at 96 h post-transfection and used for the remaining experiments. Incorporation of the *gp64* gene into the bacmids was confirmed by PCR using bacmid DNA as a template (data not shown) and immunoblot analysis of GP64 proteins isolated from transfected cells and partially purified virions (Supplementary Fig. 2D).

To determine whether the GP64 protein mutations that altered membrane fusion activity also affected the infectivity and productivity of rAcMNPVs, plaque assay was carried out in Sf9 cells for four rAcMNPVs: rAcMNPV$^{Ac\text{-}GP64wt}$, rAcMNPV$^{Bm\text{-}GP64wt}$, rAcMNPV$^{Ac\text{-}GP64H155Y}$ and rAcMNPV$^{Bm\text{-}GP64Y153H}$, encoding wt Ac-GP64, wt Bm-GP64, Ac-GP64^{H155Y} and Bm-GP64^{Y153H}, respectively. Culture media were harvested from bacmid-transfected Sf9 cells at 96 h post-transfection and diluted to 1×10^{-3} or 1×10^{-5} with TC100 medium and inoculated into Sf9 cells. The inoculated cells were then overlaid with agarose in TC100 medium and plaque formation was examined under a fluorescence microscope at 6 days after inoculation. The results revealed the formation of obvious plaques in the cultures inoculated with rAcMNPV$^{Ac\text{-}GP64wt}$, while rAcMNPV$^{Bm\text{-}GP64wt}$ produced very small plaques (Fig. 6). These

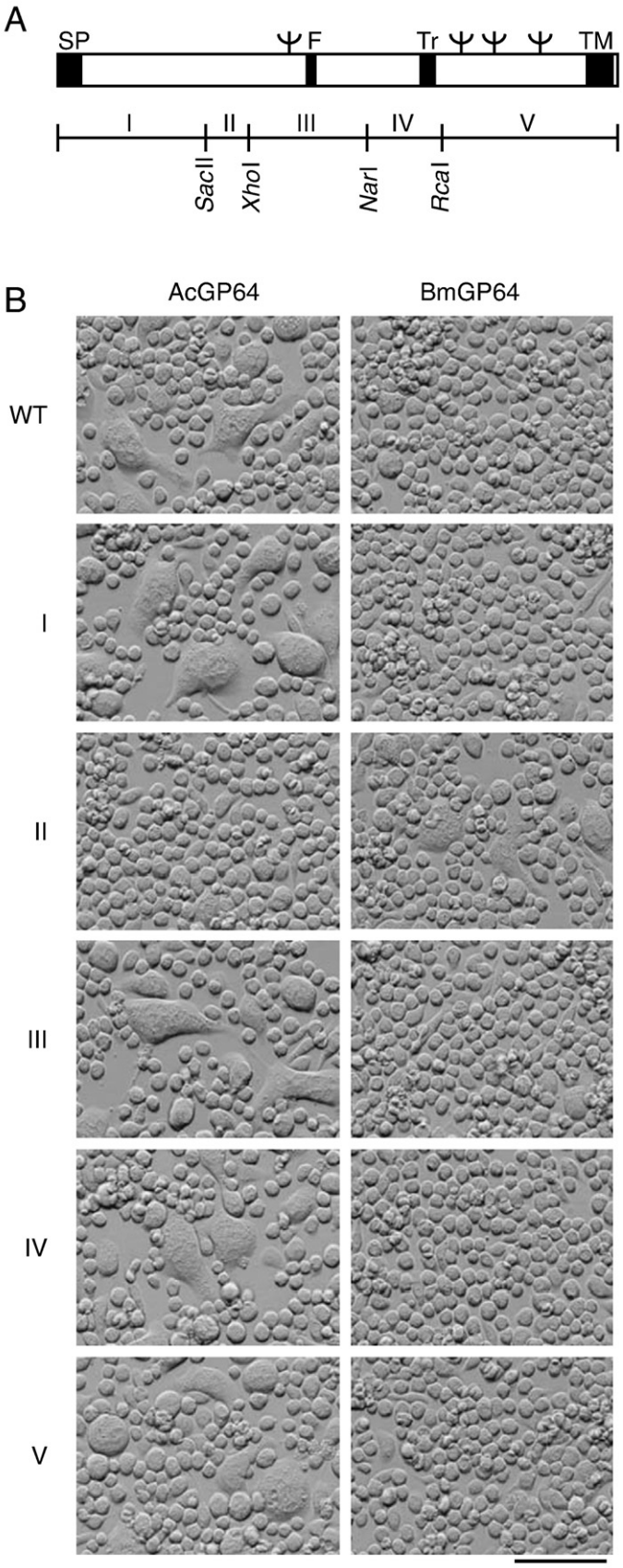

Fig. 4. Membrane fusion activitiy of chimeric Ac-GP64 and Bm-GP64 transiently expressed in Sf9 cells. (A) Schematic maps of GP64 protein (upper box) and gene (lower horizontal line). Roman numerals I–V between the vertical lines indicate the regions substituted between the *ac-gp64* and *bm-gp64* genes. The location of restriction endonuclease sites (vertical lines) used for construction of chimeric GP64 protein vectors is shown, together with the names of the restriction endonucleases. Black shaded boxes indicate signal peptides (SP), fusion domain (F), oligomerization domain (Tr) and transmembrane domain (TM), and Ψ indicates *N*-glycosylation sites. (B) Syncytium formation of Sf9 cells expressing chimeric Ac-GP64 and Bm-GP64. For the syncytium formation assay in Sf9 cells transfected with chimeric *gp64* genes, see the legend for Fig. 2. WT, wild-type GP64 proteins. Scale bar = 100 μm.

results indicate that the multiplication and the cell-to-cell spread of progeny viruses is severely restricted in rAcMNPV$^{Bm\text{-}GP64wt}$-inoculated cells compared to rAcMNPV$^{Ac\text{-}GP64wt}$-inoculated cells. In addition, rAcMNPV$^{Bm\text{-}GP64Y153H}$ produced obvious but slightly smaller plaques, while only very small plaques were seen in the cells inoculated with rAcMNPV$^{Ac\text{-}GP64H155Y}$. These findings suggest that, in addition to altering low-pH-triggered membrane fusion activity, amino acid substitutions in GP64 proteins also affect the infectivity and productivity of recombinant viruses in cell culture.

To further characterize rAcMNPVs with respect to their infectivity and productivity, Sf9, High Five and BmN-4 cells were inoculated with rAcMNPVs and reporter EGFP expression was examined at 0, 24, 48, 72 and 96 h post-infection (Supplementary Fig. 3). The results demonstrated that in Sf9 and High Five cells, Ac-GP64 H155Y substitution resulted in a marked reduction in rAcMNPV infectivity, while Bm-GP64 Y153H substitution increased rAcMNPV infectivity. In BmN-4 cells, the reduction in infectivity due to Ac-GP64 H155Y substitution and the increase in infectivity due to Bm-GP64 Y153H substitution remained relatively small.

Sensitivity of gp64 recombinant AcMNPV infection to ammonium chloride

We have shown that low-pH-triggered membrane fusion activity at specific low pH is significantly higher in Ac-GP64 than in Bm-GP64, and that Bm-GP64 requires more acidic pH to trigger fusion activity (see Fig. 2). To provide further evidence that the altered infectivity observed in rAcMNPVs reflects altered GP64-mediated membrane fusion activity between viral envelopes and endosomal membranes, we examined the effects of ammonium chloride on viral infection. BmN-4 cells were infected with rAcMNPV$^{Ac\text{-}GP64wt}$, rAcMNPV$^{Bm\text{-}GP64wt}$, rAcMNPV$^{Ac\text{-}GP64H155Y}$ and rAcMNPV$^{Bm\text{-}GP64Y153H}$ and cultured in TC100 medium containing increasing concentrations of ammonium chloride, and reporter EGFP expression was examined at 24 h post-infection. The results demonstrated that ammonium chloride treatment inhibits EGFP expression of rAcMNPVs in a dose-dependent manner, indicating that rAcMNPV$^{Bm\text{-}GP64wt}$ and rAcMNPV$^{Ac\text{-}GP64H155Y}$ were more sensitive to ammonium chloride than rAcMNPV$^{Ac\text{-}GP64wt}$ and rAcMNPV$^{Bm\text{-}GP64Y153H}$ (Fig. 7). Relative

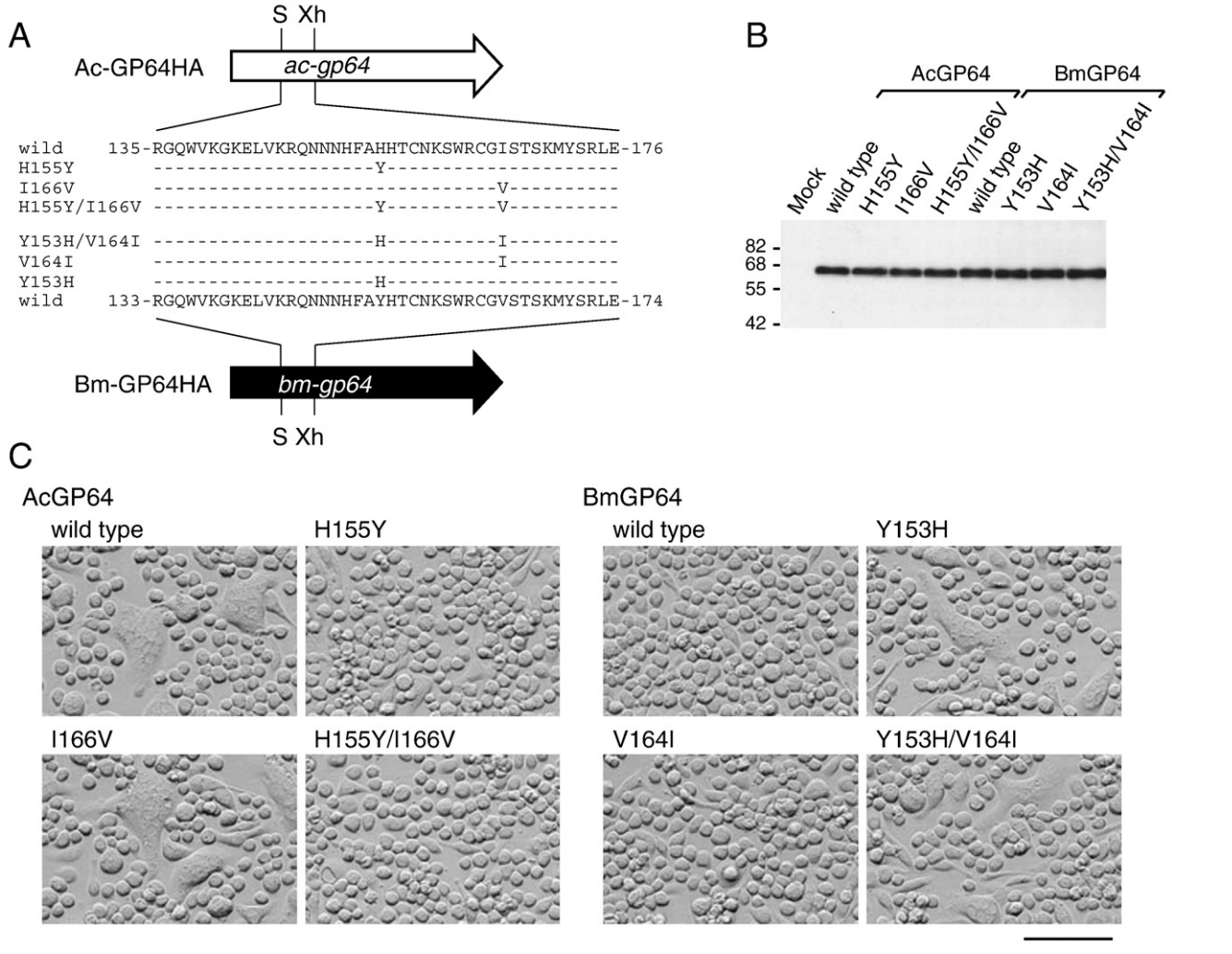

Fig. 5. Membrane fusion activity of point-mutated Ac-GP64 and Bm-GP64 transiently expressed in Sf9 cells. (A) Schematic diagram of GP64 protein substitution constructs. Partial amino acid sequences of GP64 proteins containing single and double amino acid substitutions, together with those of wild-type Ac-GP64 (upper panel) and Bm-GP64 (lower panel), are presented. Substituted amino acids are indicated for each GP64 protein substitution construct. S and Xh indicate restriction endonuclease sites for *Sac*II and *Xho*I, respectively. (B) Immunoblot analysis of polypeptides from Sf9 cells expressing point-mutated GP64 proteins. Sf9 cells were transfected with respective GP64 protein expression vectors and polypeptides isolated from transfected cells were analyzed at 24 h post-transfection by immunoblotting. Point-mutated Ac-GP64HA and Bm-GP64HA expressed in the transfected cells were probed with anti-GP64 monoclonal antibody. (C) Syncytium formation of Sf9 cells expressing point-mutated Ac-GP64 and Bm-GP64. For the syncytium formation assay for Sf9 cells transfected with point-mutated *gp64* genes, see the legend for Fig. 2. Scale bar = 100 μm.

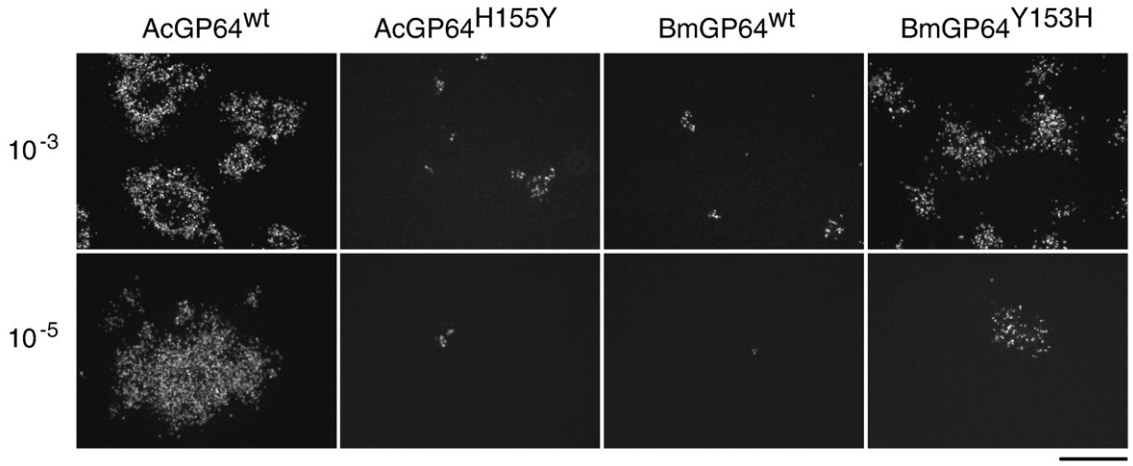

Fig. 6. Plaque assay for GP64 recombinant AcMNPVs. GP64 recombinant bacmid DNAs containing the *egfp* gene and each of the *ac-gp64*^{wt}, *ac-gp64*^{H155Y}, *bm-gp64*^{wt} and *bm-gp64*^{Y153H} genes were transfected into Sf9 cells and the BV-containing culture media harvested at 96 h post-transfection. Culture media were diluted 1:1,000 (upper panel) and 1:100,000 (lower panel) and subjected to plaque assay. Plaques produced by each recombinant virus were examined under a fluorescence microscope 6 days after infection. Scale bar = 500 μm.

to the control, which did not receive ammonium chloride treatment (0 mM), EGFP expression levels in rAcMNPV$^{Bm\text{-}GP64wt}$ and rAcMNPV$^{Ac\text{-}GP64H155Y}$ were decreased to less than 20% in the presence of 10 mM ammonium chloride, while those in rAcMNPV$^{Ac\text{-}GP64wt}$ and rAcMNPV$^{Bm\text{-}GP64Y153H}$ remained at approximately 80% and 60%, respectively, at the same concentration of ammonium chloride. In the presence of 25 mM ammonium chloride, the multiplication of all rAcMNPVs decreased to approximately 10% of that observed at 0 mM. These results suggest that the infection of rAcMNPV$^{Bm\text{-}GP64wt}$ and rAcMNPV$^{Ac\text{-}GP64H155Y}$ is more sensitive to the inhibition of endosomal acidification by ammonium chloride than that of rAcMNPV$^{Ac\text{-}GP64wt}$ and rAcMNPV$^{Bm\text{-}GP64Y153H}$, and that the lower infectivity of rAcMNPV$^{Bm\text{-}GP64wt}$ and rAcMNPV$^{Ac\text{-}GP64H155Y}$ is caused by the decreased sensitivity of Bm-GP64^{wt} and Ac-GP64^{H155Y} to endosomal acidification.

Discussion

In this study, we compared the low-pH-triggered membrane fusion activity of GP64 proteins encoded by the closely related AcMNPV and BmNPV, and demonstrated that the membrane fusion activity of Bm-GP64 was significantly lower than that of Ac-GP64. Using syncytium formation assay, we showed that, upon exposure to a specific acidic pH, BmNPV-infected BmN-4 cells formed only a few syncytia, while AcMNPV-infected BmN-4 cells formed significantly abundant and larger syncytia containing many nuclei. As BmNPV-infected BmN-4 cells contained more GP64 proteins than AcMNPV-infected BmN-4 cells, the reduced membrane fusion activity observed in BmNPV-infected BmN-4 cells was most likely not caused by reduced GP64 protein accumulation in infected cells.

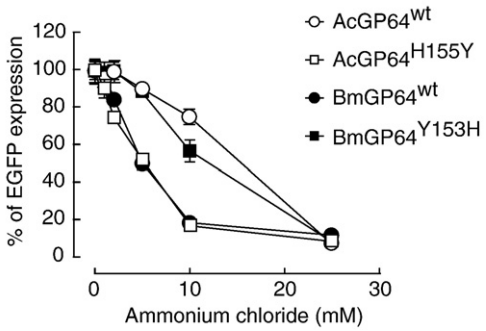

Fig. 7. Effects of ammonium chloride treatment on GP64 recombinant AcMNPV infection of BmN-4 cells. Pre-chilled BmN-4 cells were infected with rAcMNPV$^{Ac\text{-}GP64wt}$ (AcGP64^{wt}), rAcMNPV$^{Ac\text{-}GP64H155Y}$ (AcGP64^{H155Y}), rAcMNPV$^{Bm\text{-}GP64wt}$ (BmGP64^{wt}) and rAcMNPV$^{Bm\text{-}GP64Y153H}$ (BmGP64^{Y153H}) at 4 °C for 60 min. The infected cells were then washed three times with TC100 medium and cultured in the presence of ammonium chloride at a concentration of 0, 1.25, 2.5, 5, 10 or 25 mM. At 24 h post-infection, cells were harvested and lysed in cell lysis buffer and EGFP expression was examined using a fluorospectrometer. EGFP expression is presented as relative (%) to that in the presence of 0% ammonium chloride. Error bars indicate the standard deviations of the means from three determinations.

The fact that uninfected Sf9 cells transiently expressing Bm-GP64 exhibited less extensive fusion in response to low pH than those expressing Ac-GP64 suggests that GP64 protein is indeed involved in the observed discrepancy in membrane fusion activity. Analysis using cells transiently expressing GP64 protein also revealed that Bm-GP64 required lower pH for syncytium formation than Ac-GP64. In addition, Bm-GP64 expressed transiently in Sf9 cells was detected at the cell surface in the form of two trimers, similar to Ac-GP64. Taken together, these data indicate that the reduced low-pH-triggered membrane fusion activity of Bm-GP64 is likely due to its functional insufficiency in membrane fusion reactions compared to that of Ac-GP64. These data also indicate that BmN-4 cells are more sensitive to low-pH-dependent membrane fusion than Sf9 cells, although the cause of this difference between BmN-4 and Sf9 cells cannot be inferred from the available data.

Sequence comparison data demonstrated that 18 amino acid residues differed between Bm-GP64 and Ac-GP64, in addition to the two amino acid deletions previously reported in Bm-GP64 (Ayres et al., 1994; Gomi et al., 1999). Analyses using a series of GP64 proteins containing substitution mutations revealed that, among these amino acid substitutions, only the histidine-to-tyrosine substitution had a substantial negative effect on membrane fusion activity. Syncytium formation assay revealed that Ac-GP64 requires a histidine residue at position 155 (H155), corresponding to Y153 in Bm-GP64, to mediate membrane fusion activity at a level higher than that observed in Bm-GP64. An Ac-GP64 mutant with a tyrosine substitution at H155 (H155Y) exhibited dramatically reduced membrane fusion activity, while Bm-GP64 with a converse Y153H mutation exhibited significantly increased membrane fusion activity. In support of these results, immunocytochemistry of the major capsid protein VP39 (cf., Katou et al., 2006) showed that group I NPVs (AcMNPV, OpMNPV and HycuMNPV), which had a conserved histidine residue at position 155 or the analogous positions in their

GP64 proteins, were able to migrate into the nuclei of various lepidopteran cell lines (Sf9, BmN-4, Tn368, High Five, Se301 and SpLi) (unpublished). In contrast, with the exception of homologous cell line BmN-4, BmNPV was unable to enter the nuclei of any of the cell lines examined, despite successful viral internalization into the cells (unpublished). These results demonstrate that a single amino acid substitution may be responsible for the differences in membrane fusion activity observed between Bm-GP64 and Ac-GP64, and suggest that Ac-GP64 H155 may somehow facilitate the low-pH-triggered membrane fusion reactions between viral envelopes and endosomal membranes.

Ac-GP64 H155 has not been previously implicated in any of the important functions of GP64 protein. Ac-GP64 H155 is not localized to either the fusion domain or within the oligomerization domain, both of which have been identified as playing key roles in membrane fusion activity (Monsma and Blissard, 1995). Rather, we found that Ac-GP64 H155 is located within another conserved region found in the majority of group I NPV GP64 proteins (Supplementary Fig. 4). Recent analyses of Ac-GP64 revealed a putative receptor-binding domain in the region spanning amino acids 21 to 159 and identified two amino acid residues, F153 and H156, as essential for receptor-binding and low-pH-triggered membrane fusion (Zhou and Blissard, 2008). These analyses with H155V and H155A substitutions also demonstrated that H155 is not critical for BV binding to host cells, syncytium formation activity and viral multiplication of AcMNPV in Sf9 cells (Zhou and Blissard, 2008). It is also demonstrated that H155F substitution shows similar fusion activity to the wt (Kadlec et al., 2008). Thus, more detailed analyses are required to explore the functional role of Ac-GP64 H155 in membrane fusion reactions. Specific histidine residues have been demonstrated to play a critical role in low-pH-triggered fusion reactions in other enveloped viruses, such as the Semliki Forest virus and vesicular stomatitis virus, suggesting that the protonation of histidine residues may be involved in membrane fusion (Carneiro et al., 2003; Chanel-Vos and Kielian, 2004; Qin et al., 2009).

GP64 has also been shown to be essential for virus multiplication, viral attachment to cells, membrane fusion during viral entry and efficient virion budding required for cell-to-cell infection (Hefferon et al., 1999; Monsma et al., 1996; Oomens and Blissard, 1999). Our analyses using rAcMNPVs revealed that a single amino acid substitution in the GP64 protein alters low-pH-triggered membrane fusion activity, significantly affecting the multiplication and cell-to-cell spread of progeny viruses. Plaque assay revealed that rAcMNPV$^{Ac\text{-}GP64H155Y}$ and rAcMNPV$^{Bm\text{-}GP64wt}$ did not produce obvious plaques, while rAcMNPV$^{Bm\text{-}GP64Y153H}$ produced clear plaques, although slightly smaller than those produced by rAcMNPV$^{Ac\text{-}GP64wt}$. These findings indicate that the multiplication and cell-to-cell spread of progeny viruses decrease upon H155Y substitution in Ac-GP64 and increase upon Y153H substitution in Bm-GP64. Experiments performed in the presence of ammonium chloride further revealed that rAcMNPV$^{Bm\text{-}GP64wt}$ and rAcMNPV$^{Ac\text{-}GP64H155Y}$, which exhibited reduced membrane fusion activity, are more sensitive to ammonium chloride treatment than rAcMNPV$^{Ac\text{-}GP64wt}$ and rAcMNPV$^{Bm\text{-}GP64Y153H}$, which have a higher membrane fusion activity. Given that ammonium chloride treatment prevents acidification of endosomes, these results, together with the observation that both BmNPV and AcMNPV readily enter the cell cytoplasm, indicate that single amino acid substitutions in the GP64 protein affect viral multiplication during nucleocapsid release into the cytoplasm from the endosome of infected cells, which requires membrane fusion between virus envelopes and endosomal membranes.

The envelope glycoproteins of two tick-borne arboviruses, the Thogoto (THO) and Dhori (DHO) viruses, share an approximately 30% overall identity in amino acid sequence with the GP64 proteins of AcMNPV and *Orgyia pseudotsugata* MNPV (Morse et al., 1992). These glycoproteins and GP64 proteins share several highly conserved regions, including the region in which Ac-GP64 H155 is located. Interestingly, Bm-GP64 and the glycoproteins from the THO and DHO viruses also share a tyrosine residue at the position corresponding to Ac-GP64 H155 (Supplementary Fig. 4). Studies employing syncytium formation assay have shown that transient expression of the THO virus glycoprotein GP75 is unable to mediate low-pH-triggered membrane fusion in Sf9 cells. In contrast, low-pH-triggered membrane fusion is readily observed in Chinese hamster ovarian cells transiently expressing GP75 protein, suggesting that the GP75 protein is somehow incompatible with Sf9 cells (Lung et al., 2003). Whether substitution of the THO virus GP75 tyrosine residue with histidine residue enables GP75 to increase low-pH-triggered membrane fusion activity in Sf9 cells remains unclear. It is also unknown whether retaining the tyrosine residue in place of the histidine residue in the envelope protein at the expense of higher membrane fusion activity is beneficial for these viruses.

Materials and methods

Cells and viruses

BmN-4 cells (Maeda, 1989) and BM-N cells (Volkman and Goldsmith, 1982) isolated from *Bombyx mori* and Sf9 cells from *Spodoptera frugiperda* were cultured at 28 °C in TC100 medium (JRH Biosciences) supplemented with 10% fetal bovine serum (FBS). AcMNPV E2 (Smith and Summers, 1978), BmNPV N9 (Nagamine et al., 1989), and *gp64* recombinant AcMNPVs generated from the bacmid (Invitrogen) were used. A working stock of BmNPV was obtained in BM-N cells, whereas working stocks of AcMNPV and recombinant AcMNPVs were propagated in Sf9 cells.

Transfection

DNA in 150 µl of serum-free SF-900II medium (Invitrogen) was mixed with the same volume of 10% Lipofectin reagent (Invitrogen) in a polystyrene tube, incubated at room temperature for 30 min and diluted to 1.5 ml with serum-free SF-900II medium. Monolayer cultures consisting of 0.8×10^6 cells in 12.5 cm^2 flasks (Falcon 3018) or 35-mm dishes (Falcon 3001) were overlaid with the DNA mixture and incubated for 4–5 h at 28 °C. The transfected cells were washed three times with SF-900II medium and incubated in 2.5 ml of TC100 medium.

Construction of expression vectors

The expression vectors pHyHr6IE1/AcGP64HA and pHyHr6IE1/BmGP64HA were constructed from pHyHr6IE1/EGFP (Ishikawa et al., 2004) by replacing its *egfp* open reading frame (ORF) with those of *ac-gp64* and *bm-gp64*, respectively, tagged with hemagglutinin (HA) at the C-terminus (see Supplementary Fig. 1A). For gene replacement, *ac-gp64* and *bm-gp64* ORFs were amplified by PCR using genomic DNAs from AcMNPV and BmNPV, respectively, as templates and specific paired primers (Supplementary Table 1). PCR products tagged with HA were inserted into pBluescript II (Stratagene) and digested with *Nco*I/*Xho*I and *Xho*I/*Xba*I. These DNA fragments were simultaneously inserted into the *Nco*I–*Xba*I sites of pHyHr6IE1/EGFP.

For chimeric mutagenesis, *ac-gp64* and *bm-gp64* ORF sequences were separated into five regions by the restriction endonucleases *Nar*I, *Rca*I, *Sac*II and *Xho*I, and each region was substituted with the analogous regions from the ORF of either *ac-gp64* or *bm-gp64*. The resultant chimeric-mutated *gp64* ORFs were inserted into *Nco*I–*Xba*I sites of pHyHr6IE1/EGFP. Vector construction was verified using nucleotide sequence analysis.

For site-directed mutagenesis of *ac-gp64* and *bm-gp64*, specific primers were designed and *gp64* genes were amplified by PCR using vectors containing *ac-gp64ha* or *bm-gp64ha* as a template and paired primers in appropriate combinations (Supplementary Table 1). PCR

products were self-ligated and the six site-directed mutated *gp64* genes obtained were verified by nucleotide sequence analysis. The mutated *gp64* genes were inserted into the *Nco*I–*Xba*I sites of pHyHr6IE1/EGFP.

Syncytium formation assay

The membrane fusion activities of Ac-GP64, Bm-GP64 and mutated GP64 proteins were determined using syncytium formation assay as described previously (Blissard and Wenz, 1992). Semiconfluent monolayer cultures of Sf9 cells (1×10^6) in 12.5 cm^2 flasks were transfected for 4 h with 1 or 2 μg of GP64 protein expression vectors using Lipofectin (Katou et al., 2001). At 24 h post-transfection, cells were exposed to TC100 medium at pH ranging from 3.75 to 6.0 for 5 min, and incubated for 4 h in standard TC100 medium at pH 6.2. Syncytium formation assay of BmN-4 cells infected with AcMNPV and BmNPV was carried out in the same manner as for the transfected Sf9 cells. Syncytium formation was examined using light microscopy at 4 h after the low pH treatment.

Immunocytochemistry

Immunocytochemistry was carried out as described previously (Katou et al. 2006). Sf9 cells (1×10^6) seeded onto 22-mm coverslips (Matsunami, Osaka, Japan) placed in 6-well plates (Falcon 3046) were maintained at 28 °C overnight. The cells were then transfected with either pHyHr6IE1/AcGP64HA or pHyHr6IE1/BmGP64HA and cultured at 28 °C for 24 h. Transfected cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS: 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 10 min at room temperature and were washed three times with PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The fixed cells were then permeablized in 0.2% Triton X-100 in PBS and washed three times with PBS. The cells were incubated in PBS containing 5% bovine serum albumin (BSA) for 30 min and immunolabelled for 60 min with anti-GP64 monoclonal antibody (Clontech) diluted to 1:2000 in PBS containing 2% BSA and 0.1% tween 20 (PBS-BT), washed three times with PBS-BT, and reacted with AlexaFluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probes) in PBS-BT. Cells were then washed three times with PBS-BT and incubated with propidium iodide (PI) and RNase A in PBS for 30 min at 37 °C and mounted in FluoroGuard (BioRad). Cells were imaged using a confocal laser-scanning microscope (Zeiss LSM510).

Cell surface enzyme-linked immunosorbent assay (cELISA)

cELISA was performed as described previously (Li and Blissard, 2009) with minor modifications. Briefly, Sf9 cells (1.5×10^5 cells/well) in a 24-well plate were transfected with pHyHr6IE1/AcGP64HA or pHyHr6IE1/BmGP64HA, incubated for 24 h and fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature. The fixed cells were blocked by 1% gelatin in PBS for 2 h at 27 °C and reacted with anti-GP64 monoclonal antibody (Clontech) diluted to 1:1000 in PBS containing 0.5% gelatin for 45 min at 27 °C, followed by reaction with HRP-conjugated goat anti-mouse IgG (Zymed) diluted to 1:750 in PBS containing 0.5% gelatin for 45 min at 27 °C. The peroxidase activity was determined using QuantaBlu™ fluorogenic peroxidase substrate kit (PIERCE) according to the manufacturer's instructions. A 100 μl aliquot of the reaction mixture was each transferred to a 96-well plate and accumulation of fluorescent product was monitored using Fluoroskan Ascent (Thermo Scientific) with an excitation wavelength of 325 nm and an emission wavelength of 420 nm.

Disruption of ac-gp64 gene in the bacmid

To generate *gp64* recombinant AcMNPVs, the Bac-to-Bac bacmid system was used and the *ac-gp64* gene was initially deleted from the bacmid as described previously (Kitagawa et al., 2005; Lung et al., 2002, 2003). Disruption of the *gp64* gene in the bacmid was performed by homologous recombination in Sf9 cells (Kitagawa et al., 2005). The *zeocin*-resistant gene from pIZ/V5-His (Invitrogen) was inserted into the *Spe*I–*Bgl*II site of pAc64Eco/Nhe containing the *Eco*RI–*Nhe*I fragment of AcMNPV genome, in which the *ac-gp64* gene and flanking regions were located. The resulting plasmid, pAc64Eco/Nhe[Zeocin], was linearized by digestion with *Eco*RI and *Sca*I, and 500 ng of the linearized *Eco*RI–*Sca*I fragment containing the *zeocin*-resistant gene and 500 ng of bMon14272 (bacmid) were co-transfected into 1×10^6 Sf9 cells. At 48 h post-transfection, DNA was purified from the transfected cells and transformed into *Escherichia coli* DH10B competent cells (Invitrogen). Bacmid DNA was then purified from *E. coli* colonies resistant to zeocin and kanamycin and the recombination of the *gp64* locus was verified by Southern hybridization analysis using the entire *ac-gp64* gene fragment as a probe. A colony transformed with the *gp64*-null bacmid was additionally transformed with the helper plasmid pMon7124 (Invitrogen) that encoded the Tn7 transposon to generate the *E. coli* strain DH10B/64KO-Bac/helper.

Donor plasmid construction and generation of gp64 recombinant AcMNPV

For the transposition of wt and mutated *gp64* genes into the *gp64*-null bacmid, donor vectors containing the *gp64* and *egfp* genes under the control of the *ac-gp64* and *ac-ie1* promoters, respectively, were constructed from pFastBac1 (Invitrogen). The region containing the *ac-ie1* gene was amplified by PCR using the AcMNPV genome as a template and two oligonucleotides, AcIE1pl and AcIE1PA1, as the paired primers (Supplementary Table S1). The PCR product was then inserted into the *Eco*RV site of pBluescript II (stratagene) to generate pBS/AcIE1. To excise the *ac-ie1* ORF from pBS/AcIE1, pBS/AcIE1 was used as a template for PCR using AcIE1R and AcIE1PA2 as the paired primers (Supplementary Table S1). This PCR product was self-ligated and digested with *Acc*I to obtain the cassette for the *ac-ie1* promoter and the polyA signal. This cassette was then inserted into the *Acc*I site of pFastBac1, generating pFastBac[IE1prom]. The *gp64* promoter was amplified by PCR using the AcMNPV genome as a template and AcGP64aI and AcGP64pR1 as the paired primers (Supplementary Table S1). This PCR product was digested with *Sal*I and *Not*I and inserted into the *Sal*I–*Not*I site of pFastBac[IE1prom], generating pFastBac[IE1prom/GP64prom]. For the construction of pFastBac[EGFP/GP64prom], pBac[3xP3-EGFPafm] (Horn and Wimmer, 2000) was digested with *Nco*I and *Not*I and end-filled with T4 DNA polymerase. The resulant fragment containing the *egfp* gene was then inserted into the *Sac*I site of pFastBac[IE1prom/GP64prom] which had previously been digested with *Sac*I and end-filled with the T4 DNA polymerase. For the insertion of pFastBac[EGFP/GP64prom] downstream of the *ac-gp64* promoter, four HA-tagged wt and point-mutated *gp64* genes were obtained from previously described expression vectors by restriction endonuclease digestion, and these *gp64* genes were inserted into the *Nco*I–*Xho*I sites of pFastBac[EGFP/GP64prom], generating the *gp64* donor vectors pFastBac[Ac64wt], pFastBac[Ac64H155Y], pFastBac[Bm64wt] and pFastBac[Bm64Y153H].

These four *gp64* donor vectors were used for the transformation of DH10B/64KO-Bac/helper to introduce *gp64* genes into the *gp64*-null bacmid, and transposition of the *gp64* genes into the *gp64*-null bacmid was verified by PCR. The bacmids were transfected into Sf9 cells to generate *gp64* recombinant AcMNPVs. Transfected cells and partially purified virions were subjected to immunoblot analysis to confirm the expression of GP64 proteins. To produce *gp64*-null AcMNPV, pFastBac/AcIE1p/AcGP64p was used and the generated bacmid was transfected into Sf9 cells.

Ammonium chloride treatment

Pre-chilled BmN-4 cells were infected with *gp64* recombinant AcMNPV at 4 °C for 60 min, washed three times with fresh TC100 medium and cultured in the presence of different concentrations (0, 1.25, 2.5, 5, 10, and 25 mM) of ammonium chloride. At 24 h post-infection, the infected cells treated with ammonium chloride were lysed in cell lysis buffer (MBL) and EGFP expression was quantified using a fluorescence microplate reader (Fluoroskan Ascent microplate fluorometer, ThermoElectron Corporation).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Katou et al., 2006). Polypeptides from the infected and transfected cells were resolved by SDS-polyacrylamide gel electroporesis and transferred onto an Immobilon-P transfer membrane (Millipore). The membrane was immunolabelled with monoclonal antibodies against Ac-GP64 (Clontech) and HA (HA. 11; Babco) and reacted with HRP-conjugated goat anti-mouse IgG antibody (Zymed). Polyclonal antibody against AcMNPV/BmNPV VP39 was raised in rabbits against a partial amino acid sequence of AcMNPV/BmNPV VP39 as described previously (Katou et al., 2006) and used for immunoblot analysis, together with HRP-conjugated goat anti-rabbit IgG antibody (Zymed). Signals of GP64, HA and VP39 were detected by ECL Western blotting detection reagents (Amersham Biosciences). For the analysis of GP64 proteins for oligomerization state, polypeptides were resolved by non-reducing SDS-polyacrylamide electrophoresis as described previously (Monsma and Blissard, 1995) and processed for immunoblotting as described above.

Plaque assay

Plaque assay of rAcMNPVs harboring *egfp* and mutated *gp64*s was perfomed in Sf9 cells as described previously (Katou et al., 2006; Nagamine et al., 1989). Semiconfluent monolayer cultures consisting of 0.8×10^6 cells were prepared in 35-mm culture dishes and inoculated with 200 µl of virus suspensions to be examined. After a 60-min adsorption period, the inoculum was removed from the cultures and replaced by the overlay medium containing 0.75% SeaPlaque GTG agarose (FMC Bioproducts) in TC100 medium. These cultures were tightly sealed by parafilm, incubated for 6 days at 28 °C and examined for plaque formation under a fluorescence microscope.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.04.028.

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