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Analysis of Fusogenic Activity of *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) gp64 Envelope Glycoprotein

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The baculovirus gp64 glycoprotein is a major component of the envelope of budded virus (BV) and has been shown that it plays an essential role in the infection process, especially virus-cell membrane fusion. We have cloned *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) *gp64* gene and expressed transiently in transfected insect cells. The cells expressing gp64 protein were examined for membrane fusion activity by using a syncytium formation assay under various conditions. The optimal conditions required for inducing membrane fusion are 1) from pH 4.0 to 4.8 2) 15 min exposure of cells to acidic pH 3) at least 1 μg of gp64 cloned plasmid DNA per 3×10^6 cells 4) and an exposure of cells to acidic pH at 72 h post-transfection. In order to investigate the role of hydrophobicity of the gp64 glycoprotein for the membrane fusion, the two leucine residues (amino acid position at 229 and 230) within hydrophobic region I were substituted to alanine by PCR-derived site-directed mutagenesis and the membrane fusion activity of the mutant was analysed. The gp64 glycoprotein carrying double alanine substitution mutation showed no significant difference in fusion activity. This result suggested that minor changes in hydrophobicity at the amino acid position 229 and 230 does not affect the acid-induced membrane fusion activity of the gp64 glycoprotein.

Key words: AcMNPV, gp64, membrane fusion, hydrophobicity

Enveloped animal viruses enter the host cell either by direct fusion between viral envelope and plasma membrane or by cell-mediated endocytosis followed by fusion between viral envelope and endosomal membrane. The viral envelope protein located in virus envelope or infected cell membrane mediates fusion for both viral envelope and cell membrane. In the influenza virus, the best-characterized example of viruses that enter cells by endocytosis, fusion activity of the hemagglutinin protein, is activated by the acidification of the endosome, resulting in fusion of the viral envelope with the endosome membrane (3).

Viral fusion protein activity is most often triggered by acidic pH within the endosome. Irreversible changes in conformation of the fusion protein induced by acidic condition is required for membrane fusion (11). Viral fusion proteins often contain hydrophobic domains or fusion peptides that are important for membrane fusion activity. These hydrophobic domains presumably interact with the hydrophobic lipid interior of the host cell membranes and are typically conserved within virus families.

The baculovirus *gp64* envelope glycoprotein is the major envelope associated protein of budded virus (BV). During infection, the gp64 envelope glycoprotein is synthesized and transported to the plasma membrane (21). As progeny viruses bud out from the plasma membrane, they acquire a plasma membrane-derived envelope containing the gp64 protein (1, 8). The native gp64 is a phosphoglycoprotein and is found on the surfaces of infected cells and virus envelope as a homo-oligomers (21, 19).

Monoclonal antibodies directed against gp64 glycoprotein can neutralize budded virion infectivity (8, 10). It was also demonstrated that a neutralizing monoclonal antibody directed gp64 glycoprotein, AcV1, does not interfere with binding or the uptake of virions into host cells but blocks infection at a later stage (20). This result indicated that gp64 is responsible for both binding of virus to the cell surface and for fusion of the viral envelope with the endosomal membrane after endocytosis. This fusion results in uncoating of the viral nucleocapsid into the cytoplasm of the cell and the virus replication

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Table 1. Sequence and position of primers used for PCR. The primer sequence were designed from the published sequence of AcMNPV gp64 (23)

Primer	Sequences (5'→3')	Size	Position (5'→3')
P1	TAGCCATCGTGATCGCCGTC	20 nt	−292∼−273
P2	TGCGGTTCTTTTAGTGGGTTA-TGGTG	26 nt	+1926→+1901
M1	TGCGTGTGCGGCCATTAAAGA-TGACA	26 nt	+732→+757
M2	TGTCATCGGGAATGGCCGCA-CACGCA	26 nt	+757→+732

is initiated.

The gene encoding the gp64 glycoprotein has been identified and sequenced in two baculoviruses, *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) and AcMNPV. Although the regions of gp64 involved in the membrane fusion have not been definitely identified, it was shown that two functional domains of OpMNPV gp64 glycoprotein are responsible for the membrane fusion activity (14). In AcMNPV, it is possible that the gp64 glycoprotein has hydrophobic regions that are responsible for fusion activity like OpMNPV. The amino acid sequence of gp64 glycoprotein is highly conserved between AcMNPV and OpMNPV (78%). Furthermore, the predicted ectodomains are approximately 83% conserved in amino acid sequence. Potential N-glycosylation sites, cysteine residues, and potential acylation sites are also conserved (17).

In this study, we developed a system for transient expression of the gp64 glycoprotein in uninfected Sf21 cells and examined the fusion activity of the AcMNPV *gp64* glycoprotein in the absence of other viral proteins. We have cloned AcMNPV gp64 gene and determined the optimal conditions for inducing membrane fusion in gp64 transfected insect cell. In addition, in order to investigate the role of hydrophobicity for membrane fusion, the two leucine residues (amino acid position at 229 and 230) within hydrophobic region were mutated to alanine by PCR and the membrane fusion activity of the mutant was analysed.

Materials and Methods

Virus and Cell

Spodoptera frugiperda (Sf21) cells were used for transfection studies and for AcMNPV DNA preparation. Sf21 cells were cultured at 27℃ in Grace's medium (Gibco) supplemented with 8% fetal bovine serum and antibiotics (18). For virus propagation, monolayer of Sf21 cells (3×10^6 cells/ 60×15 mm dish) were infected with AcMNPV

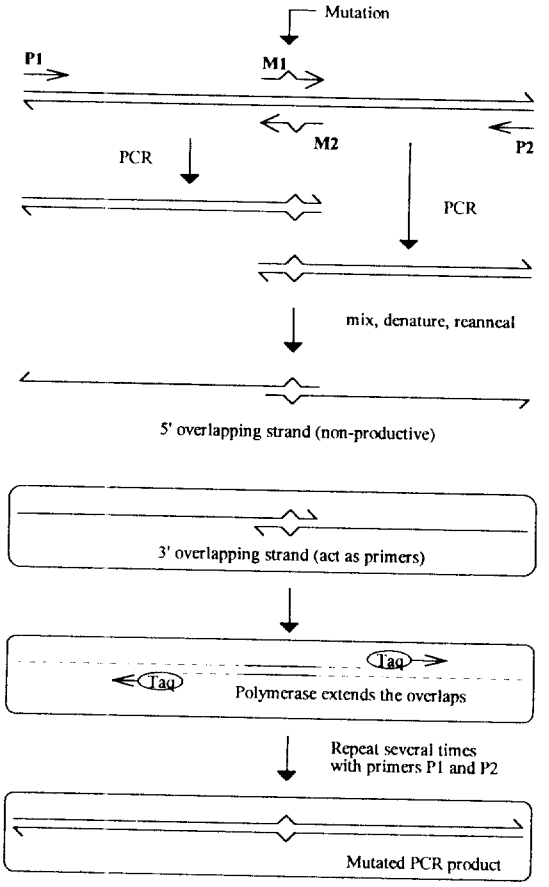


Fig. 1. Schematic representation of the strategy for PCR-derived site-directed mutagenesis.

at 27℃ for 1 h with gentle rocking, and incubated at 27℃ for 36 h after the replacement of virus infection media with fresh Grace's media.

Polymerase chain reaction (PCR)

Sequence and location of the primers used for PCR are listed in Table 1. PCR was performed in a 100 μl reaction mixture containing 20 pmol of each primer, 20 mM Tris-HCl, pH 8.7, 40 mM KCl, 1 mM $MgCl_2$, 200 μM each of the four dNTPs, 1 μg of template AcMNPV DNA and 2 units of *Thermus caldophilus* (*Tca*) DNA polymerase (15). The reaction mixture covered with 50 μl mineral oil to prevent evaporation was subjected to the following reaction cycle. After initial denaturation at 95℃ for 5 min, denaturation at 95℃ for 1 min, annealing at 55℃ for 2 min and polymerization at 67℃ for 3 min were performed for 30 cycles, followed by final polymerization at 67℃ for 7 min. PCR products were then separated on 0.9% agarose gel electrophoresis.

Construction of pGP64

PCR product was cloned directly into pT7Blue vector

(Novagene). Ligation was performed in 10 μl reaction mixture contaning 20 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.5 mM ATP, and 2~3 Weiss units of T4 DNA ligase for 4 h at 16℃.

Site-directed mutagenesis

The concept of joining two DNA fragments together by overlapping extension provides the key to introducing mutations into the centre of a PCR fragment, as shown in Fig. 1. PCR was used to generate substitution mutations near the overlapping ends of the targeted fragments. When these fragments are combined in an overlap extension reaction, the mutation is placed inside the product molecule. Sequence and location of the primers used for mutagenesis are listed in Table 1.

PCR was performed in two separate tubes. One contained M1 (mismatch primer for mutation) and P2 primer, the other contained M2 (mismatch primer for mutation) and P1 primers. PCR was performed in 100 μl reaction mixture as described above.

After the amplification reaction, the PCR products were electroeluted for purification. In a new tube, the reaction mixtures, containing 100 ng of the first two PCR products, 20 mM Tris-HCl, pH 8.7, 40 mM KCl, 1 mM $MgCl_2$, 200 μM each of the four dNTPs, and 2 units of *Tca* DNA polymerase, were mixed for the overlap extension reaction. After initial denaturation at 95℃ for 5 min, denaturation at 95℃ for 1 min, annealing at 45℃ for 2 min and polymerization at 67℃ for 3 min were performed in a thermal cycler for 10 cycles. In this reaction, the first two PCR products acted as primers. After adjusting annealing temperature to 50℃, 20 pmol of primers P1, P2, and 2 units of *Tca* DNA polymerase were added to the reaction tube and additional 20 cycles of reactions were performed.

Cloning of the mutated PCR product

Both the mutated PCR product and pGP64 were digested with *Xho*I and *Bgl*II. The 1154-bp *Xho*I-*Bgl*II restriction fragment of the PCR product bearing the desired mutation was ligated into the *Xho*I-*Bgl*II digested pGP64 fragment and the resulting recombinant was named pGP 64-mu. The mutation in pGP64-mu was confirmed by sequencing.

Transfection

Transfection was performed in 25 cm^2 culture flasks containing 3×10^6 Sf21 cells by calcium phosphate precipitation method (18). pGP64 DNA in 0.95 ml of 1X HEBS/salmon sperm DNA (15 μg/ml salmon sperm DNA, 0.137 M NaCl, 6 mM D-glucose, 5 mM KCl, 0.7

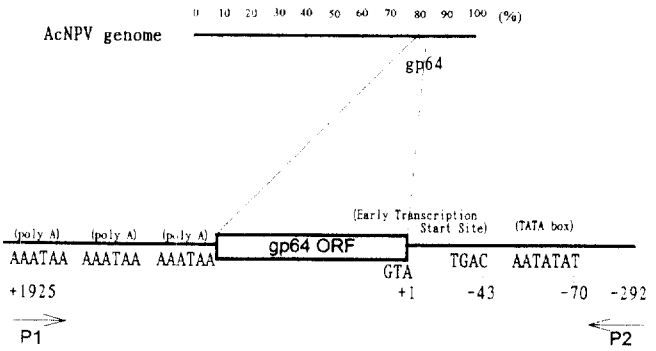


Fig. 2. Schematic representation of the structure and location of gp64 gene on AcNPV genome (23). The 5′ and 3′ untranslated regions are described as thin line and the promoter and poly A tailing signal sequences are inserted. The binding sites and direction of the primers used for PCR are indicated by arrow.

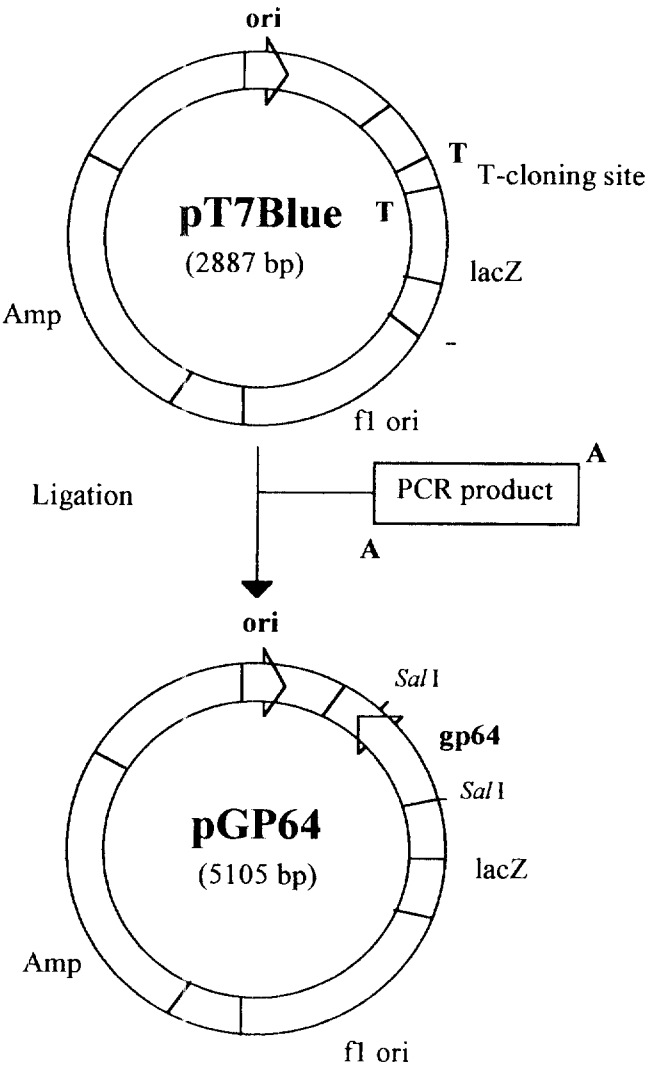


Fig. 3. Schematic representation of gp64 cloning strategy and physical map of pGP64. The 2.2 kb-long PCR product was cloned into the T-cloning site of the pT7Blue vector.

mM Na_2HPO_4, 20 mM HEPES, pH 7.0) was precipitated at room temperature for 30 min after the addition of 50 μl of 2.5 M $CaCl_2$. One ml of the precipitated DNA mixture was added slowly to the flasks. After 4 h of incubation at 27℃, it was replaced with fresh Grace's media and incubated 27℃ till membrane fusion assay was performed.

Membrane fusion assay

Transfected Sf21 cells were rinsed twice with Grace's medium without serum and then incubated in medium with pH 4.5 for 10 min at room temperature. The acidic medium was replaced with fresh Grace's media at pH 6.1 (11) and were incubated at 27℃ for 12 h. Membrane fusion was assayed by the formation of syncytium observed under the microscope.

Results

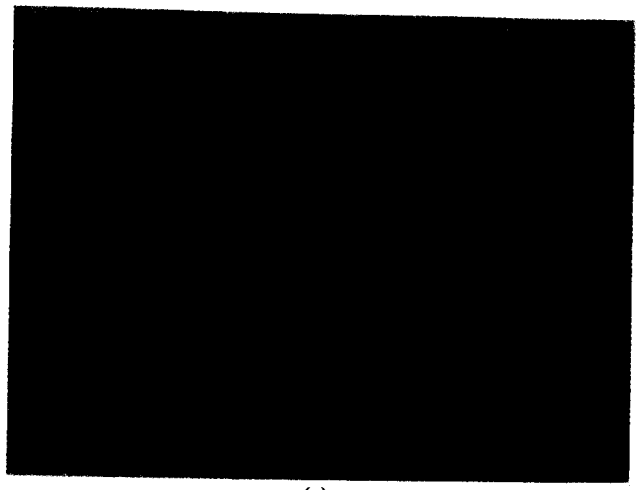
Construction of pGP64

The baculovirus gp64 glycoprotein is required for budded virus entry into host cells (8, 17) and uncoating through acid-mediated membrane fusion. To investigate gp64-mediated membrane fusion, AcMNPV *gp64* gene was cloned and expressed transiently in Sf21 cells. The entire coding region of *gp64* as well as 5′ and 3′ flanking sequences were amplified by PCR using primers binding at −292 and +1926 (Table 1) (23). The amplified 2.2 kb DNA fragment includes 292 nucleotides upstream from the AUG codon of *gp64*, entire *gp64* ORF, and approximately 390 nucleotides downstream from the termination codon of the *gp64* ORF including the polyadenylation signal (Fig. 2).

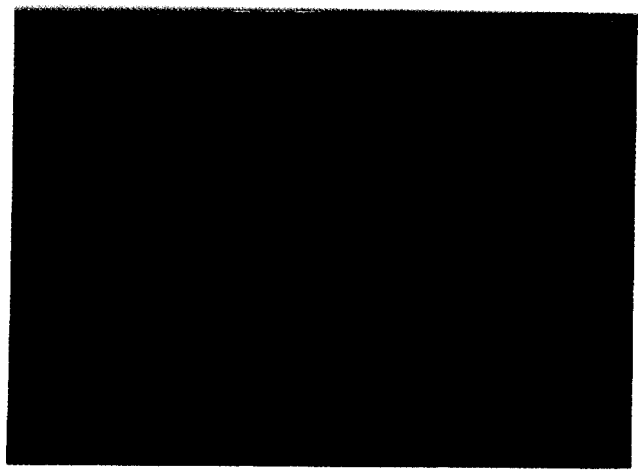
The PCR product was cloned into the T-cloning site of the pT7Blue vector and the resultant recombinant was named pGP64 (Fig. 3). The pGP64 has two *Sal*I sites; one is located at the cloning site of the pT7Blue T-vector and the other is located at downstream of the *gp64* gene.

gp64-mediated membrane fusion

Previous studies showed that the gp64 glycoprotein is readily detected on the surface of baculovirus-infected cells (11). Therefore, we used Sf cells infected with AcMNPV as positive control for gp64-mediated fusion activity. Infected cells (72 h post-infection) were incubated in Grace's medium adjusted to a pH of either 4.5 or 6.1 and observed for syncytium formation. Cell fusion was readily observed in AcMNPV-infected Sf cells shifted to pH 4.5, but not when the pH was maintained at pH 6.1 (Data not shown). Although some membrane



(a)



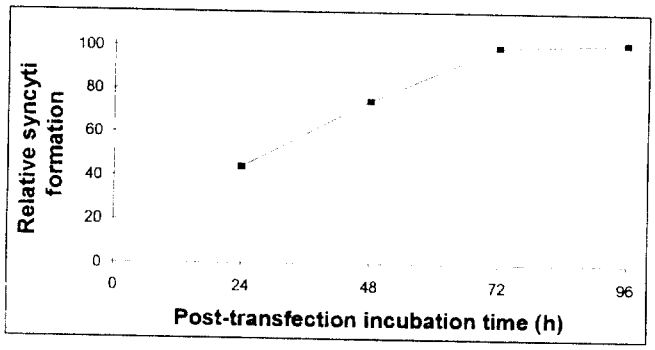
(b)

Fig. 4. AcMNPV gp64-mediated membrane fusion and pH dependence of syncytium formation in cells expressing gp64. SF21 cells were transfected with plasmid pBluscript II (A) or pGP64 (B). At 72 h post-transfection, cells were exposed to pH 4.5 for 10 min and syncytium formation was observed under the microscope. No cell fusion was observed in pBluescript II transfected cells (A) but syncytia were observed in pGP64 transfected cells (B).

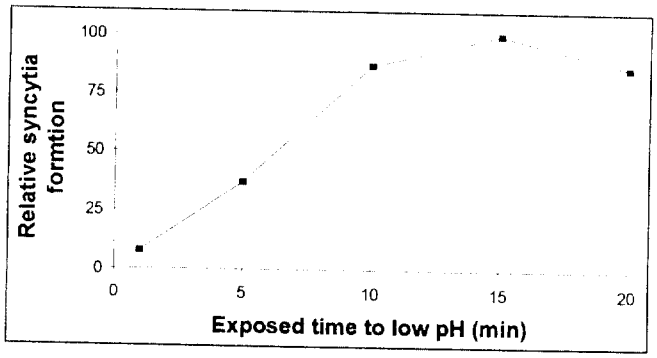
fusion were observed at earlier times, a substantial degree of fusion had occured by 4 h after exposing the cells to the acidic pH. Fused cells were distinguished from cell aggregates by the loss of the normal plasma membrane between adjacent cells and the formation of polynucleate syncytia.

To determine whether gp64 glycoprotein (expressed in the absence of other viral protein) is sufficient to induce membrane fusion, pGP64 transfected Sf21 cells were incubated in Grace's media for 72 h, exposed briefly to acidic pH, and then observed for syncytium formation under the microscope.

The results of the experiment to examine gp64-me-

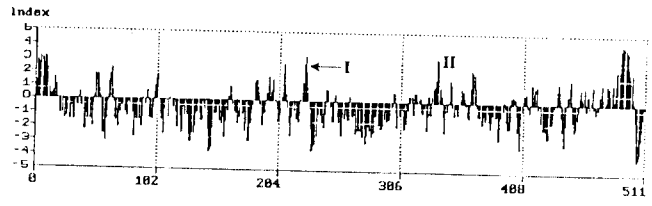


(a)

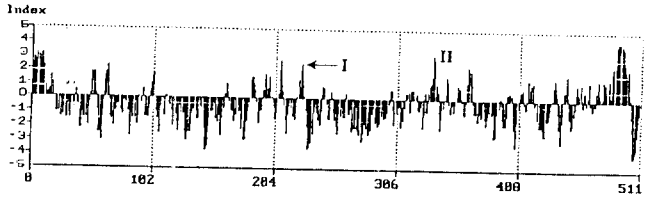


(b)

Fig. 5. Relative level of syncytia formation in pGP64 transfected cells. After transfection, cells were incubated for various time length and exposed to pH 4.5 (A). Transfected cells were incubated for 72 h and exposed to pH 4.5 for various time length (B).



(a)



(b)

								229	230			
Thr	Arg	Gln	Ile	Lys	Ala	Ala	Cys	Leu	Leu	Ile	Lys	WT
ACG	CGC	CAA	ATA	AAA	CGT	GCG	TGT	CTG	CTC	ATT	AAA	
-	-	-	-	-	-	-	-	Ala	Ala	-	-	Mutant
								gcG	gcC			

(c)

Fig. 6. Hydrophobicity profile of AcMNPV gp64 glycoprotein. Kyte hydrophobicity profiles for both wild type (A) and mutant (B) AcMNPV gp64 glycoprotein were obtained by using the PROSIS program. Two hydrophobic regions in the ectodomain are indicated. The vertical scale indicates Kyte hydrophobicity values, and number on the horizontal scale represent gp64 glycoprotein amino acid positions. The nucleotide and amino acid sequences of the part of hydrophobic region I where the two leucine residues are mutated into alanines are shown (C).

diated membrane fusion in Sf21 cells is shown in Fig. 5. As a control, Sf21 cells were transfected with the pBluescript II DNA and exposed to pH 4.5 at 72 h post-transfection. No cell fusion was observed in pBluscript II transfected cells (Fig. 4A) and in untransfected Sf21 cells (Data not shown). However, pGP64 transfected Sf21 cells were found to be able to mediate membrane fusion after the cells were exposed to acidic media (pH 4.5) (Fig. 4B). These results indicated that the gp64 glycoprotein is sufficient to mediate membrane fusion and that the fusion is dependent on acidic pH.

Optimal conditions required for membrane fusion

To determine optimum conditions required for *gp64*-mediated membrane fusion in insect cells, several parameters for fusion activity were examined. Sf21 cells were transfected with increasing quantities of pGP64 (0. 5, 1, 5, 10 μg of pGP64 DNA per 2.5×10^6 Sf21 cells) and were exposed to pH 4.5 at 72 h post-transfection. One μg of pGP64 DNA per 2.5×10^6 cells was sufficient for getting efficient membrane fusion activity. Since transfection with upto 10 μg of pGP64 DNA did not increase syncytium formation, 1 μg of pGP64 DNA was used for transfection in the rest of the experiments.

To determine optimal incubation period after transfection, Sf21 cells (2.5×10^6 cells) transfected with 1 μg of pGP64 plasmid were incubated for 24, 48, 72, 96 h, respectively. Time zero was defined as the time when plasmid DNAs were added to the cells. The cells were exposed to the acidic pH at indicated time points and membrane fusion activity was measured. Although exposing the pGP64 transfected Sf21 cells to acidic pH at 24 h post-transfection could have induce syncytium formation, maximum level was reached at 72 h post-transfection (Fig. 5A).

The exposure length at acidic pH might be critical for inducing gp64 mediated membrane fusion. The pGP 64 transfected Sf21 cells were exposed to pH 4.5 at 72 h post-transfection for various time periods. Five min of exposure was sufficient to induce membrane fusion but syncytium formation was reached to maximum level at 15 min exposure and decreased at 20 min exposure (Fig. 5B). In a previous study, when cells expressing OpMNPV *gp64* were incubated at pH values below 4.7 for long periods (4 h), fusion was not readily observed. This is presumably due to adverse effects on the cell

Table 2. pH value required for gp64-mediated membrane fusion*

pH	Syncytium formation
6.0	−
5.5	−
5.3	−
5.0	−
4.8	+
4.5	+
4.0	+

* Duplicate plates of transfected cells were exposed to Grace's medium at indicated pH values for 15 min and then returned to pH 6.1. Membrane fusion was evaluated syncytium formation observed under the microscope.

caused by prolonged exposure to low pH (2).

For viral fusion proteins such as the influenza hemagglutinin protein, membrane fusion is initiated by a pH-induced conformational change in fusion protein that results in the activation of the fusion domain (2, 13, 24) followed by a rapid induction of membrane fusion. The maximal rate for hemagglutinin-mediated fusion with cellular membranes occurred from pH 4.9 to 5.2 (5). To determine the required pH for gp64-mediated membrane fusion, we also examined the pH range required for syncytium formation. To examine precise requirements of the pH for induction of *gp64*-mediated membrane fusion, the transfected Sf21 cells were exposed to various pH range and the fusion activity was examined. Cells transfected with 1 μg of pGP64 were incubated for 72 h, shifted to the given pH values for 15 min, and scored for syncytium formation. No fusion activity was detected at pH values greater than 5.0 (Table 2). This result suggests that the AcMNPV gp64 glycoprotein is a pH-dependent membrane fusion protein and BV entry into the host cell is via endocytosis.

Mutagenesis of *gp64* in hydrophobic region I

The two hydrophobic region of OpMNPV are important functional domains for membrane fusion activity (14). In AcMNPV, it is possible that the gp64 glycoprotein has hydrophobic regions that are responsible for fusion activity like OpMNPV. We examined the predicted hydrophobic profile of AcMNPV gp64 glycoprotein (Fig. 6A) and selected two regions of highest local hydrophobicity in the ectodomain for functional analysis. The first hydrophobic region is composed of 6 hydrophobic residues from amino acids 226 to 231 (Fig. 6A, I, and 6C) and is highly conserved between the AcMNPV, OpMNPV, and *Choristoneura fumiferana* multicapsid nuclear

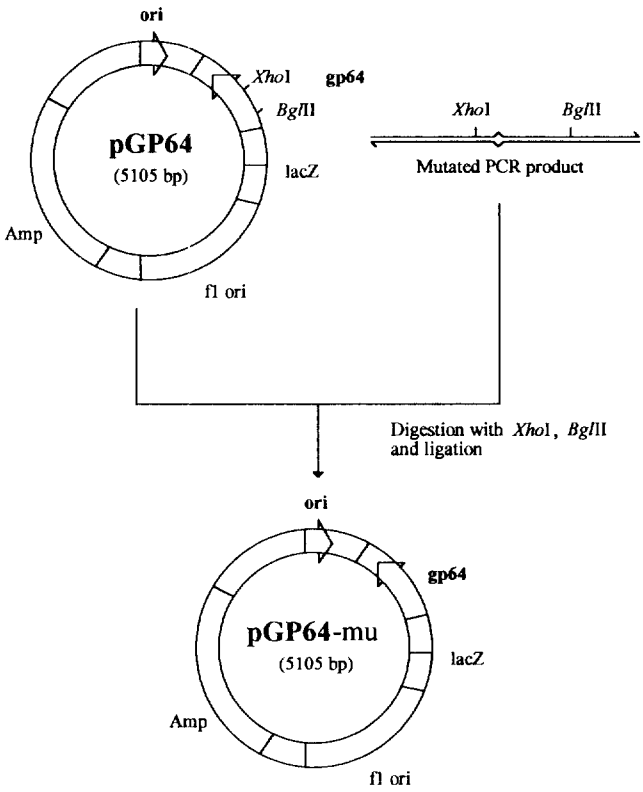


Fig. 7. Schematic representation of the strategy for subcloning of the mutated PCR product. To construct pGP64-mu, the mutated PCR product and pGP64 were digested with *Xho*I and *Bgl*II. The restriction fragment bearing the desired mutation was ligated to the digested pGP64 plasmid.

polyhedrosis virus (CfMNPV) gp64 proteins. The second hydrophobic region is located from amino acids 333 to 341 (Fig. 7A, II) and lies within a conserved portion of the protein which is predicted to form an amphipathic alpha-helix.

The peak of hydrophobicity of region I is contributed by three amino acids, Cys-228-Leu-229-Leu-230 (Fig. 6C). To examine the role of the hydrophobic leucine residues at position 229 and 230, *gp64* mutant with substitution of leucine to alanine at amino acid position 229 and 230 was constructed and named pGP64-mu (Fig. 6A, 6C , and 7). Substitution of leucine to alanine would reduce the overall hydrophobicity of region I but would not expect to change the protein conformation drastically. The pGP64-mu transfected Sf21 cells were examined for acid-induced membrane fusion. No significant changes in fusion activity was observed after treatment of cells at pH 4.5. This result suggests that minor changes of hydrophobicity within region I may not affect the fusion activity.

Discussion

In general, viral fusion proteins are integral membrane glycoproteins and usually contain a stretch of relatively hydrophobic domains or fusion peptides that are important for membrane fusion activity. The fusion peptide of a number of viral fusion proteins such as hemagglutinin of influenza virus, the F protein of simian virus 5, the E1 protein of semliki forest virus, and the gp41 protein of human immunodeficiency virus have been characterized by using mutational analysis of cloned genes (6, 7, 9, 12, 16).

The AcMNPV gp64 glycoprotein fusion domain located at hydrophobic region I proposed in this study is an internal amino acid sequence and is similar in several respects to the internal fusion domains of semliki forest virus (SFV), E1 glycoprotein and vesicular stomatitis virus (VSV) G protein (25). The amino acid sequences of the SFV and VSV fusion domains are highly conserved within the alphaviruses and vesiculoviruses, respectively, as is hydrophobic region I within the three known baculoviruses gp64 proteins; AcMNPV, OpMNPV and CfMNPV. Site-directed mutagenesis of particularly conserved residues in the SFV or VSV fusion domains abolished fusion activity without affecting transport to the cell surface, whereas substitutions of other conserved residues altered the pH optima for fusion activity to more acidic values (25).

The current understanding of the mechanism of protein-mediated membrane fusion is based primarily on the studies of the fusogenic activity of influenza virus hemagglutinin (HA) glycoprotein (3, 22, 24). The fusion peptide of HA contains 24 hydrophobic amino acids located at the NH_2 terminus. Exposure to acidic pH induces an irreversible conformational change in the HA molecule to make its fusion competent. In the fusion-competent conformation, the previously buried hydrophobic fusion peptide of HA is exposed and relocated so that it can interact with the target membrane. Similar to the proposed interaction of the influenza virus HA fusion peptide with the adjacent membrane, hydrophobic region I of baculovirus AcMNPV gp64 glycoprotein may directly interact with membrane lipids during initial stages of fusion reaction.

A spring loaded mechanism of influenza virus HA protein was proposed (3). A sequence with a high propensity for forming a coiled coil was identified adjacent to the hydrophobic fusion peptide. At acidic pH, this sequence forms an extended coiled coil structure that exposes the highly hydrophobic NH_2- terminal peptide and induces fusion by moving the fusion peptide closer to the target membrane. In AcMNPV, the sequence for forming a coiled coil was identified within hydrophobic region II (Fig. 7). Hydrophobic region II is located near the C-terminus end of a strongly predicted amphipathic alpha-helix and extends approximately 302 to 337 amino acids. The amino acid sequence of the predicted alpha-helix contains a long heptad repeat (or leucine zipper), exhibiting one methionine and five leucine residues at every seventh position (14). It is typical structure found in alpha-helices that can form coiled coil.

In several other viral membrane fusion proteins, alpha-helices appear to play direct roles in membrane fusion. Many retrovirus, as well as influenza virus, including the human and simian immunodeficiency viruses, contain a leucine zipper-like repeat in a highly conserved region of the external domain of the transmembrane glycoprotein. Site-directed mutagenesis of these virus glycoprotein indicates that the leucine zippers found in these fusion protein are required for membrane fusion activity (4).

To identify and analyze functional domains in the AcMNPV gp64 glycoprotein, oligomerization, transportation, and surface localization of the mutated gp64 glycoprotein within hydrophobic region I as well as hydrophobic region II should be analyzed.

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