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Oligomerization of the Substitution Mutants of *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) *gp64* Glycoprotein

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The baculovirus gp64 glycoprotein is a major component of the envelope protein of budded virus (BV). It has been shown that the gp64 glycoprotein plays an essential role in the infection process, especially fusion between virus envelope and cellular endosomic membrane. Recently we reported optimal conditions required for gp64-mediated membrane fusion in pGP64 DNA transfected *Spodoptera frugiperda* (Sf9) cells (H. J. Kim and J. M. Yang, *Jour. Microbiology*, 34, 7-14). In order to investigate the role of hydrophobicity within the fusion domain of the gp64 glycoprotein for membrane fusion, 13 mutants which have substitution mutation within hydrophobic region I were constructed by PCR-derived site-directed mutagenesis. Each mutated gp64 glycoproteins was transiently expressed by transfecting plasmid DNA into *Spodoptera frugiperda* (Sf9) cells. Oligomerization of the transiently expressed gp64 glycoproteins was analysed by running them on SDS-polyacrylamide gel electrophoresis under non-reducing condition followed by immunoblotting. All of the mutant gp64 glycoproteins except cysteine-228 were able to form trimers. These results suggest that hydrophobic region I of the gp64 may not be responsible for the oligomerization of the gp64 glycoprotein.

Key words: AcNPV, gp64, mutagenesis, oligomerization

Enveloped viruses generally enter the host cells either by direct fusion between viral envelope and cell membrane or by cell-mediated endocytosis followed by fusion between viral envelope and endosomal membrane. Viral fusion proteins located in the virus envelope or in the infected cell membrane mediate fusion for both viral envelope and host cell membrane. Hemagglutinin (HA) protein in the influenza virus envelope is one of the best characterized viral fusion proteins which is activated by the acidification of the endosome, resulting in the fusion of the viral envelope with the endosome membrane (3,5).

Most commonly, viral fusion proteins have hydrophobic domains (fusion peptides) that are important for viral fusion activity. The hydrophobic lipid interior of the cell membrane presumably interacts with these hydrophobic amino acid residues of fusion proteins. Fusion peptides are typically conserved within but not necessarily between virus families and are located at the internal or N-terminus of the fusion protein.

Baculoviruses, enveloped animal virus containing large double-stranded DNA as a genome, infect invertebrates, mostly insect cells. The baculovirus gp 64 envelope glycoprotein is the major envelope fusion protein in budded virus (BV). During infection, the gp64 is synthesized and transported to the plasma membrane of the host cell (17). Progeny viruses acquire a plasma membrane-derived envelope containing the gp64 protein while budding out from the plasma membrane (1, 7). The native gp64 is a phosphoglycoprotein that is expressed on the surface of infected cells and virus envelope as a homooligomers (16, 17).

The gene encoding gp64 fusion protein has been identified and sequenced in three baculoviruses, *Orgyia pseudotsugata* multiple nuclear polyhedrosis virus (OpMNPV), CfMNPV and AcMNPV. Although the functional domains of gp64 in these baculoviruses have not been completely identified, two functional domains of OpMNPV gp64 are shown to be responsible for the fusion activity (12). The amino acid sequence homology between AcMNPV and OpMNPV is 78% and the predicted ectodomains for fusion activity are about 83% conserved in amino acid sequences. Potential N-glycosylation sites, cysteine residues, and potential acylation sites are also conserved. Thus, it is possible that the gp64 of AcMNPV also contains hydrophobic regions that are responsible for fusion activity similar to that of OpMNPV.

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In a previous study, we developed a system for transient expression of the gp64 glycoprotein by using the gp64 cloned plasmid transfected Sf9 cells and examined the fusion activity of the gp64 glycoprotein by a syncytium formation assay under various conditions. It was demonstrated that exposure of Sf9 cells to acidic pH from 4.0 to 4.8 was required for the maximal membrane fusion activity mediated by gp64 (2). The fusion activities of the two gp64 mutant proteins, of which each of the two leucine residues (amino acid positions at 229 and 230) within hydrophobic region I was substituted to alanine, were not significantly different with that of wild type gp64 protein (9).

Mutant gp64 proteins constructed by amino acid substitution within hydrophobic region or other conserved regions may change the oligomerization state of the proteins and result in an abnormal oligomer formation which may affect the fusion activity. Thirteen different mutant gp64 proteins, of which amino acid sequence near (amino acid positions at 224, 225, 232, and 233) and within hydrophobic region I (amino acid positions at 228, 229, and 230) were substituted, were constructed by PCR-derived site-directed mutagenesis. The mutated gp64 plasmid DNAs were transfected into Sf9 cells and oligomerization of the transiently expressed gp64 glycoproteins were investigated.

Materials and Methods

Virus and Cell

Spodoptera frugiperda (Sf9) cells were used for transfection studies and for preparation of AcNPV DNA. Cells were cultured at 27°C in Grace's medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics (15). For virus propagation, monolayer of Sf9 cells (3×10^6 cells/60×15 mm dish) were infected with AcNPV at 27°C for 1 h with gentle rocking, and incubated at 27°C for 36 h after the replacement of virus infection media with fresh Grace's media.

Site-directed mutagenesis

To obtain mutated gp64 plasmid, overlapping extension method using PCR technology was performed as described (9). Sequence and location of the primers used for mutagenesis are listed in Table 1.

Polymerase chain reaction

PCR was performed in two separate tubes. One contained M1 (mismatch primer for mutation) and

Table 1. Sequence and position of primers used for PCR derived site-directed mutagenesis

Primer	Sequence (5' →3')	Size	Position (5' →3')
P1	TAGCCATCGTGATCGCCGTC	20	-292 →-273
P2	TGCGGTTCTTTTAGTGGGTTATGGTG	26	+1926 →+1901
I224E-1	CAAGAAAAAGCTGCGTGTCTGCTCATT	27	+667 →+694
I224E-2	AATGAGCAGACAGCGAGCTTTTTCTTG	27	+694 →+667
K225L-1	CAAATACTAGCTGCGTGTCTGCTCAT	26	+667 →+693
K225L-2	ATGAGCAGACACGCAGCTAGTATTTG	26	+693 →+667
K225E-1	CAAATAGAAGCTGCGTGTCTGCTCAT	26	+667 →+693
K225E-2	ATGAGCAGACACGCAGCTTCTATTTG	26	+693 →+667
K225R-1	CAAATAAGAGCTGCGTGTCTGCTCAT	26	+667 →+693
K225R-2	ATGAGCAGACACGCAGCTCTTATTTG	26	+693 →+667
C228A-1	AAAAGCTGCGGCTCTGCTCATTAAAGA	27	+672 →+699
C228A-2	TCTTTAATGAGCAGAGCCGCAGCTTTT	27	+699 →+672
L229AL230A-1	TGCGTGTGCGGCCATTAAAGATGACA	26	+678 →+694
L229AL230A-2	TGTCATCGGGAATGGCCGCACACGCA	26	+694 →+678
K232E-1	GCTGCGTGTCTGCTCATTGAAGATGAC	27	+676 →+693
K232E-2	GTCATCTTCAATGAGCAGACACGCAGC	27	+693 →+676
K232L-1	GCTGCGTGTCTGCTCATTCTAGATGAC	27	+676 →+693
K232L-2	GTCATCTAGAATGAGCAGACACGCAGC	27	+693 →+676
K232R-1	GCTGCGTGTCTGCTCATTAGAGATGAC	27	+676 →+693
K232R-2	GTCATCTCTAATGAGCAGACACGCAGC	27	+693 →+676
D233K-1	GCTGCGTGTCTGCTCATTAAAAAAGAC	27	+676 →+693
D233K-2	GTCTTTTTTAATGAGCAGACACGCAGC	27	+693 →+676
D233L-1	GCTGCGTGTCTGCTCATTAAACTTGAC	27	+676 →+693
D233L-2	GTCAAGTTTAATGAGCAGACACGCAGC	27	+693 →+676
D233E-1	GCTGCGTGTCTGCTCATTAAAGAAGAC	27	+676 →+693
D233E-2	GTCTTCTTTAATGAGCAGACACGCAGC	27	+693 →+676

P2 primer, the other contained M2 (mismatch primer for mutation) and P1 primers. PCR reaction mixture (100 μl) contains 20 pmol of each primer, 8 μl of each of 2.5 mM dNTPs, 100 ng of template (pGP 64), 10 μl of 10X PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin) and 2.5 units of *Taq* DNA polymerase (TaKaRa). The reaction mixture covered with 50 μl mineral oil to prevent evaporation was subjected to the following reaction cycle. After initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, annealing at 50°C for 2 min and polymerization at 72°C for 3 min were performed for 30 cycles, followed by final polymerization at 72°C for 7 min. PCR products were then separated on 0.9% agarose gel electrophoresis. 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 $MgCl_2$, 8 μl of four 2.5 mM dNTPs, 2.5 units of *Taq* DNA polymerase and 0.5 μl of *TaqStart* antibody (Clontech) (to prevent non-specific polymerization at lower temperature) were mixed for the overlap extension reaction. After the reaction mixture was prepared, PCR was performed under the same condition as described above.

Construction of the mutated pGP64

Both the mutated PCR product and pGP64 DNA were digested with *Xho*I (Amersham) and *Bgl*II (NEB). 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. The mutations in mutated pGP64 was confirmed by sequencing.

DNA sequencing

Sequencing was performed by the dideoxy chain termination procedure. Template DNA was prepared using the Wizard™ miniprep DNA purification system kit (Promega). One to two micrograms of purified DNA was denatured by adding 0.1 volumes of 2 N NaOH, 2 mM EDTA and was incubated for 1 h at 37°C. The mixture was neutralized by adding 0.1 volumes of 2 M ammonium acetate (pH 4.6) and the DNA was precipitated with 2-4 volumes of 100% ethanol (-70°C, 20 min). After washing the pelleted DNA with 70% ethanol, it was redissolved in distilled water. All procedure of sequence determination was followed as described in the supplied sequencing kit (Sequenase Version 2.0 DNA sequencing kit, United States Biochemicals).

Transfection

Transfection was performed in a 25 cm^2 culture dish containing 3×10^6 cells/ml by calcium phosphate precipitation method (15). 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 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°C, it was replaced with fresh Grace's media.

Oligomerization assay

Transfected cells at 60 h post-transfection were rinsed twice in phosphate-buffered saline (pH 7.4), and lysed with buffer containing 25 mM Tris-Cl (pH 6.8), 1% SDS, 15% glycerol, and 37.5 mM iodoacetamide (12). Cell lysates were separated on 7.5% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes (Schleider & Schuell) and blocked overnight at 4°C in blocking buffer (0.05% Tween-20, 5% skim milk in PBS, pH 7.5). Membranes were washed in the same buffer without skim milk and incubated with MAb 64R9 (kindly supplied by Dr. JaRue S. Manning, University of California, Davis) at 1:80 dilution in blocking buffer on a shaker for 1 h at room temperature (RT). After washing a 1:2,000 diluent of anti-mouse IgG conjugated with horseradish peroxidase (HRPO) (Caltag Laboratories) was added to membrane for 45 min at RT. Membranes were washed five times in 0.05% Tween-20 in PBS (pH 7.5) with shaking and incubated in chemiluminescence detection reagents (Amersham) for 1 min at RT. Blots were exposed to Hyperfilm-ECL (Amersham) and developed.

Results and Discussion

Fusion activity of gp64 glycoprotein

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 immunodificiency virus have been characterized by using mutational analysis of the cloned genes (5, 6, 8, 11, 13).

The baculovirus gp64 glycoprotein is required for budded virus entry into host cells (7, 14) and for uncoating through acid-mediated membrane fusion. To investigate gp64-mediated membrane fusion,

the entire coding region of gp64 as well as 5' and 3' flanking sequences were amplified by PCR. The PCR product was cloned into pT7Blue, and the resultant recombinant was named as pGP64. Sf9 cells transfected with pGP64 plasmid DNA was able to induce membrane fusion after the cells were exposed to acidic condition (9).

Two hydrophobic regions within OpMNPV gp64 glycoprotein are considered as an functional domains for membrane fusion activity (12). It is also possible that the AcNPV gp64 glycoprotein has hydrophobic regions that are responsible for membrane fusion activity. We examined the predicted hydrophobic profile of AcMNPV gp64 glycoprotein and selected two regions of highest local hydrophobicity in the ectodomains for functional analysis. The first hydrophobic region is composed of 6 hydrophobic amino acid residues located at 226 to 231 (Fig. 1A) and is highly conserved between the AcMNPV, OpMNPV, and CfMNPV gp 64 glycoproteins. The second hydrophobic region is located at amino acids 302 to 337 (Fig. 1A) and lies within a conserved portion of the protein which is predicted to form an amphipathic alpha-helix.

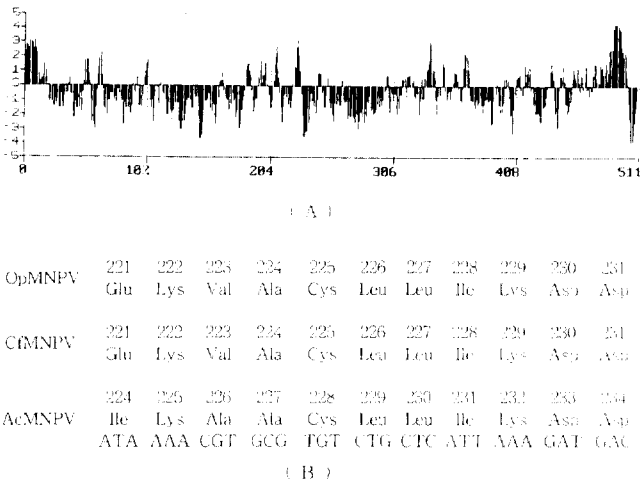

Fig. 1. Hydrophobicity profile of AcMNPV gp64 glycoprotein. Kyte hydrophobicity profiles for AcMNPV gp64 glycoprotein shown in (A) was obtained by using the PROSIS program. Two hydrophobic regions in the ectodomains are indicated. The vertical scale indicated Kyte hydrophobicity values, and number on the horizontal scale represent gp64 glycoprotein amino acid positions. The nucleotide and amino acid sequences of the part of hydrophobic region I are shown (B).

Construction of mutant gp64

Previous studies have shown that the gp64 glycoprotein is readily detected at the surface of baculovirus-infected cells as a multimer (10). Since most of the glycoproteins in enveloped viruses are inserted to the envelope as a multimer, oligomerization of these protein is essential to exhibit biological function such as membrane fusion.

Six amino acid residues at the hydrophobic region I in OpNPV and CfNPV gp64 glycoprotein are surrounded by hydrophilic amino acids (Glu-221-Lys-222 and Lys-229-Asp-230) (Fig. 1B). Since Ile instead of Glu residue is located at position 224 of AcNPV gp64 glycoprotein, Ile-224 was substituted to Glu-224 and the mutant gp64 was named as gp 64-I224E (I224E in short).

It is unusual that two hydrophilic amino acids are located next to the hydrophobic region. In order to investigate the role of lysine at 225, it was substituted to leucine, glutamate or arginine and the mutants were named K225L, K225E, K225R, respectively. Polar, charged lysine residue substitution to non-polar, uncharged leucine results in drastic alteration in hydrophobic nature of the region. Substitution to arginine would retain the hydrophilic nature of the region. Substitution to glutamate would not change the hydrophilic character of the region but the net charge would drastically be altered.

In order to study the role of hydrophilic amino acids at 232 and 233, K232E (Lys-232 to Glu-232), K232L(Lys-232 to Leu-232), K232R (Lys-232 to Arg-232),D233K (Asp-233 to Lys-233), D233L (Asp-233 to Leu-233), and D233E (Asp-233 to Glu-233) mutants were constructed. Double substitution mutants, I231VD233L (Ile-231 and Glu-233 to Val-231 and Leu-233) was also constructed.

The peak of hydrophobicity of region I is contributed by three amino acids, Cys-228-Leu-229-Leu-230 (Fig. 1B). 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 (L229AL230A). Substitution of leucine to alanine would reduce the overall hydrophobicity of region I but was not expected to change the protein conformation drastically.

Cys-228 was considered to be involved in disulfide bond formation and to play an important role for polymerization since mutant gp64 glycoprotein with substitution of cysteine at 228 to serine was not able to form oligomer in OpMNPV (12). It was suggested that the disulfide bridge formed by Cys-228 might be involved in polymerization. To study the role of the Cys-228 residue for oligomerization, C228A (Cys-228 to Ala-228) mutant was constructed. Nucleotide sequences of all the mutants were determined and the substituted nucleotides were confirmed as shown in Fig. 2. Location of the amino acid residues in wild type and substituted amino acids in mutants are sum-

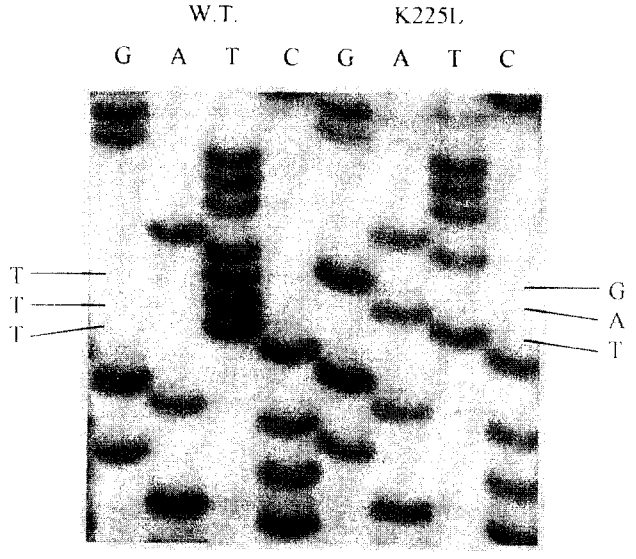

Fig. 2. Sequencing gel electrophoresis of wild type gp64 and mutant gp64-K225L.

	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237
	I	R	Q	I	K	A	A	C	L	L	I	K	D	D	K	N	N
K224E				E													
K225L					I												
K225E					E												
K225R					R												
C228A								A									
L229AL230A									A	A							
K232E												E					
K232L												L					
K232R												R					
D233K													K				
D233L													L				
D233E													E				
I231VD233L											I		L				

Fig. 3. Mutations in the hydrophobic region I of gp64 glycoprotein. The amino acid of the peptide encompassing residues 221 to 237 of gp64 glycoprotein of AcMNPV are shown. The amino acids under the wild type sequence are the replaced amino acids in each mutants.

marized in Fig. 3.

Oligomerization of gp64 glycoprotein

Substitution of particular amino acid residues within the conserved hydrophobic region I of gp64 would alter the oligomerization pattern without affecting fusion activity. Therefore, oligomerization of the mutant gp64 was performed prior to analysis of fusion activity.

To check the oligomerization state of mutated gp 64 glycoproteins, each mutated gp64 plasmid DNA was transfected into Sf9 cells. The transiently expressed gp64 glycoproteins were examined for oligomeriztion activity by separation on a SDS-PAGE under non-reducing condition and detected by immunoblotting with monoclonal anti-gp64 antibody (64R9). Wild type as well as all of the mutant gp64 glycoproteins except C228A mutant were able to

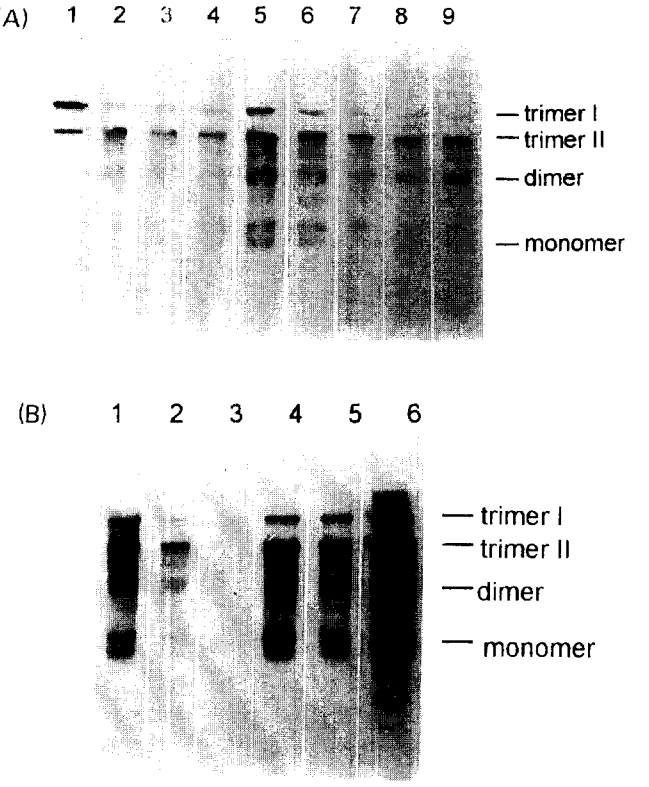

Fig. 4. Oligomerization of gp64 glycoproteins transiently expressed in gp64 plasmid DNA transfected Sf9 cells. Cell lysates were separated on 7.5% SDS-PAGE under non-reducing conditions. Immunoblotting to detect gp64 glycoproteins was performed as described in Material and Methods.(A) lane 1, K232R; lane 2, K232E; lane 3, K232L; lane 4, K225R; lane 5, K225L; lane 6, K225E; lane 7, D233K; lane 8, D233E; lane 9, D233L. (B) Lane 1, I231VD233L; lane 2, I224E; lane 3, C228A; lane 4, L229AL230A; lane 5, Wild type gp64; lane 6, Sf9 cell lysates infected with AcMNPV.

form oligomer as shown in Fig. 4. This result suggests that structural and hydrophobic changes within hydrophobic region I did not affect the oligomerization of the gp64 glycoprotein.

Cys-228 was proposed as an amino acid to form disulfide bond with the resulting protein conformation playing an important role in oligomerization (12). C228A mutant gp64 glycoprotein was unable to form oligomer (Fig. 4B, lane 3) implying that Cys-228 may be involved in oligomerization. To identify and analyze functional domains in the AcMNPV gp64 glycoprotein, transportation, surface localization, and fusogenic activity of the mutated gp64 glycoprotein within hydrophobic region I as well as hydrophobic region II need to be performed.

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