JOURNAL OF VIROLOGY, July 2004, p. 6946–6954 Vol. 78, No. 13
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.13.6946–6954.2004

Functional Analysis of the Putative Fusion Domain of the Baculovirus Envelope Fusion Protein F

Marcel Westenberg, Frank Veenman, Els C. Roode, Rob W. Goldbach, Just M. Vlak,* and Douwe Zuidema

Laboratory of Virology, Wageningen University, 6709 PD Wageningen, The Netherlands

Received 7 November 2003/Accepted 25 February 2004

Group II nucleopolyhedroviruses (NPVs), e.g., *Spodoptera exigua M*NPV, lack a GP64-like protein that is present in group I NPVs but have an unrelated envelope fusion protein named F. In contrast to GP64, the F protein has to be activated by a posttranslational cleavage mechanism to become fusogenic. In several vertebrate viral fusion proteins, the cleavage activation generates a new N terminus which forms the so-called fusion peptide. This fusion peptide inserts in the cellular membrane, thereby facilitating apposition of the viral and cellular membrane upon sequential conformational changes of the fusion protein. A similar peptide has been identified in NPV F proteins at the N terminus of the large membrane-anchored subunit F_1. The role of individual amino acids in this putative fusion peptide on viral infectivity and propagation was studied by mutagenesis. Mutant F proteins with single amino acid changes as well as an F protein with a deleted putative fusion peptide were introduced in *gp64*-null *Autographa californica M*NPV budded viruses (BVs). None of the mutations analyzed had an major effect on the processing and incorporation of F proteins in the envelope of BVs. Only two mutants, one with a substitution for a hydrophobic residue (F152R) and one with a deleted putative fusion peptide, were completely unable to rescue the *gp64*-null mutant. Several nonconservative substitutions for other hydrophobic residues and the conserved lysine residue had only an effect on viral infectivity. In contrast to what was expected from vertebrate virus fusion peptides, alanine substitutions for glycines did not show any effect.

The members of the *Baculoviridae* family are large, enveloped, double-stranded DNA viruses that are exclusively pathogenic for arthropods, predominantly insects of the order *Lepidoptera* (1). Baculoviruses are classified into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). The NPVs can be phylogenetically subdivided into group I and II NPVs (6, 10, 12, 13). The budded virus (BV) phenotype of group I NPVs contains a GP64-like major envelope glycoprotein. This protein is involved in viral attachment to host cells (11), triggers low-pH-dependent membrane fusion during BV entry by endocytosis (4, 20, 35, 42), and is required for efficient budding from the cell surface (27, 29).

In contrast, BVs of group II NPVs and GVs lack a homolog of GP64. The low-pH-dependent membrane fusion during BV entry by endocytosis is triggered in this case by a so-called F protein (17, 32). F homologs are also found as envelope proteins of the insect errantiviruses while cellular homologs are found in the fruit fly *Drosophila melanogaster* and in the African malaria mosquito *Anopheles gambiae* (25, 36). Unlike GP64, the F proteins are structurally similar to fusion proteins from several vertebrate viruses such as orthomyxoviruses and paramyxoviruses. Recently, it has been shown that the GP64 protein in BVs of *Autographa californica M*NPV (Ac*M*NPV) can be replaced by the F protein of group II NPVs (24), implying that F is functionally analogous to GP64.

Like several mammalian viral envelope fusion proteins, the baculovirus F protein has to be posttranslationally cleaved by a proprotein convertase (furin) to become fusogenic (43). Also, for some errantiviruses it has been shown that the envelope protein is posttranslationally cleaved (31, 38, 39). Cleavage seems to be a general mechanism for viruses to activate their fusion proteins (21). In a number of virus families this cleavage occurs in front of a strongly hydrophobic sequence, the so-called fusion peptide (44, 45). These fusion peptides are believed to translocate upon cleavage to the top of the protein and to insert into the target membrane after exposure to low pH or receptor binding. This translocation facilitates the apposition of viral and cellular membranes upon further conformational changes of the fusion protein (21). Comparison of available F protein sequences reveals a conserved strongly hydrophobic domain with the consensus sequence ΦGXΦBΦΦGXKΦΦΦGXΦDXXDXXXΦ, where Φ represents hydrophobic amino acids, B stands for an aspartic acid or an asparagine residue, and X represents any amino acid (Fig. 1). This domain is preceded by a furin-like cleavage site in the F proteins from group II NPVs, GVs, and errantiviruses, whereas this domain is more or less absent in the remnant F protein from group I NPVs and in the cellular homologs (36). This strongly hydrophobic sequence at the N terminus of the membrane-anchored *Spodoptera exigua M*NPV (Se*M*NPV) F_1 fragment has all the characteristics of a fusion peptide (45). It is well conserved within the virus family (Fig. 1), when modeled in α-helix, it displays one face with a hydrophobic index (H.I.) of about 0.9, according to the normalized consensus scale of Eisenberg (8), and a back face with hydrogen bonding potential, and it contains glycines on one side of the helix (Fig. 2A).

The role in the fusion process of the three conserved

* Corresponding author. Mailing address: Wageningen University, Laboratory of Virology, Binnenhaven 11, 6709 PD Wageningen, The Netherlands. Phone: 31-317-483090. Fax: 31-317-484820. E-mail: just.vlak@wur.nl.

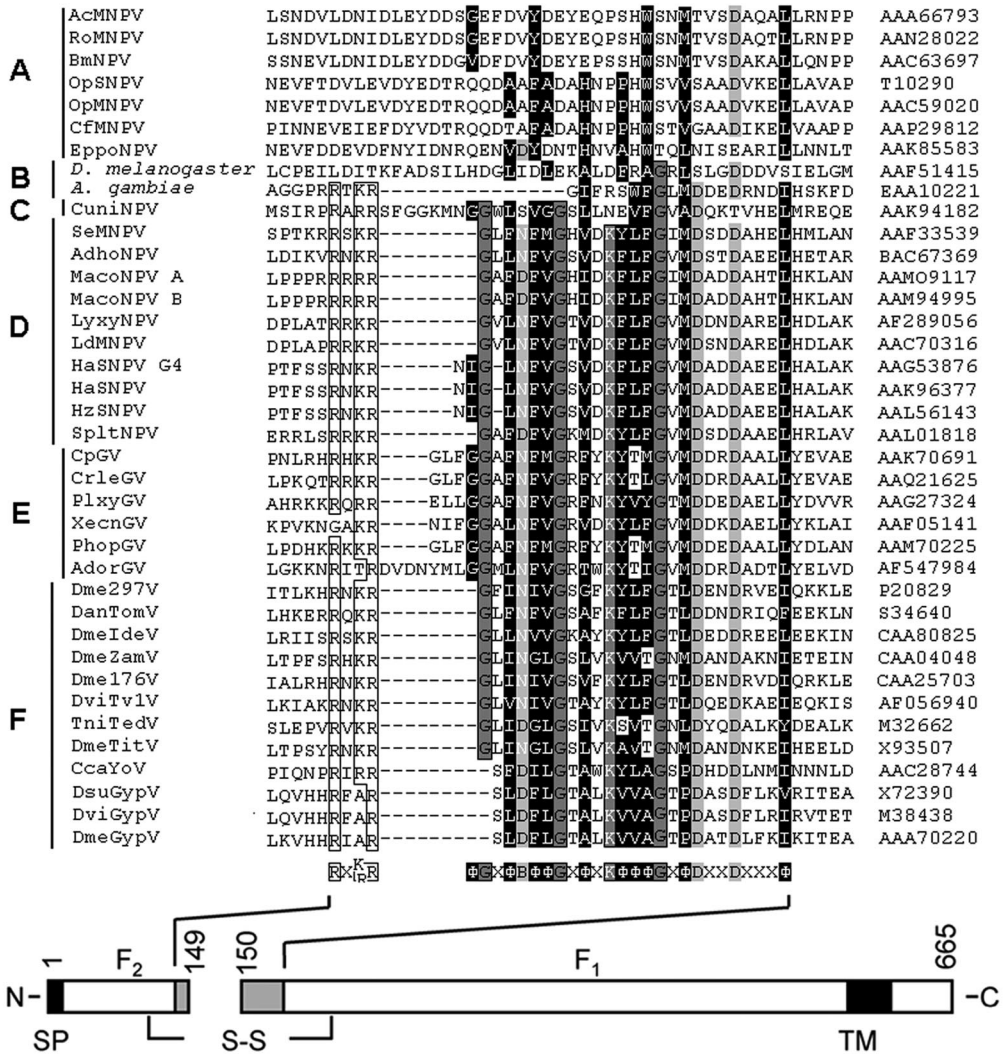

FIG. 1. Amino acid sequence alignment of the Se*M*PNV F protein domains near the protease cleavage site with the corresponding domain of the F proteins of group I NPVs (A), dipterans (B), lepidopteran NPVs (C), group II NPVs (D), GVs (E), and errantiviruses (F). Virus and dipteran abbreviations and GenBank accession numbers of the F proteins are shown on the left and right, respectively. The conserved amino acids are boxed. The consensus sequence is shown below the alignment, in which B represents D or N, Φ indicates a hydrophobic amino acid (H.I., ≥0.12), and X represents any amino acid. A schematic presentation of the Se*M*NPV F protein, with the consensus sequence shown in gray boxes, is shown at the bottom. N, N terminus; C, C terminus; SP, signal peptide; TM, transmembrane domain; S-S, disulfide bridge.

charged amino acids in the putative fusion peptide, immediately downstream of the proprotein convertase cleavage site, of the *Lymantria dispar M*NPV (Ld*M*NPV) F protein has already been studied by site-directed mutagenesis (33). In this study, site-directed mutagenesis was used to investigate the role in viral propagation and infectivity of the conserved glycines and lysine as well as the hydrophobic amino acids in the putative fusion peptide of the Se*M*NPV F protein. Several conservative and nonconservative substitutions were introduced in the fusion domain, and their impact on virus propagation and infectivity was examined by using a recently developed Ac*M*NPV pseudotyping system in which the envelope fusion protein GP64 can be replaced by the heterologous envelope fusion protein F of Se*M*NPV (24). The virus propagation of mutant infectious viruses as well as the amount of BV produced gives further credence for the role of the conserved domain of the baculovirus F protein as the fusion peptide.

MATERIALS AND METHODS

Cells. *Spodoptera frigiperda* cell lines IPLB-Sf21 (41) and $Sf9^{Op1D}$ (35) (provided by G. W. Blissard, Ithaca, N.Y.) were cultured at 27°C in plastic culture flasks (Nunc) in Grace's insect medium, pH 5.9 to 6.1 (Gibco-BRL), supplemented with 10% fetal bovine serum.

Donor plasmids containing envelope protein genes. A silent mutation was introduced into the coding sequence of the Se*M*NPV F open reading frame to generate an NdeI cloning site. Therefore, nucleotide 525 in the *f* open reading frame was changed from C to T by PCR-based site-directed mutation, according to the method of Sharrocks and Shaw (37). The 5′ mutagenic primer 5′-CGAC GCTCACGAACTGCATATGCTCGCCAACACCACAA-3′ (underlined and boldface sequences represent an NdeI site and the mutation, respectively) and the 3′ primer 5′-GAGAGGCACGGGCCACGAAAGG-3′ (primer downstream of the PmeI site) were used in conjunction with plasmid pΔFBgusSe8 (24) as a

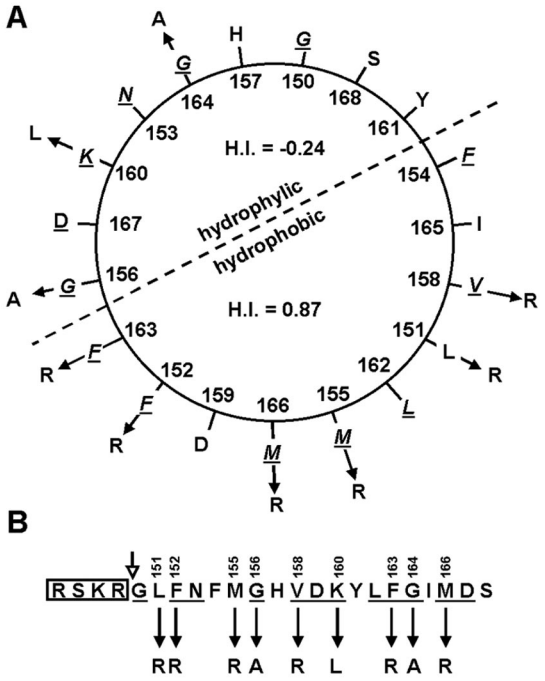

FIG. 2. (A) Helical wheel presentation of the first 21 N-terminal amino acids of the Se*M*NPV F_1 fragment. The helix is typically amphipathic, with relatively polar amino acids (H.I., ≤0.48) above the dotted line, with an average H.I. of −0.24, and except for one aspartic acid residue (D), nonpolar amino acids (H.I., >0.62) below the dotted line, with an average H.I. of 0.87. (B) Linear presentation of the first 21 N-terminal amino acids of the Se*M*NPV F_1 fragment preceded by the consensus furin recognition sequence (box). The open arrow indicates the cleavage site. Underlined amino acids are conserved as shown in Fig. 1. Solid arrows indicate the point mutations as well as the substitutions described in this study.

template with *Pfu* polymerase (Promega). The PCR product (346 bp) was agarose gel purified, and the single strand containing the mutation at the 3′ end served as a 3′ mutagenic primer in a second PCR with 5′ primer 5′-TTATGGATCCATGCTGCGTTTTAAAGTGAT-3′ (the underlined sequence represents a BamHI site) with high-fidelity Expand long-template polymerase (Roche). The second PCR product (862 bp) was cloned into pGEM-T (Promega) to generate the intermediate plasmid pGEM-SeF^{NdeI}. Plasmid pΔFBgusSeF^{NdeI} was obtained by swapping the BamHI/PmeI fragment of pGEM-SeF^{NdeI} with the same fragment of pΔFBgusSe8.

Mutations and deletions in the coding sequence of SeF, encompassing amino acids 151 to 170 were performed as follows. For every mutant, a 5′ phosphorylated primer pair (Table 1) was used with pGEM-SeF^{NdeI} as a template and *Pfu* polymerase (Promega) to amplify the entire vector. Finally, the 5′ ends of the PCR products were ligated to its own 3′ ends, generating a new restriction endonuclease site at the junction (Table 1). Clones containing the additional restriction site were sequenced to confirm the mutation. The obtained mutations in pGEM-SeF^{NdeI} were introduced into pΔFBgusSeF^{NdeI} by swapping the BamHI/NdeI fragments.

Transfection infection assay. The *gus* reporter gene controlled by the *p6.9* promoter and the mutant Se*M*NPV *f* genes under control of the *gp64* promoter from pΔFBgusSeF^{NdeI} were transposed into the *att* Tn*7* transposon integrase site of a *gp64*-null Ac*M*NPV bacmid (provided by G. W. Blissard) (24) according to the Bac-to-Bac manual (Invitrogen). Transpositions of inserts from donor plasmids were confirmed by PCR with a primer corresponding to the gentamicin resistance gene of the donor plasmid (P-gen-RV [5′-AGCCACCTACTCCCAACATC-3′]) in combination with a primer corresponding to the bacmid sequence adjacent to the transposition site (M13/pUC forward primer [5′-CCCAGTCACGACGTTGTAAAACG-3′]). Bacmid DNA, positive in the PCR, was electroporated into DH10β cells to eliminate the helper plasmid and some residual untransposed bacmid DNA.

Approximately 1 μg of DNA of each recombinant bacmid was transfected into 1.5×10^6 Sf21 or Sf9^{Op1D} cells with 10 μl of Cellfectin (Invitrogen). At 5 days posttransfection, transfected cells were stained for GUS activity according to the Bac-to-Bac protocol (Invitrogen) to monitor transfection efficiency. The supernatant was clarified for 10 min at 2,200 × *g* and subsequently filter sterilized (0.45-μm pore size). One-fourth (500 μl) of the supernatant was used to infect 2.0×10^6 Sf9 or Sf9^{Op1D} cells, respectively. At 72 h postinfection, cells were split into two portions. Cells of one portion were stained for GUS activity; the other portion was used at 10 days postinfection to monitor viral propagation. The *gp64*-positive bacmid served as a positive control for transfection and infection while the *gp64*-null bacmid was used as a negative control for transfection and infection (24). The SeF$_{R149K}$ bacmid was used as a negative control for F protein cleavage (24).

BV amplification and preparation. Viruses carrying *f* genes that rescued the *gp64*-null phenotype were amplified by infecting 1.0×10^7 Sf21 cells with 500 μl of cell supernatant from 10 days postinfection. Viruses carrying *f* genes that did not rescue the *gp64* deletion in Sf21 cells were amplified in a similar manner by using Sf9^{Op1D} cells. Cells were split every 3 to 5 days until all cells were infected. Amplified pseudotyped viruses were titrated on Sf9^{Op1D} cells by a 50% tissue culture infective dose ($TCID_{50}$) assay (30) and scored for infection by examining cells for GUS expression.

The genotypes of the pseudotyped viruses were confirmed by PCR on purified BV DNA by using primers P-SeF-mutant-FW (5′-GGCGTTGACGGTCGAGGCTAAAT-3′) and P-SeF-mutant-RV (5′-GTGCATCGCTTTTTCGGTGAGAGG-3′) to amplify a DNA fragment containing the incorporated restriction site (Table 1). The amplified DNA fragment was subsequently subjected to restriction enzyme analysis.

One-step growth experiments. To monitor infectious BV production from viruses carrying mutant *f* genes that rescued the *gp64*-null phenotype, viral growth results were generated by collecting infected cell supernatants. Sf21 cells (1.5×10^5 cells per well, 24-well plates) were infected at a multiplicity of infection (MOI) of 5.0 or 0.5 $TCID_{50}$ U/cell for 1 h at 27°C. After infection, the inoculum was removed and 0.5 ml of fresh medium was added to the cells. At 0, 24, 48, 72, 69, and 144 h postinfection, the infected cell supernatants were

TABLE 1. Primer pairs generating the desired mutation and a restriction endonuclease site[a]

Mutant	5′ primer (5′–3′)	3′ primer (5′–3′)	RE[b] site
SeF$_{\Delta 151–170}$	GCCCACGAACTGCATATGCTCGCC	GCCGCGTTTAGAGCGTCTTTTCG	NarI
SeF$_{L151R}$	CC**GC**TTTAATTTTATGGGACACGTCG	CCGCGTTTAGAGCGTCTTTTC	NotI
SeF$_{F152R}$	CCTT**CG**TAATTTTATGGGACACGTCG	CC**T**CGTTTAGAGCGTCTTTTCGTC	StuI
SeF$_{M155R}$	**GCGG**ACACGTCGACAAATATCTG	GGAAATTAAAAAGGCCGCGTTTAG	SacII
SeF$_{G156A}$	CGT**G**GACAAATATCTGTTTGGCATTATG	TGT**G**CCATAAAATTAAAAAGGCCGCGTTT	Eco72I
SeF$_{V158R}$	CGACAAATATCTGTTTGGCATTATG	**CGA**TGTCCCATAAAATTAAAAAGGCCGCGTTT	NruI
SeF$_{K160L}$	**TCT**ATATCTGTTTGGCATTATGGACAG	TCTACGTGTCCCATAAAATTAAAAAGG	BglII
SeF$_{F163K}$	**CT**TG**CG**TGGCATTATGGACAGCGAC	TA**CT**TGTCGACGTGTCCCATAAAA	ScaI
SeF$_{G164A}$	**CGA**TTATGGACAGCGACGACGCTCAC	C**GA**ACAGATATTTGTCGACGTG	NruI
SeF$_{M166R}$	**CGCGA**CAGCGACGACGCTCACGA	AATGCCAAACAGATATTTGTCGAC	NruI

[a] Underlined sequences of primer pairs generate restriction sites after blunt-ended self-ligation of PCR products. Mutations are indicated in boldface type.
[b] RE, restriction endonuclease.

collected. For each time point postinfection and each virus sample, duplicate samples were generated. The quantity of infectious BVs in the samples was determined by $TCID_{50}$ assays on $Sf9^{Op1D}$ cells. A third sample was generated for those duplicated samples for which titers differed by a factor of 2.5 or more.

Western blot analysis. BVs were amplified by infecting 1.0×10^7 Sf21 or $Sf9^{Op1D}$ cells at an MOI of 0.5 $TCID_{50}$ U/cell. Cells were split every 3 to 5 days until all cells were infected. BVs were purified from the supernatants as described previously (43). Equal amounts of BVs, determined by the Bradford method (5), were disrupted in Laemmli buffer (125 mM Tris-HCl, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue [pH 6.8]) and denatured for 10 min at 95°C. Proteins were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore) by semidry electrophoresis transfer (3). Membranes were blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 2% milk powder, followed by incubation for 1 h at room temperature with either polyclonal antibodies anti-F1 and anti-F2 (43), monoclonal antibody AcV5 (14), or polyclonal antibody anti-VP39 (40) (provided by A. L. Passarelli, Manhattan, Kans.), all at a 1:1,000 dilution in PBS containing 0.2% milk powder. After washing three times for 15 min in PBS containing 0.1% Tween-20, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated appropriate secondary antibody (Sigma, DAKO) in PBS containing 0.2% milk powder. After washing three times for 15 min in PBS containing 0.1% Tween-20, the signal was detected by enhanced chemiluminescence technology as described by the manufacturer (Amersham).

Computer-assisted analysis. Protein comparisons with entries in the updated GenBank and EMBL were performed with the FASTA and BLAST programs (2, 34). Sequence alignments were performed with the program ClustalW (EMBL European Bioinformatics Institute, http://www.ebi.ac.uk) and edited with the Genedoc Software (28). The α-helix predictions were performed with the Protean software of DNASTAR by using the method of Garnier et al. (9).

RESULTS

Pseudotyping *gp64*-null Ac*M*NPV with *f* mutants. To examine the role of the N terminus of the Se*M*NPV F_1 fragment in viral propagation and infectivity, mutational analysis on this domain was performed. The hydrophilic arginine was substituted for hydrophobic amino acids (Fig. 2 and Table 1). Five of these hydrophobic amino acids (F^{152}, M^{155}, V^{158}, F^{163}, and M^{166}) are conserved and one (L^{151}) is nonconserved among F homologs of group II NPVs, GVs, and errantiviruses (Fig. 1). Alanine was substituted for glycines (G^{156} and G^{164}), and a hydrophobic leucine was substituted for the conserved lysine (K^{160}). Each mutation was marked by the presence of a new restriction site. Furthermore, an F mutant with a deletion in the conserved region (amino acids 151 to 170) of the fusion peptide was also constructed. It was then determined whether the F protein mutants could rescue BV propagation of a *gp64*-null Ac*M*NPV bacmid relative to the native F protein (24) when transfected into Sf21 cells. Mutant *f* genes under control of the Ac*M*NPV *gp64* promoter and a *gus* reporter gene downstream of the baculovirus *p6.9* promoter were inserted simultaneously by Tn*7*-based transposition (23) into the *gp64*-null Ac*M*NPV bacmid. Sf21 cells were transfected with the constructed bacmids together with various control bacmids: *gp64*-null Ac*M*NPV bacmids containing (i) no envelope fusion protein gene (ΔF) (negative control), (ii) Ac*M*NPV *gp64* (positive control), (iii) Se*M*NPV *f*, (iv) Se*M*NPV f^{NdeI} (*f* gene with silent mutation creating an NdeI cloning site), and (v) Se*M*NPV f_{K149R} (*f* gene mutant negative in F_0 cleavage). At 5 days posttransfection, cells were stained for GUS activity (Fig. $3A^1$ to O^1). The presence of infectious BVs in the supernatant was determined by passaging the supernatants to new Sf21 cells. At 3 days postinfection, infected cells were demonstrated by their GUS activity (Fig. $3A^2$ to O^2). The bacmids SeF_{L151R}, SeF_{M155R}, SeF_{G156A}, SeF_{F152R}, SeF_{V158R}, SeF_{K160L}, SeF_{F163R}, SeF_{G164A}, and SeF_{M166R} were all able to produce infectious viruses (Fig. 3E to M), as were SeF^{NdeI} (Fig. 3C) and the positive controls, AcGP64 and SeF (Fig. 3A and B). The bacmids SeF_{F152R} and $SeF_{\Delta 151\text{-}170}$ (Fig. 3F and N) and both negative controls (ΔF and SeF_{K149R}) (Fig. 3D and O) were not able to produce infectious viruses, as expected. However, when those bacmids were transfected into $Sf9^{Op1D}$ cells, which constitutively express the *Orgyia pseudogata M*NPV GP64 protein to pseudotype Ac*M*NPV (35), infectious BVs could be demonstrated (Fig. 3P to S), indicating that the defect in BV propagation was attributable to the expression of an inactive fusion protein.

BVs carrying an envelope fusion protein gene that rescued the *gp64*-null phenotype were amplified by infecting Sf21 cells; those BVs that were not rescued were amplified by using $Sf9^{Op1D}$ cells as described in Materials and Methods. The amplified viruses were all subjected to PCR analysis to verify the introduced mutations in their *f* genes (Fig. 4). For viruses containing the mutant *f* genes, PCR fragments of similar sizes were obtained, whereas the $SeF_{\Delta 151\text{-}170}$ mutant showed a much smaller fragment (Fig. 4A). Along with the desired mutations, an additional restriction site was introduced into the *f* genes to mark the mutation (Table 1), allowing the analysis of the resulting PCR fragments by restriction enzyme analysis (Fig. 4B). From the patterns, it could be concluded that the *gp64*-null Ac*M*NPV viruses contained the correct mutations.

Western analysis of *f* gene-pseudotyped *gp64*-null Ac*M*NPV viruses. The effect of the various mutations on the processing and incorporation of the F protein in BVs was determined by Western analysis (Fig. 5). An antibody against the major nucleocapsid protein (anti-VP39) served as an internal control to determine the amount of BVs used in the analysis. Results indicated that all mutant F proteins were present in BVs. The amounts of BVs for each recombinant virus were estimated to be similar, except for SeF_{V158R} (lane 9) and SeF_{K160L} (lane 10), where less BVs were used (Fig. 5D). The Ac*M*NPV GP64 protein could be detected with the repair virus (Fig. 5C, lane 1) as well as for the viruses propagated in $Sf9^{Op1D}$ cells (Fig. 5C, lanes 4, 6, 14, and 15). Western analysis with antibodies against the Se*M*NPV F_1 and F_2 indicated that the incorporation of the F protein in the BVs was reduced for SeF_{F152R}, SeF_{M155R}, SeF_{M166R}, and SeF_{R149K} (Fig. 5A and B, lanes 6, 7, 13, and 15, respectively) compared to that of native SeF protein (Fig. 5A and B, lanes 1). Furthermore, all mutant F proteins, except SeF_{R149K} (Fig. 5A, lane 15), lacking a furin cleavage site, were properly cleaved into F_1 (Fig. 5A) and F_2 (Fig. 5B) fragments.

Viral infectivity of *f* gene-pseudotyped *gp64*-null Ac*M*NPV viruses. To characterize the effect of the mutations on viral infectivity of the mutant *gp64*-null viruses, one-step growth experiments were performed. Sf21 cells were infected at an MOI of 5.0 $TCID_{50}$ U/cell. At different time points postinfection, the amount of infectious BVs was determined (Fig. 6A). The virus titers for the native SeF and the SeF^{NdeI} mutant were not significantly different, indicating that the silent mutation to generate the NdeI site had no effect on BV infectivity and propagation. The one-step growth results for SeF^{NdeI} is therefore left out of the graph (Fig. 6). Except for SeF_{V158R}, the virus titers of the other mutants did not significantly differ from that of SeF. The virus titer of SeF_{V158R} remained significant

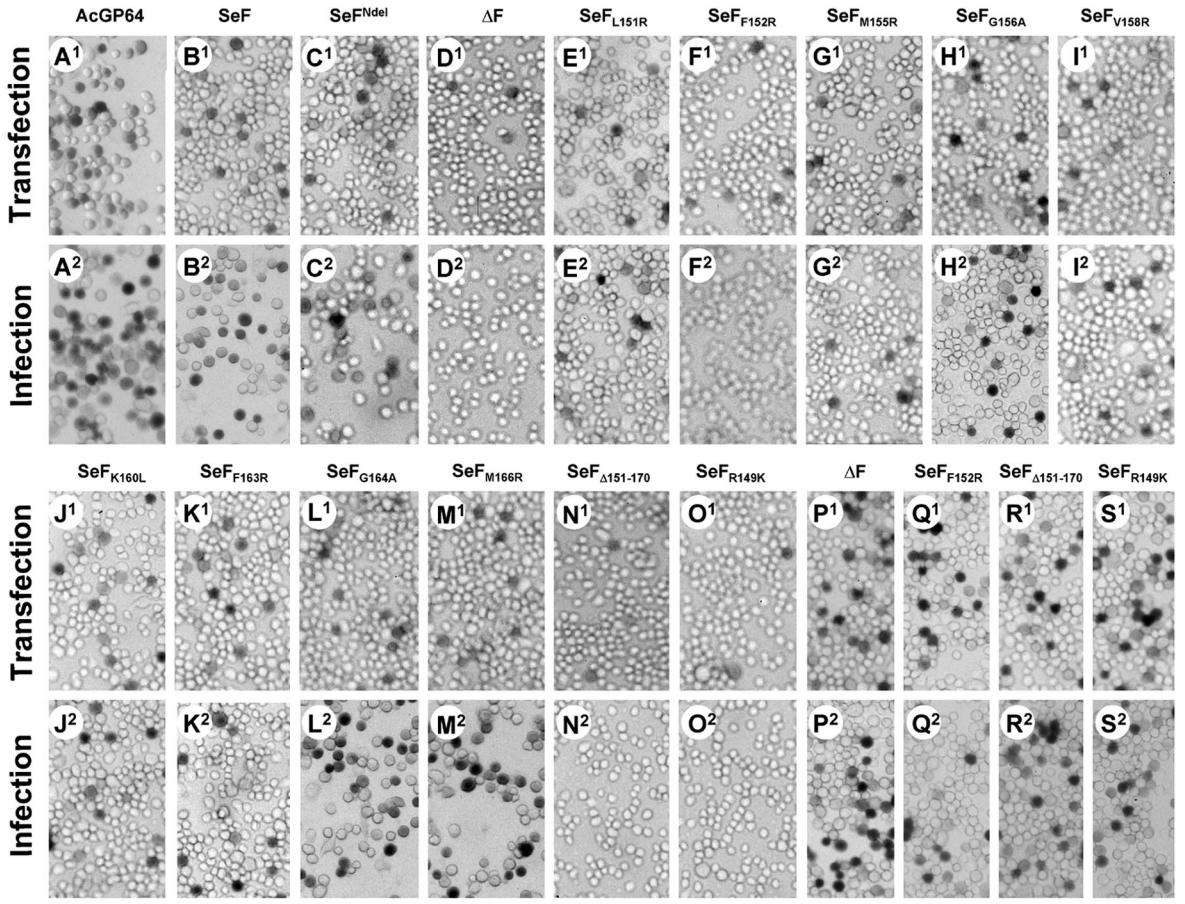

FIG. 3. Transfection-infection assays for viral propagation. Sf21 cells were transfected with indicated mutant *gp64*-null bacmids pseudotyped with *f* genes, incubated for 5 days, and stained for GUS activity (A^1 to O^1). Supernatants from the transfected cells were used to infect Sf21 cells (A^2 to O^2), which were incubated for 72 h and subsequently stained for GUS activity. Stained cells (infected cells) indicate that infectious virions were generated in the transfected cells. Bacmids that failed to propagate an infection in Sf21 cells (D^2, F^2, N^2, and O^2) were propagated in cells expressing constitutive *O. pseudogata M*NPV GP64 ($Sf9^{Op1D}$). $Sf9^{Op1D}$ cells were transfected with indicated *gp64*-null bacmids (P^1 to S^1) and incubated for 5 days; then supernatants were transferred to $Sf9^{Op1D}$ and stained for GUS activity after 72 h (P^2 to S^2). Indicated *gp64*-null Ac*M*NPV bacmids are pseudotyped with AcGP64 (Ac*M*NPV *gp64*), SeF (Se*M*NPV *f*), SeF^{NdeI} (Se*M*NPV *f* with silent mutation generating an NdeI restriction site), ΔF (no envelope fusion gene), SeF_{L151R}, SeF_{F152R}, SeF_{M155R}, SeF_{G156A}, SeF_{V158R}, SeF_{K160L}, SeF_{F163R}, SeF_{G164A}, SeF_{M166R} (Se*M*NPV *f* with mutations causing amino acid substitution in the putative fusion peptide as indicated in the subscript), $SeF_{\Delta 151\text{-}170}$ (Se*M*NPV *f* with a deletion of the segment encoding amino acids 151 to 170), and SeF_{R149K} (Se*M*NPV *f* with mutations causing an amino acid substitution in the furin cleavage site).

lower than that of the authentic SeF. This indicates that the mutations had hardly any effect on the amount of BV produced over time. A different behavior was seen when Sf21 cells were infected at an MOI of 0.5 $TCID_{50}$ U/cell (Fig. 6B), so that not all cells were infected in the first round. In this experiment, the effect of the mutations on viral infectivity could be determined, since the amount of BV production over time was not altered, except for SeF_{V158R}. Also, this time the virus titers of SeF^{NdeI} were almost identical to that of SeF (data not shown). In the situation of 0.5 $TCID_{50}$ U/cell, only the one-step growth results for SeF_{L151R}, SeF_{G164A}, and SeF_{M166R} were similar to those for SeF. The titer of SeF_{G156A} shows a drop between 48 and 72 h postinfection, but at 144 h postinfection, the titer was elevated to levels similar to that of SeF. The virus titers of SeF_{M155R}, SeF_{V158R}, SeF_{K160L}, and SeF_{F163R} showed a dramatic decrease compared to that of SeF. Thus, mutations in the fusion peptide affect viral infectivity (Fig. 6B), presumably the dynamics of viral spread, rather than viral production (Fig. 6A).

DISCUSSION

Posttranslational cleavage is a general mechanism to activate the fusion proteins of enveloped viruses (21). Recently, it has been shown that this is also the case for the baculovirus F protein, where two subunits are generated, F_1 and F_2 (24, 43). Cleavage of envelope fusion proteins usually occurs in front of a conserved hydrophobic region, the fusion peptide. The N terminus of the Se*M*NPV membrane-anchored domain (F_1) contains striking similarities with the fusion peptides of other viruses: (i) the domain is well conserved among its functional homologs, (ii) it is relatively hydrophobic, (iii) it can be modeled as an amphipathic helix, and (iv) it contains conserved glycines at one side of the helix (Fig. 1 and 2). The helical

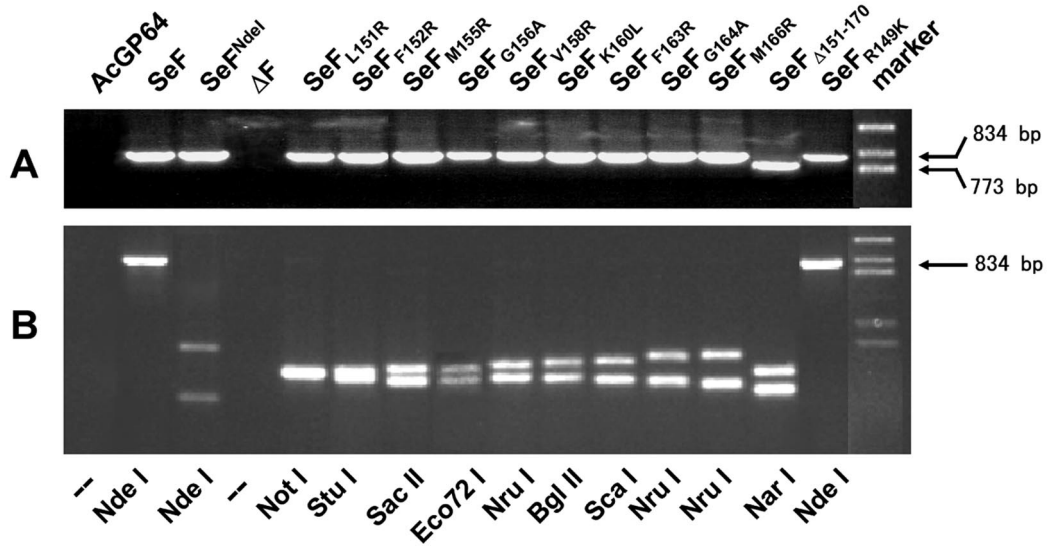

FIG. 4. PCR and restriction enzyme analyses of purified BV DNA to verify the genotype of indicated pseudotyped vAcGP64$^-$ viruses as described in the legend to Fig. 3. (A) PCR with *f* gene-specific primer pairs used to examine mutant viral DNAs amplifying an 834-bp fragment, when the virus contains the *f* gene, except for $SeF_{\Delta 151\text{-}170}$, where a 773-bp fragment was amplified. (B) Restriction enzyme analysis of PCR-amplified DNA fragments. Mutant F genes have, in addition to the incorporated NdeI site, an additional restriction site, which is used to distinguish the viruses (Table 1). The SeF_{R149K} mutant has neither a NdeI site nor an additional restriction site.

nature of the corresponding region of Ld*M*NPV F has recently been determined by circular dichroism (M. Pearson and G. F. Rohrmann, Abstr. 22nd Annu. Meet. Am. Soc. Virol., abstr. W53-5, 2003).

However, there are also some striking differences with vertebrate viral fusion peptides. Fusion peptides of the latter are rich in alanines while the first alanine (residue 22) in the Se*M*NPV F_1 N terminus is found outside the conserved region. Another difference is that N-terminal fusion peptides of most vertebrate viral fusion proteins are generally apolar, whereas the Se*M*NPV F_1 N terminus contains six polar amino acids (N^{153}, H^{157}, D^{159}, K^{160}, D^{167}, and S^{168}). Other baculovirus F proteins may have up to nine polar amino acids in this region (Fig. 1). However, the fusion peptide of influenza hemagglutinin also contains two to three polar residues (26). It is very well possible that the polar amino acids force the N terminus of F_1 to insert in the membrane in a more perpendicular angle, with the polar amino acids to the hydrophilic side of the phospholipids compared to other fusion peptides.

The importance of the Se*M*NPV F_1 N terminus for virus infection and propagation was investigated by a series of amino acid substitutions. Ac*M*NPV virions, lacking *gp64*, were pseudotyped with mutant *f* genes and assayed (results are summarized in Table 2). A similar experimental system has been

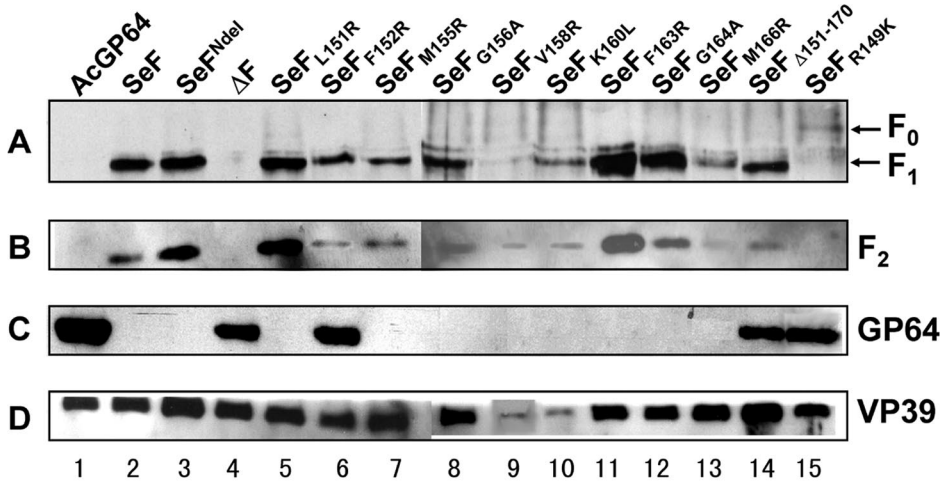

FIG. 5. Western blot analysis of *gp64*-null BVs pseudotyped with indicated (mutant) *f* genes as described in the legend to Fig. 3. Infectious BVs were generated in Sf21 cells (lanes 1 to 3, 5, 6, and 8 to 13), and BVs, which are not able to propagate in Sf21 cells, were propagated in $Sf9^{Op1D}$ cells (lanes 4, 7, 14, and 15). Blots were probed with antibodies anti-F1 (A), anti-F2 (43) (B), and anti-GP64 (monoclonal antibody AcV5) (14) (C). (D) An anti-nucleocapsid antibody (anti-VP39) was used as an internal control for each preparation of purified BVs (40).

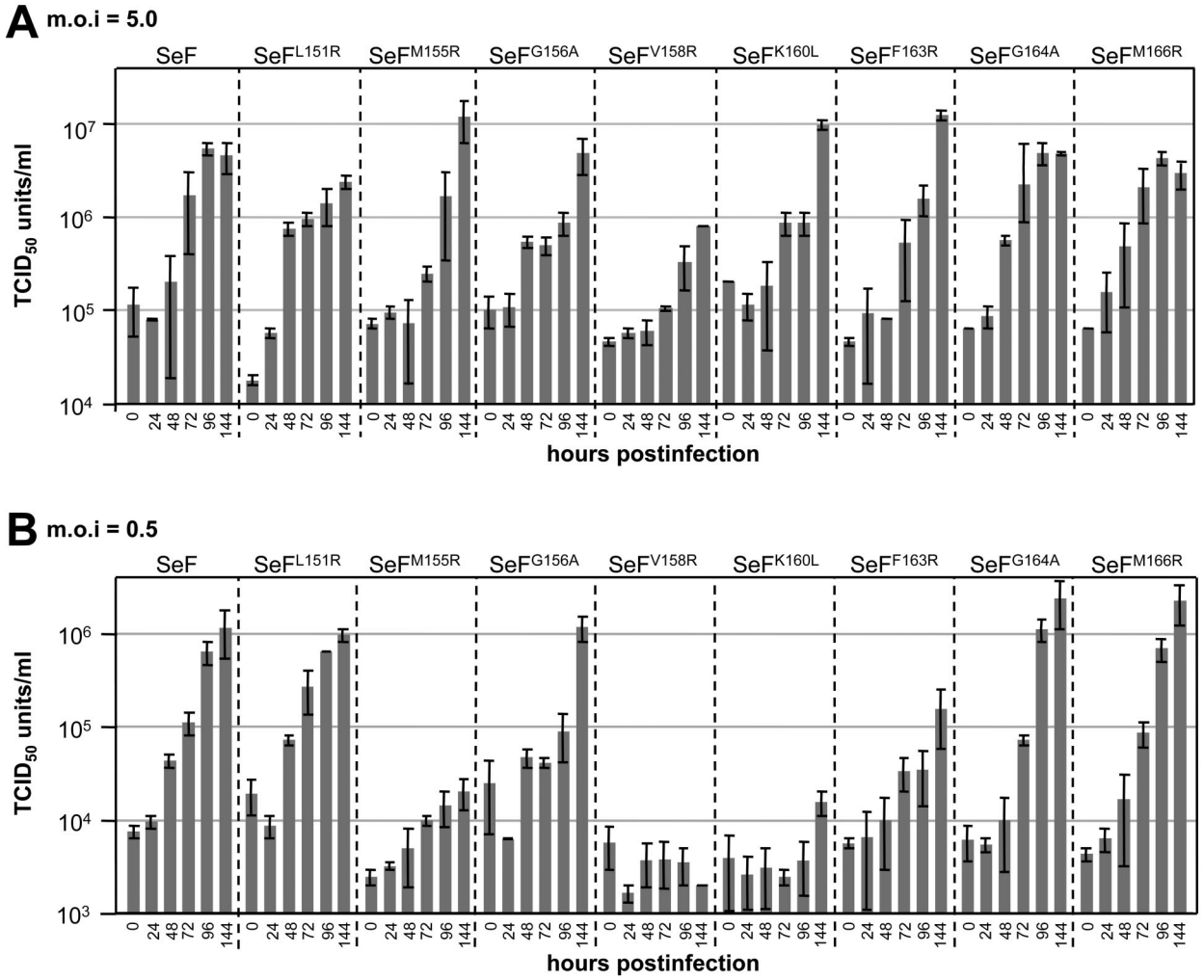

FIG. 6. One-step growth results are shown for *gp64*-null Ac*M*NPV viruses pseudotyped with wild-type SeF and SeF mutants as described in the legend to Fig. 3. Sf21 cells were infected with an MOI of 5.0 $TCID_{50}$ U/cell (A) or 0.5 $TCID_{50}$ U/cell (B), and supernatants were harvested at the indicated times postinfection and titrated on $Sf9^{Op1D}$ cells. Each data point represents the average of the results from two or three independent infections. Error bars represent standard deviations.

used to analyze the effect of mutations in the Ebola virus glycoprotein fusion peptide through pseudotyping of the vesicular stomatitis virus lacking its own fusion protein (18). The F protein with a deletion of the N terminus ($SeF_{\Delta 151\text{-}170}$) was not able to produce infectious virus (Fig. 3), in spite of its ability to be incorporated in BVs and the occurrence of the posttranslational cleavage (Fig. 5). This suggests that this domain is not involved in virion assembly but plays an important role in baculovirus entry into cells.

Glycines, as well as their conserved positions in the fusion peptides, seem to be important for the function of viral fusion proteins (7). It is supposed that glycine residues may provide the proper balance of amphipathicity necessary for merging virus and cell membranes or may be important for an oblique insertion of the fusogenic peptide into the target membrane (15). In this study, the specific role of the two conserved glycine residues in the putative fusion peptide of Se*M*NPV F was addressed by converting these into alanines. These substitutions might increase the stability of the possible α-helical conformation of the F_1 N terminus. The G-to-A mutations in the Sendai virus fusion peptide led to increased fusion activity of the fusion protein (16) while for the Semliki Forest virus E1 protein it caused fusion at a lower pH (22). With the Se*M*NPV F protein, the G-to-A mutations showed no notable effect. However, these results are in line with results obtained for the Ebola virus glycoprotein fusion peptide (18), where one of the mutations of the glycines also had no effect on the virus titer and the incorporation of the fusion protein in virions.

Arginine was substituted for conserved as well as nonconserved hydrophobic residues in the fusion peptide of the Se*M*NPV F protein. Introduction of a polar residue in the hydrophobic face of the amphipathic helix is expected to result in either a shorter (SeF_{L151R} or SeF_{M166R}), narrower (SeF_{F152R}, SeF_{M155R}, or SeF_{F163R}), or disrupted (SeF_{V158R}) hydrophobic face. The alterations can possibly disturb the helical conformation or the insertion of the helix in the host membrane and, hence, may have an effect on infectivity. Leucine was substituted for the conserved polar lysine

TABLE 2. Summary of obtained results[a]

gp64-null bacmid	Rescue	PCR	Western blot analysis with:		Viral propagation at an MOI of:	
			F incorporation	F cleavage	5.0	0.5
Controls						
AcGP64	+++	√	NA	NA	+++	+++
SeF	++	√	++	+	++	++
SeF_{R149K}	−	√	+	−	NA	NA
ΔF	−	√	NA	NA	NA	NA
Mutants						
SeF^{NdeI}	++	√	++	+	++	++
SeF_{L151R}	++	√	++	+	++	++
SeF_{F152R}	−	√	+	+	NA	NA
SeF_{M155R}	+	√	+	+	++	+
SeF_{G156A}	++	√	++	+	++	++
SeF_{V158R}	+	√	++	+	+	+
SeF_{K160L}	+	√	++	+	++	+
SeF_{F163R}	+	√	++	+	++	+
SeF_{G164A}	++	√	++	+	++	++
SeF_{M166R}	++	√	++	+	++	++
$SeF_{\Delta 151–170}$	−	√	++	+	NA	NA

[a] √, in agreement with the expectancy; NA, not applicable; +++, good; ++, fair; +, bad; −, none.

(SeF_{K160L}), which decreases the hydrogen bonding potency of the back face of the helix. Despite all the substitutions, there was no notable effect on incorporation of F proteins in BVs and on the processing of the mutant F proteins (Fig. 5). Only SeF_{F152R} was not able to produce infectious virus (Fig. 3), suggesting a critical role of this amino acid in fusion. Similar results with an F-to-R conversion have been obtained for the Ebola glcyoprotein (18).

The virions pseudotyped with the SeF_{M155R}, SeF_{F163R}, SeF_{V158R} (hydrophilic substitutions), and SeF_{K160L} (hydrophobic substitution) genes were all impaired in their virus propagation dynamics (Fig. 6B). For SeF_{M155R}, this could be caused by a reduced incorporation of F protein in BVs (Fig. 5, lane 7). In contrast, when cells were infected with a higher dose, only the titer of virus pseudotyped with SeF_{V158R} was significantly lower than that of native SeF (Fig. 6A). Such a V-to-R conversion has been shown to reduce the fusion activity of the murine leukemia virus fusion protein (19). However, Western analysis suggested that the amount of BVs produced is extremely low (Fig. 5, lane 9). It is very well possible that this is due to a defect in transport of the protein to the cell membrane, caused by incorrect folding of the protein.

The mutations L151R and M166R did not result in a significant drop in virus titers, although the incorporation of SeF_{M166R} in BVs was somewhat affected. The L^{151} and M^{166} residues are the first and the last hydrophobic amino acids of the putative fusion peptide, and this suggests that the F protein can properly function with a smaller hydrophobic face. However, this does not imply that the borders of the putative fusion peptide of Se*M*NPV F are well defined, because the two conserved aspartic acid residues D^{167} and D^{170} also appeared to be important for the fusogenic activity of the Ld*M*NPV F protein (33).

The N-terminal domain of the Se*M*NPV F_1 subunit is most likely involved in the entry and infectivity of BVs, and further credence is given for the role of this domain as a fusion peptide. Future biochemical studies involving the three-dimensional structure of the fusion peptide should indicate how the active fusion peptide is folded and how the behavior of the site-specific mutant fusion peptides can be explained. Experiments to this end are in progress.

REFERENCES

1. **Adams, J. R., and J. T. McClintock.** 1991. Nuclear polyhedrosis viruses of insects, p. 87–204. *In* J. R. Adams and J. R. Bonami (ed.), Atlas of invertebrate viruses. CRC Press, Boca Raton, Fla.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York, N.Y.
4. **Blissard, G. W., and J. R. Wenz.** 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J. Virol. **66:** 6829–6835.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Bulach, D. M., C. A. Kumar, A. Zaia, B. Liang, and D. E. Tribe.** 1999. Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. J. Invertebr. Pathol. **73:**59–73.
7. **Cross, K. J., S. A. Wharton, J. J. Skehel, D. C. Wiley, and D. A. Steinhauer.** 2001. Studies on influenza haemagglutinin fusion peptide mutants generated by reverse genetics. EMBO J. **20:**4432–4442.
8. **Eisenberg, D.** 1984. Three-dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. **53:**595–623.
9. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:**97–120.
10. **Hayakawa, T., G. F. Rohrmann, and Y. Hashimoto.** 2000. Patterns of genome organization and content in lepidopteran baculoviruses. Virology **278:** 1–12.
11. **Hefferon, K. L., A. G. Oomens, S. A. Monsma, C. M. Finnerty, and G. W. Blissard.** 1999. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. Virology **258:**455–468.
12. **Herniou, E. A., T. Luque, X. Chen, J. M. Vlak, D. Winstanley, J. S. Cory, and D. R. O'Reilly.** 2001. Use of whole genome sequence data to infer baculovirus phylogeny. J. Virol. **75**:8117–8126.
13. **Herniou, E. A., J. A. Olszewski, J. S. Cory, and D. R. O'Reilly.** 2003. The genome sequence and evolution of baculoviruses. Annu. Rev. Entomol. **48**:211–234.
14. **Hohmann, A. W., and P. Faulkner.** 1983. Monoclonal antibodies to baculovirus structural proteins: determination of specificities by Western blot analysis. Virology **125:**432–444.
15. **Horth, M., B. Lambrecht, M. C. Khim, F. Bex, C. Thiriart, J. M. Ruysschaert, A. Burny, and R. Brasseur.** 1991. Theoretical and functional analysis of the SIV fusion peptide. EMBO J. **10:**2747–2755.
16. **Horvath, C. M., and R. A. Lamb.** 1992. Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. J. Virol. **66:**2443–2455.
17. **IJkel, W. F. J., M. Westenberg, R. W. Goldbach, G. W. Blissard, J. M. Vlak, and D. Zuidema.** 2000. A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. Virology **275:**30–41.
18. **Ito, H., S. Watanabe, A. Sanchez, M. A. Whitt, and Y. Kawaoka.** 1999. Mutational analysis of the putative fusion domain of Ebola virus glycoprotein. J. Virol. **73:**8907–8912.
19. **Jones, J. S., and R. Risser.** 1993. Cell fusion induced by the murine leukemia virus envelope glycoprotein. J. Virol. **67:**67–74.
20. **Kingsley, D. H., A. Behbahani, A. Rashtian, G. W. Blissard, and J. Zimmerberg.** 1999. A discrete stage of baculovirus GP64-mediated membrane fusion. Mol. Biol. Cell **10:**4191–4200.
21. **Klenk, H. D., and W. Garten.** 1994. Activation of viral spike proteins by host proteases, p. 241–280. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Levy-Mintz, P., and M. Kielian.** 1991. Mutagenesis of the putative fusion domain of the Semliki Forest virus spike protein. J. Virol. **65:**4292–4300.
23. **Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins.** 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. **67:**4566–4579.
24. **Lung, O., M. Westenberg, J. M. Vlak, D. Zuidema, and G. W. Blissard.** 2002. Pseudotyping *Autographa californica* multicapsid nucleopolyhedrovirus

(Ac*M*NPV): F proteins from group II NPVs are functionally analogous to Ac*M*NPV GP64. J. Virol. **76:**5729–5736.
25. **Malik, H. S., S. Henikoff, and T. H. Eickbush.** 2000. Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. Genome Res. **10:**1307–1318.
26. **Martin, I., J. M. Ruysschaert, and R. M. Epand.** 1999. Role of the N-terminal peptides of viral envelope proteins in membrane fusion. Adv. Drug Deliv. Rev. **38:**233–255.
27. **Monsma, S. A., A. G. Oomens, and G. W. Blissard.** 1996. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J. Virol. **70:**4607–4616.
28. **Nicholas, K. B., H. B. Nicholas, Jr., and D. W. Deerfield.** 14 October 2002. Genedoc: analysis and visualization of genetic variation. EMBnet News **4**:14. [Online.] http://www.hgmp.mrc.ac.uk/embnet.news/vol4_2/genedoc.html.
29. **Oomens, A. G., and G. W. Blissard.** 1999. Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. Virology **254:**297–314.
30. **O'Reilly, D. R., L. K. Miller, and V. A. Luckow.** 1992. Baculovirus expression vectors: a laboratory manual. W. H. Freeman and Co., New York, N.Y.
31. **Ozers, M. S., and P. D. Friesen.** 1996. The Env-like open reading frame of the baculovirus-integrated retrotransposon TED encodes a retrovirus-like envelope protein. Virology **226:**252–259.
32. **Pearson, M. N., C. Groten, and G. F. Rohrmann.** 2000. Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the *Baculoviridae*. J. Virol. **74:**6126–6131.
33. **Pearson, M. N., R. L. Russell, and G. F. Rohrmann.** 2002. Functional analysis of a conserved region of the baculovirus envelope fusion protein, LD130. Virology **304:**81–88.
34. **Pearson, W. R.** 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol. **183:**63–98.
35. **Plonsky, I., M. S. Cho, A. G. Oomens, G. Blissard, and J. Zimmerberg.** 1999. An analysis of the role of the target membrane on the Gp64-induced fusion pore. Virology **253:**65–76.
36. **Rohrmann, G. F., and P. A. Karplus.** 19 February 2001, posting date. Relatedness of baculovirus and *gypsy* retrotransposon envelope proteins. BMC Evol. Biol. **1:**1. [Online.] http://www.biomedcentral.com.
37. **Sharrocks, A. D., and P. E. Shaw.** 1992. Improved primer design for PCR-based, site-directed mutagenesis. Nucleic Acids Res. **20:**1147.
38. **Song, S. U., T. Gerasimova, M. Kurkulos, J. D. Boeke, and V. G. Corces.** 1994. An env-like protein encoded by a *Drosophila* retroelement: evidence that gypsy is an infectious retrovirus. Genes Dev. **8:**2046–2057.
39. **Tanda, S., J. L. Mullor, and V. G. Corces.** 1994. The *Drosophila* tom retrotransposon encodes an envelope protein. Mol. Cell. Biol. **14:**5392–5401.
40. **Thiem, S. M., and L. K. Miller.** 1990. Differential gene expression mediated by late, very late and hybrid baculovirus promoters. Gene **91:**87–94.
41. **Vaughn, J. L., R. H. Goodwin, G. J. Tompkins, and P. McCawley.** 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). In Vitro **13:**213–217.
42. **Volkman, L. E., and P. A. Goldsmith.** 1985. Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. Virology **143:**185–195.
43. **Westenberg, M., H. Wang, W. F. IJkel, R. W. Goldbach, J. M. Vlak, and D. Zuidema.** 2002. Furin is involved in baculovirus envelope fusion protein activation. J. Virol. **76:**178–184.
44. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52:**675–697.
45. **White, J. M.** 1992. Membrane fusion. Science **258:**917–924.