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A Functional F Analogue of *Autographa californica* Nucleopolyhedrovirus GP64 from the *Agrotis segetum* Granulovirus▽

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The envelope fusion protein F of *Plutella xylostella* granulovirus is a computational analogue of the GP64 envelope fusion protein of *Autographa californica* nucleopolyhedrovirus (AcMNPV). Granulovirus (GV) F proteins were thought to be unable to functionally replace GP64 in the AcMNPV pseudotyping system. In the present study the F protein of *Agrotis segetum* GV (AgseGV) was identified experimentally as the first functional GP64 analogue from GVs. AgseF can rescue virion propagation and infectivity of *gp64*-null AcMNPV. The AgseF-pseudotyped AcMNPV also induced syncytium formation as a consequence of low-pH-induced membrane fusion.

Baculovirus envelope fusion proteins play a key role in the cell-to-cell movement and systemic infection of viruses in insects via budded viruses (BVs). For the lepidopteran nucleopolyhedroviruses (NPVs), GP64 and F have not only been shown to be responsible for cell fusion upon entry but also to be essential for BV formation. *Autographa californica* nucleopolyhedrovirus (AcMNPV) GP64 plays an important role in the attachment of virions to cells (5), low-pH-dependent membrane fusion (2, 7, 17), and efficient virion budding (15). Deletion of the *gp64* gene is lethal for BV propagation; the deficiency can be rescued by *gp64* homologues such as group II NPV *F* protein genes and vertebrate virus *F* genes (10, 12, 13, 14). Relative to NPVs, most granuloviruses (GVs) exhibit a relatively narrow host range and various tissue tropisms (21). GVs lack a *gp64*-like gene but have a putative *F* gene (18). The F protein of *Plutella xylostella* GV (PlxyGV), a pathogen which causes systemic infection to the diamondback moth *P. xylostella* (Yponomeutidae) (3, 4), cannot readily rescue the infectivity of *gp64*-null AcMNPV (12). It has hypothesized that the greater evolutionary distance between GVs and lepidopteran NPVs results in a less compatible interaction with AcMNPV proteins. This may be an explanation for the inability of the GV F proteins to compensate for the absence of GP64 in AcMNPV (12). However, in the present study we show that the F protein of *Agrotis segetum* GV (AgseF; GI151564275), a pathogen which causes systemic infection to the cutworm *A. segetum* (Noctuidae) and kills the infected larva in a few days (19), could rescue the infectivity of AcMNPV lacking its own envelope fusion protein GP64.

To determine whether AgseF could substitute for the function of GP64 in AcMNPV, the *gp64* gene of AcMNPV was inactivated by replacement with a combined enhanced green fluorescent protein (EGFP)-chloramphenicol acetyltransferase (CAT) gene cassette (Fig. 1A). This modified AcMNPV bacmid allowed the selection of recombinants in *Escherichia coli* through CAT and the detection of recombinants' replication in Sf9 cells through EGFP. The heterologous *F* genes, *Spodoptera exigua* MNPV-F (SeF) and AgseF, as well as Ac*gp64* (rescue control), were inserted into the polyhedrin locus of AcMNPV by using Tn7-mediated transposition (11). The structure of the recombinant bacmids generated is shown

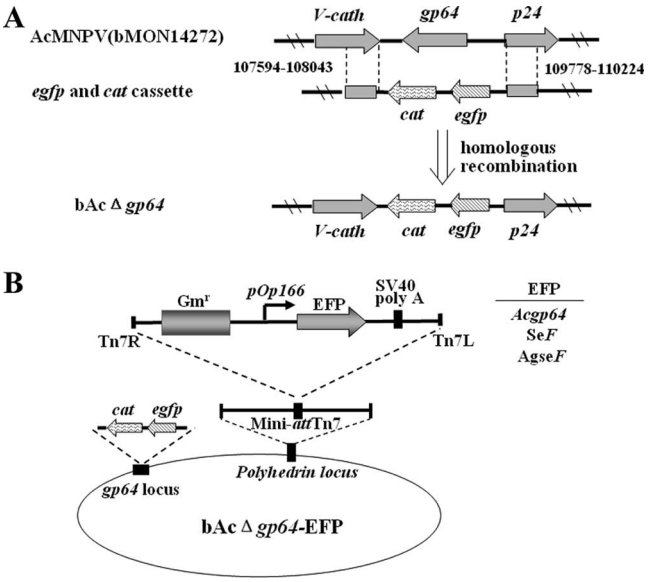

FIG. 1. (A) Generation of the bacmid AcMNPVΔgp64. *cat* and *egfp* genes flanked by *gp64* sequences (upstream nucleotides 109778 to 110224 and downstream nucleotides 107594 to 108043) were used for homologous recombination. (B) The structure of bacmids resulting in virions that are pseudotyped with F proteins. The envelope fusion protein genes (Ac*gp64*, *SeF*, and Ag*seF*) listed on the right under the control of the OpMNPV *Op166* promoter were inserted into the polyhedrin locus of AcMNPV by Tn7-mediated transposition.

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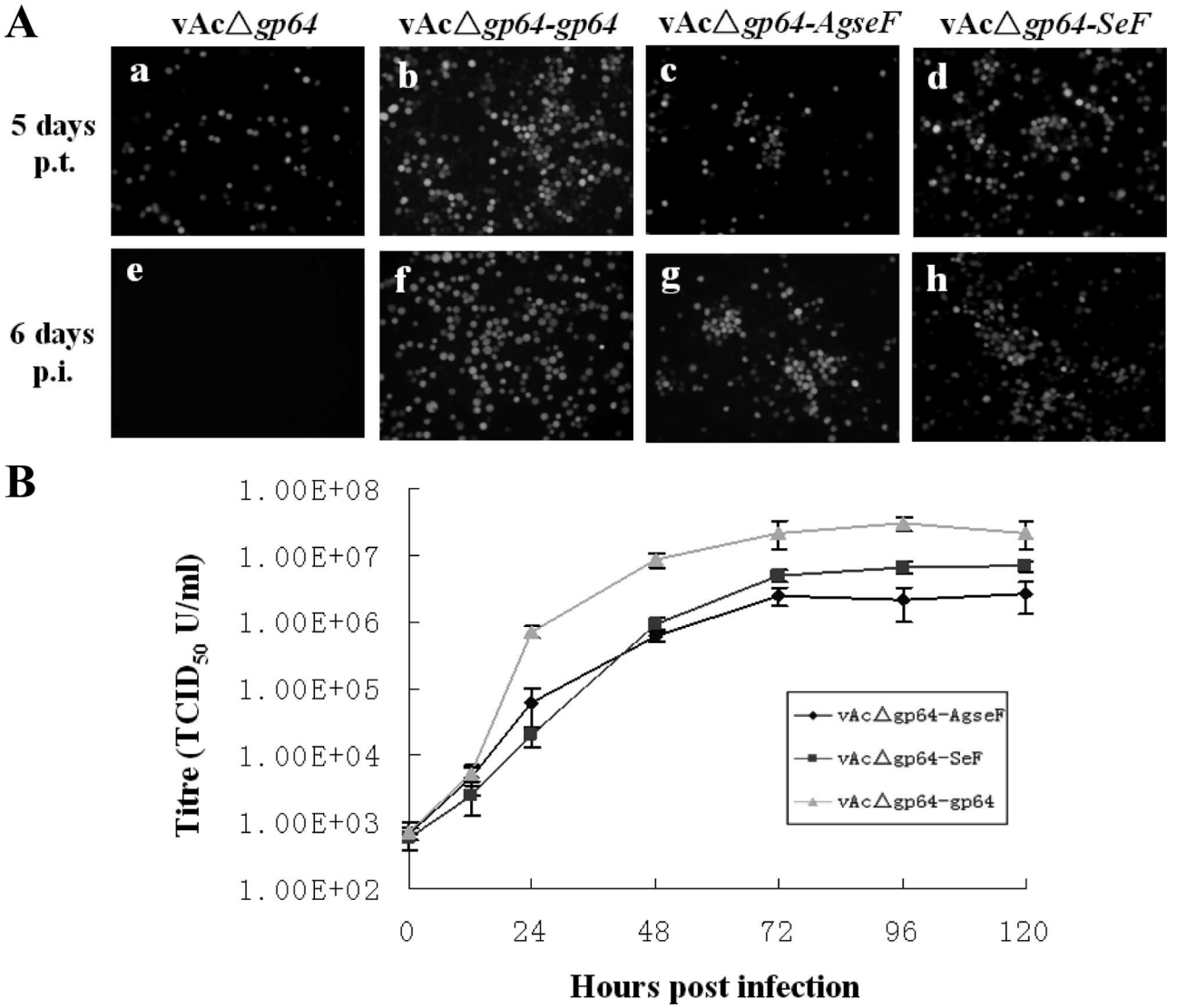

FIG. 2. (A) Transfection-infection assays of pseudotype bacmids for viral propagation. The indicated bacmids (top) were transfected into Sf9 cells (a, b, c, and d). At 5 days posttransfection, clarified supernatants were used for infection of Sf9 cells (e, f, g, and h). The transfected and infected cells were observed by using fluorescence microscopy. (B) One-step growth curve analysis of BV production. Sf9 cells were infected at an MOI of 10 $TCID_{50}$/cell by vAcΔ*gp64-SeF*, vAcΔ*gp64-AgseF*, or vAcΔ*gp64-gp64*. BVs were harvested at the indicated time points postinfection, and titers were determined on Sf9 cells. Each data point represents the average titer from three independent infections. Error bars represent standard deviations.

in Fig. 1B and includes (i) bAcΔ*gp64* (negative control), (ii) bAcΔ*gp64-gp64* (*gp64* repaired), (iii) bAcΔ*gp64-SeF* (positive control), and (iv) bAcΔ*gp64-AgseF* (substitution of *gp64* with *AgseF*). Positive clones proved to be correct by PCR and by EcoRI digestion (data not shown).

AgseF is a functional analogue of AcMNPV GP64. Using a transfection-infection assay, the effect of the *gp64* deletion and *F* insertions on BV propagation could be determined by fluorescence microscopy (Fig. 2A). Upon transfection of Sf9 cells with bAcΔ*gp64* (Fig. 2Aa), many singly infected cells were seen (2Aa). Transfer of the supernatant to healthy cells did not result in infected cells (Fig. 2Ae), indicating that, as expected, BVs were not produced (15). As a positive control, *gp64* was reinserted into bAcΔ*gp64* to give bAcΔ*gp64-gp64* (Fig. 1B) and the resulting virus (vAcΔ*gp64-gp64*) rescued infectious BV production, since the supernatant of the primary transfection (Fig. 2Ab) was able to infect Sf9 cells efficiently (Fig. 2Af).

To determine whether the F protein of AgseGV is a functional analogue of AcMNPV GP64 protein, the bacmid bAcΔ*gp64-AgseF* (Fig. 1B) was transfected into Sf9 cells (Fig. 2A), and at 5 days posttransfection 0.5 ml of the supernatant was used to infect a fresh dish of Sf9 cells. As can be seen, vAcΔ*gp64-AgseF* not only produced a primary infection upon transfection (Fig. 2Ac) but also produced infectious BVs (Fig. 2Ag). As a positive control for the functionality of F protein, bacmid bAcΔ*gp64-SeF* was used and also showed a successful transfection and infection of Sf9 cells (Fig. 2Ad and h), as has been reported previously (12). These results demonstrate that

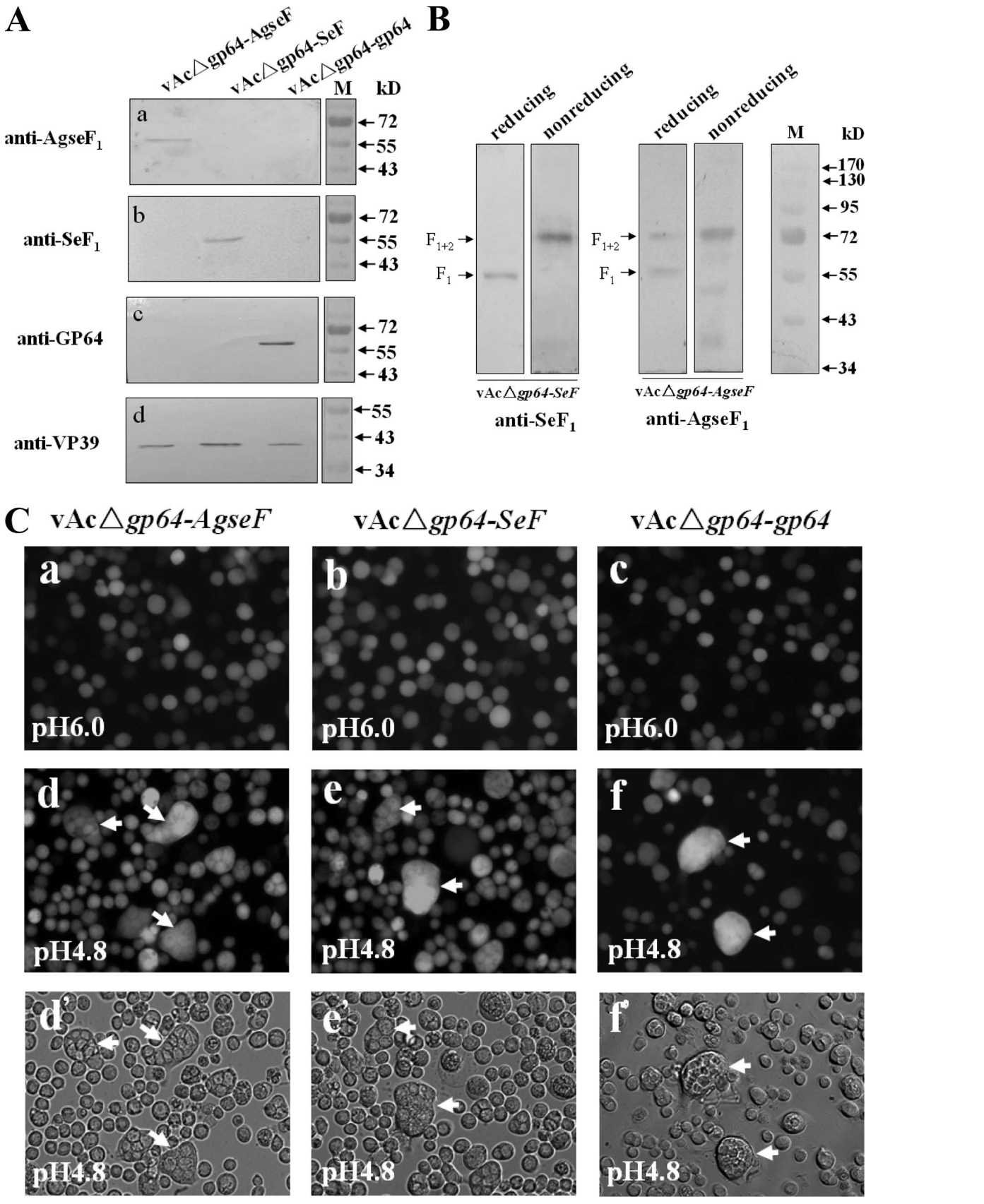

FIG. 3. Western blot analysis and baculovirus-mediated cell fusion of pseudotyped *gp64*-null AcMNPV virions. (A) BVs pseudotyped with AgseF (lane 1), SeF (lane 2), and GP64 (lane 3) were detected by anti-AgseF$_1$ (against 57-kDa cleavage product AgseF$_1$), anti-SeF$_1$ (against

deletion of the *gp64* gene of the AcMNPV bacmid can be successfully complemented by *AgseF* and result in a functional pseudotyped AcMNPV.

One-step growth curves of infectious BV production were determined and compared to BV production from *gp64*-null AcMNPV substituted with *SeF* and *gp64* (Fig. 2B). Sf9 cells were infected in parallel with vAcΔ*gp64-AgseF*, vAcΔ*gp64-SeF*, and vAcΔ*gp64-gp64* at a multiplicity of infection (MOI) of 10 50% tissue culture infective doses ($TCID_{50}$)/cell. Supernatants collected at the indicated time points postinfection were titrated by endpoint dilution on Sf9 cells. The virus titers of *gp64*-rescued AcMNPV are approximately 1 log unit higher than for AcMNPV pseudotyped with *AgseF* and *SeF*; the latter two showed similar levels of virus production (Fig. 2B). These data indicate that the efficiency of AgseF to substitute for the function of GP64 is similar to that of SeF.

Expression of AgseF in pseudotyped BV. To show the presence of AgseF, vAcΔ*gp64-AgseF*, vAcΔ*gp64-SeF*, and vAcΔ*gp64-gp64* BVs were isolated from the supernatant of infected Sf9 cells and subjected to Western blot analysis (Fig. 3A and B). With the newly prepared antibody specifically against the large subunit F_1 (amino acids 180 to 499) of AgseF according to procedures described previously (10), a band of about 57 kDa was detected in vAcΔ*gp64-AgseF* BV, which was absent in lanes with proteins from vAcΔ*gp64-SeF* and vAcΔ*gp64-gp64* BVs (Fig. 3Aa, lane 1). Similarly, a 59-kDa band was found in BVs from vAcΔ*gp64-SeF* (Fig. 3Ab, lane 2) as expected for the size of SeF_1 (6). BVs from vAcΔ*gp64-gp64* showed a band of 64 kDa (Fig. 3Ac, lane 3) representing GP64, the major envelope fusion protein of AcMNPV (2). Expression of GP64 was only detected in vAcΔ*gp64-gp64* BVs, and not in vAcΔ*gp64-SeF* BVs or vAcΔ*gp64-AgseF* BVs (Fig. 3Ac), a finding consistent with the lack of *gp64* in bAcΔ*gp64* and the ability of SeF and AgseF to compensate for GP64 in AcMNPV infectivity. Since VP39 is the major capsid protein of AcMNPV, its detection was used as an internal control for the presence and an equal amount of BVs for each of the AcMNPV pseudotyped BV samples on the gel (Fig. 3Ad, lanes 1 to 3). These results confirm that the pseudotyped AcMNPV, which rescued infectivity, contained AgseF.

Posttranslational cleavage of group II NPV F-proteins by a proprotein convertase is essential for virus infectivity and results in two subunits that are linked by a disulfide bond (6, 10, 16, 20). The disulfide bridge between $AgseF_1$ (C-terminal fragment) and $AgseF_2$ (N-terminal fragment) was examined under both reducing and nonreducing conditions (Fig. 3B). For vAcΔ*gp64-SeF*, a 59-kDa band (lane 1) and a 74-kDa band (lane 2) were detected, which correspond to SeF_1 and SeF_{1+2} (F_1 linked with F_2), respectively (6). For AgseF, a 57-kDa band (lane 3) and a 75-kDa band (lane 4) were observed that would correspond to the predict sizes of $AgseF_1$ and $AgseF_{1+2}$. From these experiments we conclude that similar to NPV F proteins, the F-protein of AgseGV is probably cleaved by furin to release an N-terminal fragment F_2 and a C-terminal membrane anchored fragment F_1 linked by a disulfide bond.

Fusogenicity of AgseF. Membrane fusion mediated by envelope fusion proteins of NPVs such as GP64, SeF, and HaF are activated by acidification (2, 6, 7, 8, 17). To determine whether low pH membrane fusion of Sf9 cells is mediated by AgseF as well, syncytium formation assays (6) were performed with vAcΔ*gp64-AgseF*-infected cells (Fig. 3Ca, d, and d′). vAcΔ*gp64-SeF* (Fig. 3Cb, e, and e′)- and vAcΔ*gp64-gp64* (Fig. 3Cc, f, and f′)-infected cells were used as positive controls for fusion. Cell-to-cell fusion was not observed upon infection of Sf9 cells by vAcΔ*gp64-AgseF* virions (Fig. 3Ca). However, after exposure to low-pH medium for 10 min, cell fusion was observed after 24 h (Fig. 3Cd and d′). Low-pH-dependent fusion was also observed when Sf9 cells were transfected with plasmids carrying only the Agse*F* gene (data not shown). This confirms that AgseF is solely responsible for the fusogenicity of vAcΔ*gp64-AgseF*.

Computational analysis did not reveal major structural differences between AgseF and PlxyF (data not shown). Both AgseF and PlxyF share all of the common features with NPV F proteins. AgseF contains a 24-residue cytoplasmic tail domain (CTD) that is similar to that of PlxyF (19 residues). Since a different length of the CTD is not an important determinant in the ability of an F protein to rescue AcMNPV *gp64*-null cells (9), the different performance of AgseF and PlxyF in the AcMNPV pseudotyping system is not likely due to a major difference in the structure of F, including CTDs. However, there are multiple smaller differences, which could explain the functional difference between PlxyF and AgseF. For example, the ends of the predicted fusion peptides are very different, with PlxyF having a glutamic acid, which is not particularly hydrophobic and could require a specialized interaction.

In summary, the AgseF has been characterized as a functional analogue of the AcMNPV GP64 protein and a homologue of baculovirus F proteins. This is the first study to experimentally identify a functional F protein from GVs. The successful substitution of GP64 with AgseF in AcMNPV and the functional characterization of AgseF provide further insight into the function of baculovirus envelope fusion proteins. Our data also emphasize the importance of studying phylogenetically distant baculovirus F proteins. In this context, it becomes interesting to see whether the nonlepidopteran baculoviruses, such as *Culex nigripalpus* (Diptera) NPV (1), also carry a functional GP64 analogue. Understanding the function of baculovirus F proteins furthers our understanding of baculovirus infection and evolution.

59-kDa cleavage product SeF_1), and anti-GP64 antisera. Anti-VP39 (nucleocapsid) antiserum was used as an internal control for each of the BV samples. (B) Posttranslational cleavage of SeF and AgseF. BVs of vAcΔ*gp64-SeF* (lanes 1 and 2) and vAcΔ*gp64-AgseF* (lanes 3 and 4) were collected from the supernatants of infected cells and separated under reducing (lanes 1 and 3) or nonreducing (lanes 2 and 4) conditions. The positions of F_1 and F_{1+2} are indicated. The outer right lane contains marker proteins for size determination. (C) Baculovirus-mediated cell fusion. Sf9 cells were infected at an MOI of 10 $TCID_{50}$/cell with either vAcΔ*gp64-AgseF* (a, d, and d′), vAcΔ*gp64-SeF* (b, e, and e′), or vAcΔ*gp64-gp64* (c, f, and f′) BV. At 48 h postinfection, cells were incubated with low pH (pH 4.8) Grace's medium for 10 min and then with the same medium but at pH 6.0. Syncytium formation was observed 24 h after the low-pH treatment. Multinucleate cells are indicated by arrows.

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