Conserved Leucines in N-Terminal Heptad Repeat HR1 of Envelope Fusion Protein F of Group II Nucleopolyhedroviruses Are Important for Correct Processing and Essential for Fusogenicity[▽]

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The heptad repeat (HR), a conserved structural motif of class I viral fusion proteins, is responsible for the formation of a six-helix bundle structure during the envelope fusion process. The insect baculovirus F protein is a newly found budded virus envelope fusion protein which possesses common features to class I fusion proteins, such as proteolytic cleavage and the presence of an N-terminal open fusion peptide and multiple HR domains on the transmembrane subunit F₁. Similar to many vertebrate viral fusion proteins, a conserved leucine zipper motif is predicted in this HR region proximal to the fusion peptide in baculovirus F proteins. To facilitate our understanding of the functional role of this leucine zipper-like HR1 domain in baculovirus F protein synthesis, processing, and viral infectivity, key leucine residues (Leu209, Leu216, and Leu223) were replaced by alanine (A) or arginine (R), respectively. By using *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) as a pseudotype expression system, we demonstrated that all mutant F proteins incorporated into budded virus, indicating that leucine substitutions did not affect intercellular trafficking of F. Furin-like protease cleavage was not affected by any of the leucine substitutions; however, the disulfide bridging and N-linked glycosylation patterns were partly altered. Single substitutions in HR1 showed that the three leucine residues were critical for F fusogenicity and the rescue of AcMNPV infectivity. Our results support the view that the leucine zipper-like HR1 domain is important to safeguard the proper folding, glycosylation, and fusogenicity of baculovirus F proteins.

Baculoviridae is a large family of enveloped DNA viruses that are pathogenic to arthropods, predominantly insects in the order *Lepidoptera* (46). Baculoviruses are divided into two genera, *Nucleopolyhedrovirus* and *Granulovirus*. Phylogenetic studies indicate that nucleopolyhedroviruses (NPVs) can be divided into two subgroups, group I and group II (21, 56). Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV) (48). ODVs are present in occlusion bodies and are able to infect midgut epithelial cells by direct membrane fusion. In contrast, BVs are adapted to propagate infection from cell to cell and enter via clathrin-mediated endocytosis (29, 48). BVs are responsible for the systemic spread of the virus in the infected insect and infected insect cell cultures.

Viral envelope fusion proteins can be sorted into at least two distinct classes, class I and class II, based on their functional characteristics (16). Class I fusion proteins are found in many disparate RNA virus families, including retroviruses, orthomyxoviruses, paramyxoviruses, arenaviruses, coronaviruses, and filoviruses. Viral fusion proteins of alphaviruses and flaviviruses have been categorized as class II (16). In the case of

baculoviruses, two distinct envelope fusion proteins were found in BVs, GP64 for group I NPVs (4) and F for group II NPVs (23, 28, 38). F proteins from group II NPVs are functional analogs to GP64 (28, 30, 32), as both entire and cytoplasmic tail-truncated F genes can rescue the infectivity of a *gp64*-null *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) (30). Group I NPVs contain a remnant F gene (Ac23 like) whose product is associated with BVs but has no fusogenic activity (33).

Unlike GP64, baculovirus F proteins have been shown to possess structural characteristics similar to class I fusion proteins from vertebrate viruses. F proteins are synthesized first as a proprotein and are later cleaved by a furin-like protease (Fig. 1), resulting in two disulfide-linked subunits, F₁ (C terminal) and F₂ (N terminal) (28, 52). Baculovirus F proteins are N-glycosylated and are found as homotrimers on the BV particle (28). A putative fusion peptide located on the N terminus of the F₁ subunit was reported to be critical to the biological function of *Spodoptera exigua* MNPV F (51). Furthermore, heptad repeat (HR) regions were predicted in baculovirus F proteins, with HR1 downstream of the fusion peptide region and HR2 upstream of the transmembrane domain (Fig. 1 and 2). In contrast to GP64 and despite their structural homology to vertebrate virus F proteins, group II NPVs are unable to infect mammalian cells. It is therefore important to study the characteristics of baculovirus F proteins, more specifically the domains that are related to F-structure and function, such as HRs.

HR motifs have the ability to form amphipathic helices and

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FIG. 1. Amino acid sequence alignment of the HearNPV F protein domains near the N-terminal heptad repeat, HR1, with the corresponding domain of F homologous proteins of group II NPVs, GVs, and group I NPVs. Virus abbreviations are shown on the left (see also reference 46).

have been detected in the transmembrane subunit of the class I viral envelope fusion proteins of RNA viruses (13). They have been shown to play an essential role in viral fusion and infectivity (9, 12, 14, 20, 34, 41, 43, 50). After receptor binding or induction by low pH, the trimeric viral fusion proteins undergo a series of conformational changes in order to allow fusion to occur (2, 7, 14, 16, 45). X-ray crystallographic studies of various viral fusion protein transmembrane subunits demonstrated that HR regions form coiled-coil trimer or six-helix bundle structures, whereby three HR1 helices form a central coiled-coil surrounded by three HR2 helices in an antiparallel orientation (8, 10, 25, 27, 35, 36, 49, 55, 57). Peptides derived from HRs were reported to be potent and specific inhibitors of membrane fusion and viral infection (5, 11, 19, 37, 42, 53).

Similar to many vertebrate viral envelope fusion proteins (1, 7, 12, 14, 34, 41, 54), an HR region with a leucine zipper-like motif (HR1) was located downstream of the fusion peptide of baculovirus (HearNPV) F (Fig. 1), but its role in baculovirus F functioning was not explored. To promote further understanding of the fusion process mediated by F in insect cells and the functional role of the proximal HR1, mutations were introduced into the predicted leucine zipper-like motif within HR1. This was done by replacing key leucines (Leu209, Leu216, and Leu223) of the zipper with alanine (nonpolar) or arginine (positively charged). A *gp64*-null AcMNPV bacmid pseudotyping system (32) and the conventional AcMNPV insect cell expression system were combined to test the effects of the substitutions on the characteristics of F protein expression and F function, including the potential to rescue *gp64*-null AcMNPV and to mediate low-pH-activated membrane fusion. The present study suggests that the conserved leucines located in the HR1 domain play an essential role in baculovirus F protein-mediated membrane fusion and viral infectivity by bringing the protein into the optimal conformation to allow proper folding and glycosylation.

MATERIALS AND METHODS

Cells and bacmid. *Spodoptera frugiperda* cell line IPLB-SF-21 (47) and Sf9^{Op1D} cells (40) were cultured at 27°C in plastic tissue culture flasks (Nunc) in Grace's insect medium (pH 5.9 to 6.1; Invitrogen) supplemented with 10% fetal bovine serum (FBS). A *gp64*-null bacmid (32) was used to study the functional role of the HR1 region of the HearNPV F protein.

Computational analysis. The sequence for the HearNPV F protein was obtained from GenBank (AF271059). Predictions of potential coiled-coil regions (Paircoil) and transmembrane domains (TMHMM) were conducted by using proteomic tools of the ExPASy Proteomics server (<http://us.expasy.org>).

Mutagenesis and bacmids. Site-directed mutagenesis of the selected leucines was performed as follows (Fig. 2). Preferred codons (underlined) of the small unchanged residue alanine (A) and charged residue arginine (R) replaced the codons for Leu209, Leu216, and Leu223 by introducing in the 5' end of mutagenesis reverse primers (R-L209R, 5'-ACGCGCGTGTGTTATTTTGGCTAAAG-3'; R-L209A, 5'-CGCCGCGTGTGTTATTTTGGCTAAAG-3'; R-L216R, 5'-ACGTTCTTCTTCTGTTGTTGAGC-3'; R-L216A, 5'-CGCTTCTTCTTCTGTTGTTGTTGAGC-3'; R-L223R, 5'-ACGACGTATGAGTTCA TCGTCGAGTTC-3'; and R-L223A, 5'-CGACGTATGAGTTCA TCGTCGAGTTC-3'). With prior 5' phosphorylation of the reverse primers and three forward primers (F-L209, 5'-ACCGAACAAGTGAAAGAAGTCTGAC-3'; F-L216, 5'-GACGATGAAGTATACGTTTGGTTC-3'; and F-L223, 5'-GTCA ACTATGAAGATCATTGCGT-3'), PCRs were performed with a template vector, pFB-F&GFP (29), a pFAST-BAC vector carrying the HearNPV *f* gene under control of its native promoter, and the *gfp* gene under control of AcMNPV *p10* promoter. Phusion polymerase (Finnzyme) was applied in the PCR. After the first purification, the mutant PCR products were treated with DpnI to eliminate template plasmid DNA. Subsequently, the 5' ends of purified PCR products were ligated to their own 3' ends, generating new plasmids containing the site-directed mutant sequences in HearNPV F. After sequence verification, the mutant F gene cassettes were cloned back into the pFB-F&GFP vector to replace the wild-type HearNPV *f* gene cassette by swapping the Bst1107I-to-HindIII fragments. This resulted in donor plasmids each carrying one of the six mutant *f* genes. These donor plasmids were used to transpose six mutant *f* genes into a *gp64*-null AcMNPV bacmid and into a wild-type AcMNPV bacmid containing the GP64 gene as well.

Competent cells containing either the *gp64*-null AcMNPV bacmid or the wild-type AcMNPV bacmid were made according to the methods described in the Bac-to-Bac manual (Invitrogen). Transpositions of site-directed mutant genes from donor plasmids to either *gp64*-null AcMNPV bacmid or wild-type AcMNPV bacmid were confirmed by diagnostic PCR using a forward primer (5'-AGCCACTACTCCCAACATC-3') from the gentamicin resistance gene in

FIG. 2. Schematic diagram of pseudotyped AcMNPV bacmid construction. The signal peptide (SP), proteolytic cleavage site (■), fusion peptide (FP), heptad repeat (HR), transmembrane domain (TM), and cytoplasmic tail domain (CTD) are shown. Selected leucines (Leu209, Leu216, and Leu223) were replaced with alanine or arginine, respectively. Donor plasmids carrying the HearNPV *f* cassette with the substitutions shown and a *p10* promoter-controlled *egfp* gene, used to mark infected cells, were used to construct pseudotyped AcMNPV bacmids.

combination with the M13 forward primer (5'-CCCAGTCACGACGTTGTAA AACG-3'). Transfection and infection assays were conducted according to the methods of Long et al. (30).

Transfection and infection assay. Transfection of AcMNPV bacmid containing HearNPV *f* or mutant *f* was performed on Sf21 cells as described previously (28, 30). Briefly, Sf21 cells were seeded in plastic petri dishes, 5 cm in diameter, with 1×10^6 cells per dish. After 24 h of incubation in Grace's insect medium supplemented with 10% FBS, cells were washed twice with Grace's insect medium. Then, cells were transfected with approximately 1 μ g bacmid DNA dissolved in 12 μ l Lipofectin (Invitrogen). Supernatants, containing BVs, were harvested 7 days posttransfection. Following an endpoint dilution assay (EPDA) on Sf21 cells to determine the 50% tissue culture infective dose (TCID₅₀) units of the obtained AcMNPVs, secondary infections were performed at a multiplicity of infection (MOI) of 5 TCID₅₀/cell. Purified AcMNPV BV samples and infected cells were subjected to Western analysis (28).

To produce HearNPV *f* or mutant *f*-pseudotyped *gp64*-null AcMNPV, transfections of these pseudotyped bacmids were performed into Sf9^{Op1D} cells constitutively expressing *Orgyia pseudotsugata* MNPV (OpMNPV) GP64, instead of Sf21 cells (see Fig. 5, below). Seven days posttransfection, supernatants containing pseudotyped *gp64*-null AcMNPV BVs carrying OpMNPV GP64 were used to infect a new batch of Sf9^{Op1D} cells to amplify these BVs. BV titers were determined with an EPDA on Sf21 cells, after which new Sf21 cells were infected by these pseudotyped *gp64*-null AcMNPV BVs derived from Sf9^{Op1D} cells at an MOI of 5 TCID₅₀/cell. Supernatants containing pseudotyped *gp64*-null AcMNPV BV derived from Sf21 cells were collected 5 days postinfection (p.i.). With an EPDA on Sf21 cells, the infectivity of obtained pseudotyped *gp64*-null AcMNPV BV samples derived from Sf21 cells from these infections was determined. The infected Sf21 cells were subjected to Western analysis (testing the presence of HearNPV F and AcMNPV VP39 and the absence of AcMNPV GP64) and to a low-pH-activated syncytium formation assay (26).

Western analysis. The expression of wild-type and mutant F proteins in Sf21 cells and their incorporation into AcMNPV BVs were examined by Western analysis using polyclonal antibodies against F₁ or F₂ (28). Sucrose-purified BVs or cellular total protein samples taken 48 h p.i. were subjected to Western analysis as previously described (28). A polyclonal antibody against AcMNPV VP39 (a gift from Pang Yi, Sun Yatsen University, Guangzhou, China) and a monoclonal antibody against the GP64 AcV5 epitope (22) were used to detect the presence of VP39 and GP64, respectively.

Budded virus production and syncytium formation assay. To investigate infectious mutant *f*-pseudotyped AcMNPV BV production, Sf21 cells were infected with pseudotyped AcMNPV, obtaining GP64 from Sf9^{Op1D} cells at an MOI of 5 TCID₅₀/cell. After 48 h, for each treatment, triplicate supernatant samples were collected and the quantity of infectious BVs from each sample was determined in an EPDA on Sf21 cells. The experiments were done in triplicate, and the results were exported to Microsoft Excel software and subjected to statistical analysis. Pseudotyping AcMNPV BV budding efficiency was further monitored by checking the average amount in 1.5 ml of released virus (pool of the triplicate supernatant samples), as measured with VP39. The infected Sf21 cells were then used for Western analysis and syncytium formation assays.

Syncytium formation (Sf21-Sf21 fusion) assays were performed upon infection of Sf21 cells with pseudotyped AcMNPV carrying GP64 from Sf9^{Op1D} cells at an MOI of 5 TCID₅₀/cell (28). Forty-eight hours after infection, cells were washed three times with 1 ml Grace's medium (pH 6.1) without FBS and treated for 5 min in 1 ml acidic Grace's medium at pH 5.0. Subsequently, the acidic medium was removed and replaced with 2 ml Grace's medium (pH 6.1) supplemented with 10% FBS. Syncytium formation was observed by light microscopy 4 h after acidification treatment.

Deglycosylation. Total proteins from AcMNPV-coding FL216R-infected Sf21 cells were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Small proteins ranging from 15 kDa to 20 kDa were

recovered from the gel by the syringe maceration extraction method (44) and denatured by boiling for 10 min in the presence of 0.5% SDS and 1% of β -mercaptoethanol. Denatured proteins were incubated overnight either in Endo H G5 buffer (50 mM sodium citrate, pH 5.5, 5 mM phenylmethylsulfonyl fluoride) containing 1 U Endo H (New England Biolabs) or in PNGase F incubation buffer (phosphate-buffered saline, pH 7.4, 20 mM EDTA, 0.5% NP-40, 5 mM phenylmethylsulfonyl fluoride) containing 1 U PNGase F (Roche). Deglycosylated proteins were separated by 12% SDS-PAGE followed by Western analysis using polyclonal antibodies against F_2 .

RESULTS

HR1 region of baculovirus F proteins. To better understand the architecture of baculovirus F proteins with respect to the fusion peptide and the downstream-located HR region (HR1), we aligned F proteins from members of group II NPV, granulovirus (GV), and group I NPV (Ac23 homologs) (33) (Fig. 1). The comparison of F proteins clearly showed the conserved furin cleavage sites, fusion peptides, and the HR1 regions. In agreement with a previous report, the areas of furin cleavage and the adjacent fusion peptide are highly conserved among F homologous proteins from group II NPVs and GVs (39, 51). Also well-conserved is the HR1 region, located immediately downstream of the fusion peptide (39). It is noteworthy that all these conserved functional domains are present in F proteins from group II NPVs and GVs, but none of these is found in the truncated F or Ac23 homologs from group I NPVs (Fig. 1). GP64 homologs are the functional envelope fusion proteins of group I NPVs, not the truncated F homologs. This alignment thus showed that F homologs from group I NPVs did not contain the hallmark elements for a functional class I fusion protein, which explains why the F homologs from group I NPVs are nonfunctional envelope fusion proteins (33). Conserved leucines are frequently found at the "d" position of the HR1 region. Such leucine zipper-like HRs are commonly identified from other class I envelope fusion proteins, e.g., from vertebrate viruses, and have been proved to be critical for fusion activity and virus infectivity (7, 12, 14, 33, 41, 54).

Expression of F protein mutants in Sf21 cells. To see whether the expression of F proteins was affected by mutations in conserved leucines in HR1, we transposed all the mutated *f* genes into an AcMNPV bacmid already encoding GP64 (Fig. 2). Transfection and infection experiments were performed to obtain AcMNPVs carrying mutant *f* genes as previously described (32). Three days postinfection, infected cells were collected and the total cellular protein from each infection was subjected to Western analysis using antibody against HearNPV F_1 and F_2 separately (Fig. 3A). Expression of GP64 (lower panel) was also tested as a loading control. Under reducing conditions, F_1 (upper panel) and F_2 (middle panel) subunits were detected in all samples, suggesting all six mutant F proteins are not only expressed but also are expressed at a similar level in AcMNPV-infected Sf21 cells as the parental F protein (left lane). The furin-like cleavage of F proteins also properly occurred with any of the substituted leucine residues in HR1, as the subunits F_1 and F_2 migrated separately in all cases.

Surprisingly, the synthesis of F_2 subunits of all the mutant F proteins was affected (Fig. 3A, middle panel). Rather than having two species of F_2 subunits (unglycosylated [ugF_2] and major glycosylated F_2 [gF_2] as minor and major bands, respectively), as in the case of parental F protein (leftmost lane),

three species of F_2 subunits were found in mutant F proteins. The third one (higher glycosylated F_2 [hgF_2]) appeared to be around 2 kDa heavier than the unglycosylated form of F_2 (Fig. 3A, middle panel). Especially in the case of F^{L216R} , a substantial amount of the heavier F_2 was detected, whereas little or no unglycosylated F_2 could be detected in this case. These results suggested that the expression and proteolytic processing of wild-type and mutant F proteins were similar but that the N-linked glycosylation pattern of the F_2 subunit is considerably changed as a consequence of the Leu substitutions in F_1 .

As the mutations were introduced in the HR1 region located in the large F_1 subunit, the molecular mass of F_2 subunits should presumably stay the same. However, for a minor portion of the F_2 subunits, the N-linked glycosylation appears to be changed. There is only one genuine N-linked glycosylation site in the F_2 subunit (31). The most likely explanation is that the N-linked glycosylation pattern of the F_2 subunit is somehow affected by the introduction of mutations in HR1 of F_1 . The large N-linked glycans from the F^{L216R} F_2 subunit might be an Endo H-sensitive form (oligomannose). To study the characteristics of the larger F_2 , a deglycosylation assay was performed on F_2 recovered from SDS-PAGE (Fig. 3B). It was found that the larger and glycosylated F_2 subunits were sensitive to PNGase treatment (lane 4), resulting in a single band with similar molecular size to the F_2 without N-linked glycosylation (lane 1). Neither the mobility of F_2 nor the glycosylation of F_2 was sensitive to Endo H treatment, suggesting that the larger N-linked glycan is not of the high-mannose type (Fig. 3B, lane 5). This observation further suggested that the mutations in conserved leucines in the HR1 region caused changes in N-linked glycan processing in the F_2 subunit, although the majority of F_2 is glycosylated as gF_2 , like the wild-type F.

Incorporation of F protein mutants in AcMNPV. To further study the effects of mutations of conserved leucine residues in the HR1 region of HearNPV F protein on trafficking, we examined the incorporation of mutated F proteins in AcMNPV BVs. Proteins of sucrose-purified AcMNPV BVs were separated by SDS-PAGE under reducing (Fig. 4A) or nonreducing (Fig. 4B) conditions and were subsequently immobilized on membranes for Western analysis. GP64 protein was detected in all cases in almost equal amounts and served as an internal control for the incorporation of F in BVs. Under reducing conditions (Fig. 4A), F_1 and F_2 subunits were detected separately in all BVs. This result is in agreement with the previous finding that the furin-like cleavage of F protein is not affected by mutating the conserved leucine residues (Fig. 3A). However, the incorporation level of the various mutant F proteins in BVs is different; in particular, a much lower amount of mutant F^{L216R} was incorporated in mature BVs. Under nonreducing conditions (Fig. 4B), both antibodies (against F_1 and F_2) detected F_0 in the case of parental F protein. F_0 was also detected in the mutant F proteins except for BVs containing the F^{L216R} protein. A significant amount of disulfide-linked oligomer was detected in the case of F^{L216A} , F^{L223A} , and F^{L216R} proteins. The F^{L216R} protein in monomeric form was hardly detected as F_0 but was present mainly as oligomers. These results indicate that correct formation of disulfide bonds is dependent on the conserved leucine residues in the HR1 region.

FIG. 3. (A) Expression of the parental F and mutant F proteins by baculovirus (AcMNPV bacmid with its *gp64* gene) in insect cells. Sf21 cells were infected by AcMNPVs carrying parental *f* and mutant *f* cassettes. Forty-eight hours p.i., infected Sf21 cells were collected and lysed in SDS-PAGE loading buffer. Total cellular proteins on a Western blot assay from each infection were probed by anti-F₁ (upper), anti-F₂ (middle), and anti-GP64 (lower) antibodies under reducing conditions. (B) Deglycosylation assays of F₂ from the F^{L216R} protein. Cellular proteins (ranging from 25 kDa to 15 kDa) from AcMNPV^{*gp64-FL216R*}-infected Sf21 cells were recovered from an SDS-PAGE gel and were treated with Endo H and PNGase F, respectively. F and F^{N104Q} (an F₂ N-linked glycan-negative mutant protein [31]) were used as controls.

HR1 of HearNPV F is important for HearNPV F to rescue *gp64*-null AcMNPV and F fusogenicity. Based on findings described in the previous sections, the mutant HearNPV F proteins were found to be expressed in Sf21 cells and to be incorporated in AcMNPV BVs. To further understand correlations between these mutations and F function, a novel testing system was established to study the role of HR1 in the functionality of F and F-mediated membrane fusion (Fig. 5). In this system, HearNPV *f*/mutant *f*-pseudotyped *gp64*-null AcMNPV bacmids were transfected into Sf9^{Op1D} cells constitutively expressing OpMNPV GP64 (38). The TCID₅₀s of the obtained pseudotyped AcMNPVs carrying both GP64 derived from Sf9^{Op1D} cells and F/mutant F were determined in an EPDA on Sf21 cells. Then, Sf21 cells were infected with each GP64-incorporated pseudotyped AcMNPV at the same MOI of 5

TCID₅₀ units/cell. Infected Sf21 cells expressing no GP64 were used for a low-pH-activated syncytium formation assay as a result of the presence of a functional F, and the TCID₅₀ of progeny pseudotyped AcMNPV without GP64 incorporation was determined in an EPDA on Sf21 cells again. A wild-type AcMNPV bacmid and a *gp64*-null AcMNPV bacmid were included in this system as a positive and a negative control, respectively.

To show the potential of this novel test system, Western analysis was carried out on pseudotype virus using HearNPV F, AcMNPV VP39, and GP64 antibodies (Fig. 6A). HearNPV F/mutant F proteins were found to be present in pseudotyped AcMNPV-infected Sf21 cells (lanes 3 to 9). AcMNPV major capsid protein VP39 was found in all cases, including the *gp64*-null AcMNPV (lane 3). The latter indicated that the GP64

FIG. 4. Incorporation of parental and mutant F proteins in AcMNPV-pseudotyped viruses. Western analysis was performed on purified AcMNPV BVs carrying both F/F mutant proteins and GP64 by using anti-F₁ (upper panel), anti-F₂ (middle panel), and anti-GP64 (lower panel) antibodies under either reducing conditions (A) or nonreducing conditions (B). GP64 detection under reducing conditions served as a proper incorporation control.

obtained from Sf9^{OP1D} cells was able to bring *gp64*-null AcMNPV BVs into Sf21 cells. GP64 was found only in wild-type AcMNPV-infected Sf21 cells (lane 1) and not in *gp64*-null AcMNPV cells (lane 2). This confirmed that Sf21 cells infected with GP64-pseudotyped AcMNPV did not produce GP64. The pseudotyped AcMNPV progeny from these infections did not incorporate GP64 (Fig. 6A, upper panel), and these viruses were suitable to test the infectivity of HearNPV *f*/mutant *f* pseudotyped AcMNPV in Sf21 cells. These Western assay re-

sults confirmed the successful establishment of a new experimental system to study baculovirus F functionality.

The HearNPV F protein is a functional analog of AcMNPV GP64 (28). To determine the influence of leucine mutations in HR1 on HearNPV F functionality, the infectious BV production at 48 h p.i. was evaluated in EPDAs. HearNPV *f*-pseudotyped AcMNPV produced much more infectious BVs than all mutant *f*-pseudotyped AcMNPVs (Fig. 6B). EPDA could not detect infectious virus from AcMNPV pseudotyped

FIG. 5. Schematic of the functional analysis of baculovirus F mutants.

with the F^{L216R} gene, suggesting that F^{L216R} is not able to rescue *gp64*-null AcMNPV. This result suggests that the conserved leucines in HR1 are critical for the functionality of HearNPV F and thus important for infectious pseudotyped AcMNPV BV production. However, this could also be the result of different efficiencies of pseudotyped AcMNPV BV budding. To this end we checked the budding efficiency of pseudotyped AcMNPV BV by testing the average amount of VP39 released in supernatant containing each pseudotyped AcMNPV BV. The results showed that F and mutant Fs mediate comparable BV budding, as evidenced by the amount of VP39 (capsid protein) (Fig. 6B, lower panel). These results further indicate that mutations in conserved leucines in HR1 do not change the production of pseudotyped AcMNPV BV, but they affect the entry.

HR regions have been reported to play a critical role in class I viral membrane fusion proteins. Baculoviruses enter host cells through clathrin-mediated endocytosis (29). F protein mediates fusion in late endosome upon low-pH activation. To find out whether these conserved leucines in baculovirus F protein HR1 are important for F fusogenicity, a low-pH-activated syncytium formation assay was conducted upon infection of Sf21 cells with *f*/mutant *f*-pseudotyped AcMNPV where GP64 was provided by the Sf9^{OP1D} cells (Fig. 7). The results showed that HearNPV with wild-type F mediated much more significant syncytium formation (Fig. 7b) than the mutant Fs (Fig. 7c to h). No syncytia were observed in the case of the F^{L216R} mutant, suggesting this mutant F protein is not able to mediate fusion. This result strongly suggests that the leucines in the HR1 region of HearNPV F are important for F-mediated fusion and implies that the baculovirus F protein HR1 region plays a critical role in virus entry.

DISCUSSION

HR regions have been widely detected in membrane fusion proteins of paramyxoviruses, influenza virus, coronaviruses, and retroviruses and have been shown to play an essential role in viral fusion and infectivity (15, 17). Structural studies of membrane fusion proteins show that HR regions located in the N terminus and C terminus of the fusion protein ectodomain can form a coiled-coil six-helix bundle structure or the fusion core (15). In the F protein from baculoviruses, a group of invertebrate DNA viruses, we found three coiled-coil domains in the F_1 subunit of the HearNPV F protein (Fig. 2). The similar architecture of baculovirus F proteins suggests that this F protein is a class I virus fusion protein (16, 39), which is usually found in vertebrate enveloped RNA viruses. In order to understand the functional role of HR domains in baculovirus F proteins, we studied the functional importance of HR1 by site-directed mutagenesis of conserved leucines and reverse genetics on three leucines at the d position in the HR1 domain. These leucines, L209, L216, and L233, are likely involved in formation of the six-helix bundle in analogy to vertebrate virus F proteins.

In order to study the importance of these three nonpolar leucines on the function of F, they were mutated into either arginine (positively charged) or alanine (nonpolar) (Fig. 2). The influences of these amino acid substitutions on F biosynthesis and incorporation into virions were studied by using the AcMNPV pseudotyping system in Sf21 cells (32). Mature baculovirus F proteins are located on BV particles as a homotrimer of F, in which F_1 and F_2 subunits are bridged by disulfide bonds (24, 51). Three F proteins oligomerize as homotrimers through noncovalent interactions (28, 52). Here we demonstrated that on BV particles substitutions of selected leucines within HR1 did not affect proteolytic processing of F but resulted in multiple disulfide-linked oligomers (Fig. 4B). In particular, the L216R mutant protein hardly produced F_{1+2} as an 80-kDa protein, which is the hallmark of mature and functional F on infectious BV particles (28, 52). This mutant also showed no fusogenicity (Fig. 7g). Both antibodies against F_1 and F_2 subunits detected similar unusual disulfide-bridged oligomers (Fig. 4B) in addition to the normal 80-kDa monomers, suggesting that the disulfide bonding between F_1 and F_2 subunits per se was not affected. Unusual disulfide bonds may be the results of illegitimate oligomerization of mutant F proteins. It is possible that these illegitimate oligomers were disulfide bond-linked homotrimers of mutant F. It is equally possible that they are hetero-oligomers consisting of mutant F and other cellular or viral proteins. Nevertheless, these results suggest that the conserved leucine zipper-like motif in HR1 is important for proper folding and disulfide bond formation of HearNPV F. A similar situation exists in paramyxoviruses, where mutations destabilize the F protein and lead to malformed multimers because of chemical cross-linking (18). In addition, less protein was detected in AcMNPV BVs with F^{L216R} than with the other F mutant and authentic HearNPV F proteins, which further suggests that a correct putative coiled-coil conformation is also an important prerequisite for F incorporation in BVs.

Like other virus envelope fusion proteins, baculovirus F is also modified by N-linked glycosylation of both F_1 and F_2

FIG. 6. *gp64*-null AcMNPV rescues the activity of HearNPV F/mutant F. (A) Western analysis of Sf21 cells infected by F-pseudotyped AcMNPV grown in Op1D cells to obtain GP64 at an MOI of 5 TCID₅₀/cell. Forty-eight hours p.i., expression of GP64, VP39, and HearNPV F/mutant F was tested. (B) BVs from the supernatant of each infection treatment were collected. The infectivity in TCID₅₀/ml was tested by using Sf21 cells. Each data point represents infectious BV production at 48 h p.i. Error bars represent standard deviations from the means of triplicate infections and titrations. Budding efficiency was monitored by testing the presence of BVs as evidenced by VP39.

subunits. It was confirmed by high-performance liquid chromatography and matrix-assisted laser desorption/ionization-time of flight mass spectrometry that the only potential N-linked glycosylation site in the HearNPV F₂ subunit (28) is genuinely occupied most likely with paucimannose-type glycans (S. P. Jongen and J. P. Kamerling, personal communication). Here we showed that replacement of selected leucine residues at the "d" position in HR1 resulted in the attachment of a heavier glycan on the F₂ subunit, with an additional molecular mass of around 2 kDa, in addition to the major paucimannose-type glycans. In particular, for the F^{L216R} protein, a significant amount of F₂ with heavier glycans was detected (Fig. 3A). Traces of F₂ with heavier glycans were also detected for the other five mutant F proteins (Fig. 3A), suggesting that even a small change in F structure may result in a different type

of glycosylation. Compared to mammalian cells, insect cells possess a truncated N-linked glycosylation pathway and hence a paucimannose type of N-linked glycans, which are frequently identified in mature glycoprotein expressed in insect cells (24). However, the glycans observed in our (mutant) F protein were not sensitive to Endo H treatment (Fig. 3B, lane 5), suggesting that they are not of the oligomannose type. It may be possible that the large N-linked glycans on F₂ are the end product of further extension on the GlcNAcMan3GlcNAc2Fuc structure. We do not know yet the detailed structure of this heavier glycan. Together with the observation of malformed disulfide bond-bridged oligomers upon substitution of leucines in the zipper, these observations also suggest that the conserved leucines in baculovirus HR1 regions might be critical to F biosynthesis. Further N-linked glycan profiling and lectin bind-

FIG. 7. Syncytium formation promoted by HearNPV F/mutant F. Sf21 cells were infected by *gp64*-null AcMNPV with F or mutant F and GP64 obtained from Op1D cells at an MOI of 5 TCID₅₀/cell (panels b to h). An AcMNPV *gp64*-null mutant served as negative control for fusogenicity. Forty-eight hours p.i., infected Sf21 cells were incubated in Grace's insect medium (pH 5.0) for 5 min. After one wash with fresh Grace's insect medium, the cells were incubated in Grace's insect medium plus 10% FBS. Syncytium formation was observed 12 h post-low-pH treatment.

ing assays to determine the glycan structure will be necessary and will contribute to the understanding of N-linked glycoprotein quality control mechanisms in general in insect cells (6). By utilizing the *gp64*-null AcMNPV pseudotyping system (30), we demonstrated that conserved leucines in the HR1 region of HearNPV F are essential for rescuing the infectivity of AcMNPV and for F fusogenicity. In addition, we showed a variable but clear effect when individual leucine residues within the HR1 region were replaced by either alanine or arginine (summarized in Table 1). Both the alanine (nonpolar) and the arginine (positively charged) substitution of Leu209 (nonpolar) resulted in a dramatic decrease of rescued AcMNPV infectivity and F fusogenicity, indicating that Leu209 is very critical for F function. Leu209 is conserved at the “d” position of the second repeat in HR1 in group II baculovirus F proteins, but not in GV F proteins. The latter is also compatible with the observation that the putative F protein from *Plutella xylostella* GV is not able to rescue the infectivity of *gp64*-null AcMNPV (32), as a threonine residue (polar, uncharged) was located at the “d” position (Fig. 1). At the “a” position in the amphipathic helix is a polar asparagine residue. Substitution of the Leu209 with either alanine or arginine probably results in two contiguous amino acid residues with small (Ala) or large (Arg)

side chains on the hydrophobic side of the coil. This possibly destabilizes the interaction of HRs during six-helix bundle formation or the prefusion stage (18) and results in strongly reduced infectivity of L209A and L209R F mutants when pseudotyped in AcMNPV. Leu216 replaced by alanine resulted in a moderate decrease of the F rescue activity and fusogenicity, whereas the replacement of Leu216 with arginine led to a complete failure for HearNPV F to rescue the infectivity of the *gp64*-null AcMNPV or to mediate the low-pH-activated fusion, although BVs were made (Fig. 6A, lane 8). According to the Berger algorithm (3), among the six mutant F proteins the replacement of Leu216 by arginine is the only one to abolish the coiled-coil structure of the HR1 region, which is in good agreement with our results showing no infectivity and no fusogenicity when F^{L216R} was tested in the AcMNPV pseudotyping system (Fig. 7g; Table 1). The replacement of Leu223 with arginine (F^{L223R}) resulted in higher infectivity than the replacement of Leu223 with alanine (F^{L223A}). This might suggest that at the location of Leu223 the size of the amino acid residue side chain is more important than its polarity. These results again suggest that HR1 in baculovirus F is a critical determinant of F function. Baculovirus BVs enter host cells through a clathrin-depen-

TABLE 1. Summary of obtained results

F protein	Coil ^a	Rescue	Fusogenicity	Cleavage	Mass of N-glycans on F ₂ (kDa)	Detection of F ₀	Incorporation
F	++	+++	+++	+	1.1	++	++
F ^{L209A}	+	+	+	+	1.1	++	++
F ^{L209R}	+	+	+	+	1.1	++	++
F ^{L216A}	++	++	++	+	1.1	++	++
F ^{L216R}	—	—	—	+	1.1/~2.0	+/-	+
F ^{L223A}	+	+	+	+	1.1	++	++
F ^{L223R}	+	++	+	+	1.1	++	++

^a Coil probability was predicted according to the Berger algorithm (3): +++, good; ++, fair; +, bad. A significant amount of F₂ from F^{L216R} carries heavier N-glycans (around 2.0 kDa), although the majority of F₂ from F^{L216R} carries paucimannose-type N-glycans (around 1.1 kDa).

dent endocytosis, and the nucleocapsids are released upon a low-pH-activated fusion (29) mediated by either GP64 (group I) or F (group II). The HR region in GP64 plays a critical role in GP64-mediated membrane fusion (26). Here, using reverse genetics, we have shown that the conserved leucine residues located in fusion peptide-proximal HR1 are important determinants of baculovirus F fusogenicity, virus infectivity, and F protein proper folding. However, they do not affect pseudotyping AcMNPV BV formation. HR1 and the leucines studied are well-conserved in the F homologous proteins from group II NPVs, whereas an HR1 region cannot be found in the F homolog from group I NPVs, which possesses the GP64 ortholog as the functional envelope fusion protein (Fig. 1) (33). Testing the fusogenicity of the putative GV F protein may require a GV pseudotyping system. Our results further implicate that the presence of the HR1 region in F homologs from group II NPVs and GVs is a hallmark for a functional baculovirus F protein.

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