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Furin Is Involved in Baculovirus Envelope Fusion Protein Activation

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The *Spodoptera exigua* multicapsid nucleopolyhedrovirus (Se*M*NPV) *Se8* gene was recently shown to encode the viral envelope fusion (F) protein. A 60-kDa C-terminal subunit (F_1) of the 76-kDa primary translation product of this gene was found to be the major envelope protein of Se*M*NPV budded virus (BV) (W. F. J. IJkel, M. Westenberg, R. W. Goldbach, G. W. Blissard, J. M. Vlak, and D. Zuidema, Virology 275:30–41, 2000). A specific inhibitor was used to show that furin is involved in cleavage of the precursor envelope fusion (F_0) protein. BV produced in the presence of the inhibitor possesses the uncleaved F_0 protein, while an F protein with a mutation in the furin cleavage site was translocated to the plasma membrane but lost its fusogenic activity. These results indicate that cleavage of F_0 is required to activate the Se*M*NPV F protein and is necessary for BV infectivity. Specific antibodies against F_1 and against the putative N terminus (F_2) of the primary translation product were used to show that the F protein is BV specific and that BVs contain both the 60- (F_1) and 21-kDa (F_2) cleavage products. In nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis both subunits migrate as a single 80-kDa protein, indicating that the subunits remain associated by a disulfide linkage. In addition, the presence of the F protein predominately as a monomer suggests that disulfide links are not involved in oligomerization. Thus, the envelope fusion protein from group II nucleopolyhedroviruses of baculoviruses has properties similar to those of proteins from a number of vertebrate viruses.

The *Baculoviridae* are a family of large, enveloped double-stranded DNA viruses exclusively pathogenic to arthropods, predominantly insects in the order *Lepidoptera* (1). Baculoviruses of the genus *Nucleopolyhedrovirus* (NPV) produce two distinct virion phenotypes: the occlusion-derived virus (ODV) and the budded virus (BV) phenotypes (53). ODVs are released from occlusion bodies due to the alkaline environment of the insect gut and are able to infect the midgut epithelial cells by direct membrane fusion (12, 13, 19). BVs infect insect cells via receptor-mediated endocytosis and are responsible for the systemic spread of the virus in the insect (17, 54). Upon acidification of the endosome, the viral and endosomal membranes fuse, thereby allowing entry of the nucleocapsid into the cytoplasm (4, 17). For group I NPVs, e.g., *Autographa californica* Ac*M*NPV, *Bombyx mori* BmNPV, and *Orgyia pseudotsugata* Op*M*NPV, this fusion is mediated by envelope fusion protein GP64 (4, 24, 39). This protein is also required for efficient budding of newly synthesized nucleocapsids at the plasma membrane (30, 33). Baculoviruses belonging to group II NPVs, e.g., Se*M*NPV (21), *Lymantria dispar* Ld*M*NPV (26), and *Helicoverpa armigera* HaSNPV (7), conspicuously lack a GP64-like protein.

Recently, a novel type of envelope fusion (F) protein was identified in BVs of group II NPVs, notably in Se*M*NPV (22) and Ld*M*NPV (37). For Se*M*NPV this F protein is encoded by ORF8 (*Se8*) (21). A C-terminal 60-kDa subunit of the 76-kDa primary translation product was found to be the major envelope protein of Se*M*NPV BV. Several protein bands with molecular sizes in the 15- to 17-kDa range were found (22), and one of these could represent the N-terminal subunit of the F protein. The involvement of furin in the cleavage was hypothesized on the basis of the presence of furin-like proprotein convertase site RSKR immediately upstream of the N terminus of the 60-kDa cleavage product (22).

Cleavage of viral fusion proteins seems to be a general mechanism to activate these proteins and to produce infectious viruses. The cleavage takes place in front of a hydrophobic sequence, the "fusion peptide." Exposure to low pH or receptor binding triggers a conformational change whereby the fusion peptide is believed to translocate to the top of the molecule and to insert into the target membrane (25). A number of fusion proteins of vertebrate viruses are cleaved by furin. These include human cytomegalovirus glycoprotein B (50), human parainfluenza virus type 3 F (36), Ebola virus glycoprotein GP (52), and avian influenza virus hemagglutinin (HA) (47). The furin-mediated cleavage yields in all these cases two subunits that are disulfide linked. Furin is localized in the *trans*-Golgi network (51) and cleaves after the consensus cleavage site R-X-R/K-R (20, 32). The arginine immediately upstream of the cleavage site is the most essential amino acid (32). For Ebola virus envelope protein GP it has been shown that changing this arginine into a lysine results in an uncleaved precursor protein (52). Cleavage of some fusion proteins can

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be inhibited by peptidyl-chloroalkylketones containing the R-X-K/R-R motif, which specifically bind to the catalytic site of furin (10, 15, 40, 47).

In this paper evidence is provided by using a furin inhibitor and mutational analysis of the furin-like proprotein convertase site that cleavage of the SeMNPV BV F protein is mediated by furin and that this cleavage is required to activate the SeMNPV F protein to render infectious BVs. Furthermore, whether both subunits of the primary translation product are present in the SeMNPV BV and whether these remain associated to each other are investigated.

MATERIALS AND METHODS

Cells, insects, and viruses. *Spodoptera frugiperda* cell line IPLB-SF-21 (48) and *Spodoptera exigua* cell line Se301 (16) were cultured at 27°C in plastic tissue culture flasks (Nunc) in Grace's insect medium, pH 5.9 to 6.1 (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS). A culture of *S. exigua* insects was maintained as described by Smits and Vlak (46). The SeMNPV isolate (11) was originally obtained from B. A. Federici (Department of Entomology, University of California) and was called SeMNPV-US1 (31). The AcMNPV-E2 strain (45) was originally obtained from M. D. Summers (Texas A&M University, College Station, Tex.).

Plasmid constructions. The regions coding for amino acids 1 (M) to 149 (R) (F_2) and amino acids 150 (G) to 579 (G) ($F_1\Delta$580-665) of ORF8 in the SeMNPV genome were amplified by high-fidelity Expand long-template PCR (Boehringer Mannheim) from plasmid pSe*Bgl*II-H (21). Primers used to amplify $F_1\Delta$580-665 were 5′-TTTGGATCCCGGCCTTTTTAATTTTATGG-3′ (underlined nucleotides generated a *Bam*HI site) and 5′-AATAAGCTTAACCGATGCTGGAAA ACCACGAAGAC-3′ (underlined and boldface nucleotides generated a *Hin*dIII site and a translation stop codon, respectively). Primers used for amplifying F_2 were 5′-TTGGGATCCTATGCTGCGTTTTAAAGTGATTGTG-3′ (underlined nucleotides generated a *Bam*HI site) and 5′-ATTAAGCTTAGCGTTTA GAGCGTCTTTTCGTCG-3′ (underlined and boldface nucleotides generated a *Hin*dIII site and a translation stop codon, respectively). The PCR products were cloned into the *Bam*HI and *Hin*dIII cloning sites of expression vector pET28a (Novagen) to generate pET28-Se$F_1\Delta$580-665 and pET28-SeF_2.

PCR-based site-directed mutation of the arginine (R149) immediately upstream of the convertase cleavage site into a lysine (K149) was performed according to the method of Sharrocks and Shaw (43). The 5′ primer 5′-TTAT GGATCCATGCTGCGTTTTAAAGTGATTGTG-3′ and the 3′ mutagenic primer 5′-CCCATAAAATTAAAAAGGCCCTTTTTAGAGCGTCTTTTCGT CG-3′ (underlined nucleotides generated the mutation) were used in conjunction with plasmid p166AcV5-Se8 (22) as a template to introduce the R149-to-K149 mutation using high-fidelity Expand long-template PCR (Boehringer Mannheim). The PCR product was agarose gel purified, and the single strand containing the mutation at the 3′ end served as a 5′ mutagenic primer in a second PCR together with 3′ primer 5′-GAGAGGCACGGGCCACGAAAGG-3′. The second PCR product was cloned into pGEM-T (Promega) to generate pGEM-Se8(K149). Plasmids p166AcV5-Se8(K149) and p166AcV5-Se8(K149)GFP were obtained by exchanging the *Bam*HI/*Stu*I fragment of pGEM-Se8(K149) with those of p166AcV5-Se8 and p166AcV5-Se8GFP (22), respectively.

Production of polyclonal antibodies. Proteins $F_1\Delta$580-665 and F_2 were expressed in *Escherichia coli* Bl21 cells containing vector pET28-Se$F_1\Delta$580-676 or pET2-SeF_2 and were purified as described previously (23). The proteins were concentrated using a Centriprep 10-kDa filter device (Amicon) and dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Protein concentrations were determined with the Bio-Rad protein assay.

Two chickens were injected intramuscularly with either 200 μg of purified $F_1\Delta$580-665 or F_2 protein using a water-in-oil adjuvant. The chickens were boosted after 4 weeks with 150 μg of purified protein. Two weeks after the booster, eggs were collected every day for 2 weeks. The egg yolk was diluted threefold (wt/wt) in PBS containing 5.25% polyethylene glycol 6000 (PEG-6000) and 0.05% NaN_3. The mixture was centrifuged for 15 min at 2,250 × *g*. The supernatant was filtered through glass wool, and PEG-6000 was added until 12% (wt/vol) was reached. After centrifugation for 15 min at 2,250 × *g*, the pellet was dissolved in water and centrifuged as before to remove debris. PEG-6000 was added to the solution until 12% (wt/vol) was reached, and centrifugation for 20 min at 2,250 × *g* was performed. The pellet containing polyclonal antibodies against fusion protein subunit F_1 (α-F_1) or F_2 (α-F_2) was dissolved in 2.5 ml of 0.9% NaCl per egg and stored at −20°C.

Purification of SeMNPV BV and ODV. Hemolymph-derived BVs were purified from SeMNPV-infected *S. exigua* fourth-instar larvae as described previously (22) with some modifications. Briefly, 3 days postinfection (p.i.) hemolymph was collected and clarified at 2,000 × *g* for 10 min at 4°C. The supernatant was passed through a 0.45-μm-pore-size filter. BVs in the filtrate were pelleted through a 25% (wt/wt) sucrose cushion made up in 0.1× TE (10 mM Tris-HCl [pH 7.5], 1.0 mM EDTA) by centrifugation at 100,000 × *g* for 90 min at 4°C and resuspended in 0.1× TE. BVs from cell culture supernatants were purified in a similar fashion. Se301 cells were infected with 0.1 50% tissue culture infective dose ($TCID_{50}$) of SeMNPV/cell. Seventy-two hours p.i. cell culture supernatants were clarified at 2,000 × *g* for 10 min at 4°C and passed through a 0.45-μm-pore-size filter. BVs in the filtrate were pelleted and resuspended as described above. ODVs were purified from polyhedra derived from SeMNPV-infected *S. exigua* fourth-instar larvae as described previously (22). The purity and integrity of BVs and ODVs were checked by electron microscopy.

Western blot analysis. For electrophoresis under reducing conditions purified SeMNPV ODVs and BVs were disrupted in Laemmli buffer (125 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8). For electrophoresis under nonreducing conditions virions were disrupted in 125 mM Tris-HCl–8% SDS–37.5 mM iodoacetamide–10% glycerol–0.001% bromophenol blue, pH 6.8. Samples were denatured for 10 min at 95°C. Proteins were electrophoresed in SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore) by semidry electrophoresis transfer (2). Membranes were blocked overnight at 4°C in PBS containing 2% milk powder, followed by incubation for 1 h at room temperature (RT) with antiserum at a dilution of 1:1,000 in PBS containing 0.2% milk power. After being washed three times for 15 min in PBS-T (PBS containing 0.1% Tween 20), the membranes were incubated for 1 h at RT with horseradish peroxidase conjugated with rabbit anti-chicken immunoglubulin (Sigma) diluted 1:50,000 in PBS containing 0.2% milk powder. After three washes for 15 min in PBS-T, the signal was detected by ECL technology as described by the manufacturer (Amersham).

Furin inhibition assay. Se301 cells (2.0×10^5) were infected with 10 $TCID_{50}$s of SeMNPV or AcMNPV/cell. At 2 h p.i. cells were washed twice with medium and incubated with medium containing a 50 μM concentration of furin cleavage inhibitor decanoylated arginyl-valyl-lysyl-arginyl chloromethylketone (dec-RVKR-cmk) (Bachem). The AcMNPV-infected cells were incubated without dec-RVKR-cmk. Cell culture supernatants were collected at 48 h p.i, and infectious BV was quantified by a $TCID_{50}$ assay (35) on Se301 cells. The SeMNPV-infected cells were washed three times with medium without FBS and assayed for the presence of active fusion proteins in the plasma membrane by a syncytium formation assay as described below.

Syncytium formation assay. Syncytium formation assays were performed by transfection of 5.0×10^5 Sf21 cells with 5 μg of DNA of p166AcV5-Se8 or p166AcV5-Se8(K149) using CellFECTIN (Gibco-BRL). Empty vector p166BRNX-AcV5 (22) was used as a negative control for the syncytium formation assay. Forty-eight hours after transfection, cells were washed three times with medium without FBS and treated for 2 min in acidic medium without FBS, pH 5.0. The acidic medium was replaced with medium with FBS, pH 6.1. Syncytium formation was recorded by light microscopy 4 h after treatment with the acidic medium. Syncytium formation was positive when at least four nuclei were present in each syncytial mass.

Fluorescence microscopy. To investigate the cellular location of the mutant SeMNPV F protein, 1.0×10^5 Sf21 cells were grown on glass coverslips and transfected with 5 μg of DNA of p166AcV5-Se8GFP or p166AcV5-Se8(K149) GFP. As a control for green fluorescent protein (GFP), expression plasmid p166AcV5-GFP (22) was used. At 48 h posttransfection the localization of GFP in the cells was examined with a Zeiss LSM510 (confocal) laser scanning microscope for fluorescence using an excitation wavelength of 488 nm and an emission bandpass filter of 505 to 530 nm.

Computer-assisted analysis. The SeMNPV F protein was analyzed for several protein motifs using different bioinformation programs. N-terminal signal sequences were found with program PSORT II (National Institute for Basic Biology [http://www.nibb.ac.jp]). Possible coiled-coil regions in the protein were predicted using the program PairCoil (version 1.0; MIT Laboratory for Computer Science [http://www.lcs.mit.edu]). Comparisons between the SeMNPV F protein and other viral fusion proteins were done with program FASTA (version 3; EMBL-European Bioinformatics Institute [http://www.ebi.ac.uk]). Sequence alignments were performed with program ClustalW (EMBL-European Bioinformatics Institute [http://www.ebi.ac.uk]).

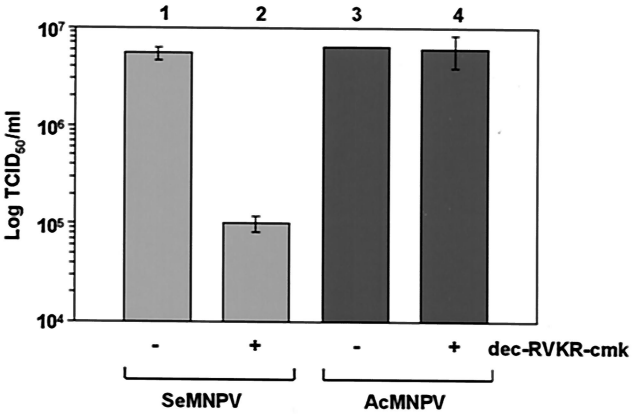

FIG. 1. Effect of furin inhibitor dec-RVKR-cmk on Se*M*NPV and Ac*M*NPV BV infectivity. Se301 cells were infected with Se*M*NPV (lanes 1 and 2) or Ac*M*NPV (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of furin inhibitor dec-RVKR-cmk. At 48 h p.i. the titer of progeny infectious BV in the cell culture supernatants was determined by $TCID_{50}$ assays. The data presented are means and standard deviations of three independent experiments.

RESULTS

Inhibition of furin-mediated cleavage. ORF8 (*Se8*) of Se*M*NPV was recently shown to encode the viral fusion (F) protein. A 60-kDa C-terminal subunit (F_1) of the primary *Se8* translation product was present in the envelopes of BVs, which were purified from hemolymph of infected *S. exigua* larvae (22). Amino acid sequence RSKR immediately upstream of the N terminus of 60-kDa subunit F_1 is consistent with a consensus cleavage site of furin-like endoproteases (22), and this suggests that F_1 is a cleavage product of a precursor, F_0, of the envelope fusion protein. To demonstrate that F_0 is subjected to proteolytic cleavage by furin and that cleavage is important for infectivity, furin inhibitor dec-RVKR-cmk was used. This inhibitor penetrates into cells and specifically binds to the catalytic site of furin (10, 15, 40, 47) and thus may inhibit the production of infectious Se*M*NPV BV.

Supernatants of Se*M*NPV-infected Se301 cells (48 h p.i.), grown in the presence or absence of dec-RVKR-cmk (50 μM), were assayed for the amount of infectious progeny BV by determining $TCID_{50}$s. In the presence of the furin inhibitor the virus titer was reduced dramatically to 2% of the titer for the control (absence of the inhibitor) (Fig. 1, lanes 1 and 2). This drop could be caused by either distortion in the BV assembly pathway, giving no new progeny BVs, or by progeny BVs that are not infectious anymore due to the presence of uncleaved fusion proteins (F_0) in their envelopes. In a similar experiment with Ac*M*NPV the furin inhibitor had no effect on the virus titer (Fig. 1, lanes 3 and 4), indicating that the furin inhibitor did not affect the formation of BV per se.

To exclude the possibility that the drop in Se*M*NPV virus titer was due to an impairment of the Se*M*NPV BV assembly pathway, progeny BVs were isolated from cell cultures incubated in the presence or absence of dec-RVKR-cmk. Western analysis with polyclonal antibodies against F_1 (α-F_1) revealed the presence of a 60-kDa protein (F_1) in both BV preparations (Fig. 2, lanes 1 and 2). BV obtained from cell cultures incubated in the presence of the furin inhibitor possessed an additional 80-kDa protein (F_0) (Fig. 2, lane 2). These experiments showed that blocking the furin-mediated cleavage of F_0 did not impair the assembly of BVs.

Se*M*NPV F protein with mutated cleavage site is unable to trigger fusion. Previously, it was demonstrated that acidification of the medium of Se*M*NPV-infected Se301 cells resulted in syncytium formation (22). To determine whether inhibition of furin-mediated cleavage would also inhibit activation of membrane fusion activity by the F protein, Se301 cells were infected with Se*M*NPV in the presence of dec-RVKR-cmk. After a pH drop to 5.0, cells were examined for their ability to form syncytia (48 h p.i.). Control cells infected in the absence of the furin inhibitor showed cell-to-cell fusion (Fig. 3A), whereas in the presence of the furin inhibitor cell-to-cell fusion was not observed (Fig. 3B). This suggests that uncleaved precursor F_0 is unable to trigger cell-to cell fusion.

To obtain direct evidence that the F_0 protein of Se*M*NPV has no fusogenic activity, consensus furin cleavage site RSKR of the F protein was mutated by changing the arginine (R) at amino acid residue 149 (upstream of the cleavage site) into a lysine (K). Whether the mutant F_{K149} protein could be transported to the plasma membrane was examined first. Sf21 cells instead of Se301 cells (Se301 cells are difficult to transfect, and cell-to-cell fusion is difficult to monitor due to the multimorphic cell phenotypes found in this cell line) were transfected with plasmids containing either the wild-type F (p166AcV5-Se8GFP) or the mutant F_{K149} (p166AcV5-Se8(K149)GFP) open reading frame, which was fused C-terminally to GFP under the control of an optimized OpMNPV *gp64* promoter to facilitate expression in insect cells (3). Both wild-type F and mutant F_{K149} proteins were predominantly localized in the plasma membrane 48 h after the transfection (Fig. 4A and B). The GFP protein alone (p166AcV5-GFP) showed a homogeneous fluorescence signal in the cytoplasm and nuclei of Sf21

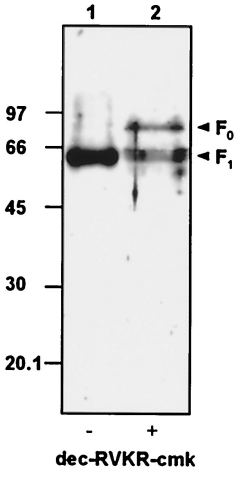

FIG. 2. Western blot analysis of proteins obtained from Se*M*NPV BV, produced in the absence (−; lane 1) or presence (+; lane 2) of furin inhibitor dec-RVKR-cmk. BVs were incubated for 10 min at 95°C in Laemmli buffer and subjected to SDS-PAGE (12% polyacrylamide). Proteins were transferred onto an Immobilon-P membrane (Millipore), incubated with α-F_1, and detected with a chemiluminescent substrate. Size standards in kilodaltons are on the left. F_0, precursor Se*M*NPV F protein; F_1, 60-kDa subunit of Se*M*NPV F protein.

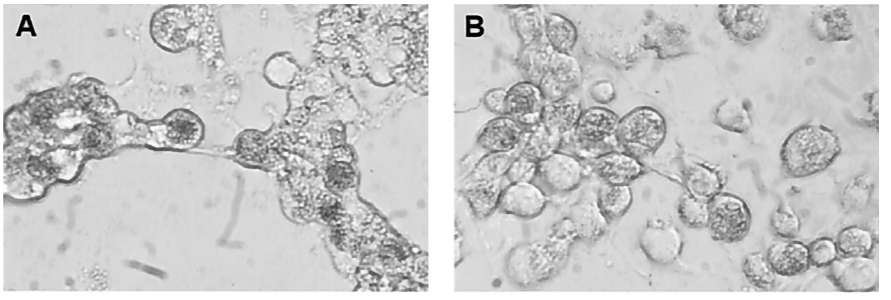

FIG. 3. Se*M*NPV-mediated fusion of Se301 cells. Cells were infected with Se*M*NPV in the absence (A) or presence (B) of furin inhibitor dec-RVKR-cmk. Forty-eight hours p.i. cells were incubated for 2 min with medium, pH 5.0. Syncytium formation was scored 4 h after dropping the pH by phase-contrast microscopy.

cells (Fig. 4C), suggesting that the mutation does not affect the transport of the F protein to the plasma membrane.

To examine whether the F_{K149} protein is able to trigger cell-to-cell fusion upon lowering the pH of the medium, Sf21 cells were transfected with similar constructs without the C-terminal GFP fusion. Cells transfected with the wild-type F construct (p166AcV5-Se8) clearly showed pH-dependent cell-to-cell fusion (Fig. 4D). The F_{K149} protein [p166AcV5-Se8(K149)] did not trigger cell-to-cell fusion (Fig. 4E), similar to what was found for the cells transfected with the empty vector (p166BRNX-AcV5) (Fig. 4F). So, it is concluded that the Se*M*NPV F protein requires cleavage by furin to become active as a fusion protein.

Se*M*NPV BV contains both F protein subunits. Cleavage of the F_0 precursor yields, besides the 60-kDa C-terminal cleavage product (F_1), a product of approximately 15 kDa (the N-terminal subunit minus the signal peptide; F_2). Several proteins in the 15- to 17-kDa range from BVs purified from hemolymph of Se*M*NPV-infected *S. exigua* larvae have been reported (22).

To determine whether Se*M*NPV BV contains both mature F subunits (F_1 and F_2) and whether cleavage of the Se*M*NPV F_0 protein occurs in tissue culture as well as in insects, BVs were purified from cell culture supernatants of Se*M*NPV-infected Se301 cells and from hemolymph obtained from Se*M*NPV-infected *S. exigua* larvae. Western analysis with polyclonal antibodies against F_1 (α-F_1) revealed the presence of a 60-kDa protein in BVs obtained from hemolymph as well as from cell culture (Fig. 5, lanes 1 and 2). Polyclonal antibodies specific against F_2 (α-F_2) detected a 21-kDa protein in both BV preparations (Fig. 5, lanes 4 and 5). Neither antibodies against F_1 nor antibodies against F_2 reacted with ODV-derived proteins (Fig. 5, lanes 3 and 6), demonstrating that both F protein subunits are BV specific.

Processing and oligomerization of the Se*M*NPV F protein. The observation that both subunits, F_1 and F_2, were present in Se*M*NPV BV raised the question of whether these subunits interacted with each other. There is a possibility that these subunits remain noncovalently associated, as found for the fusion protein of the *Retroviridae* (Env) (49), or are covalently associated by disulfide linkage, as found for fusion proteins in members of the *Orthomyxoviridae* (HA), *Herpesviridae* (gB), and *Paramyxoviridae* (F) (28, 29, 42).

To determine the type of interaction between F_1 and F_2, Se*M*NPV BV proteins were treated with iodoacetamide, which alkylates free sulfhydryl groups and thereby prevents artificial disulfide exchange during further treatment or electrophoresis (14). The proteins were denatured in the absence of reducing

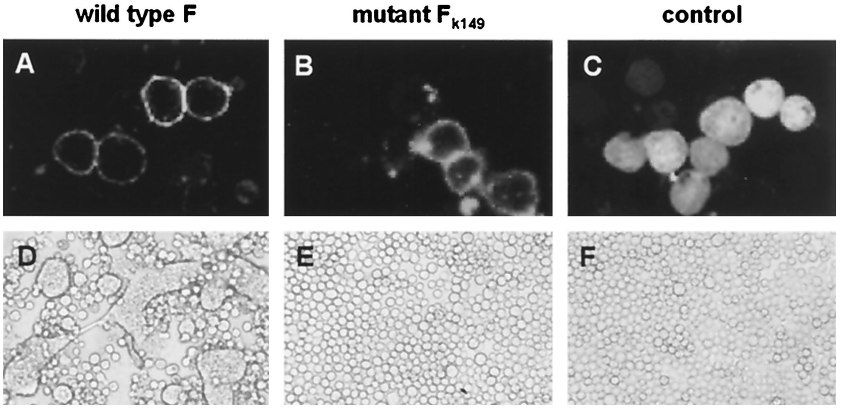

FIG. 4. Localization studies with Se*M*NPV wild-type F and mutant F_{K149}-GFP fusion proteins (A to C) and their ability to mediate cell-to-cell fusion in Sf21 cells (D to F). For the localization studies, Sf21 cells were transfected with plasmid p166AcV5-Se8GFP (A), p166AcV5-Se8(K149)GFP (B), or control plasmid p166AcV5-GFP (C). Fluorescence was examined 48 h after transfection by confocal laser scanning microscopy. For pH-dependent membrane fusion, Sf21 cells were transfected with plasmid p166AcV5-Se8 (D), p166AcV5-Se8(K149) (E), or control p166BRNX-AcV5 (F). Forty-eight hours after transfection, cells were treated for 2 min with medium, pH 5.0. Four hours after the pH drop syncytium formation was scored by phase-contrast microscopy.

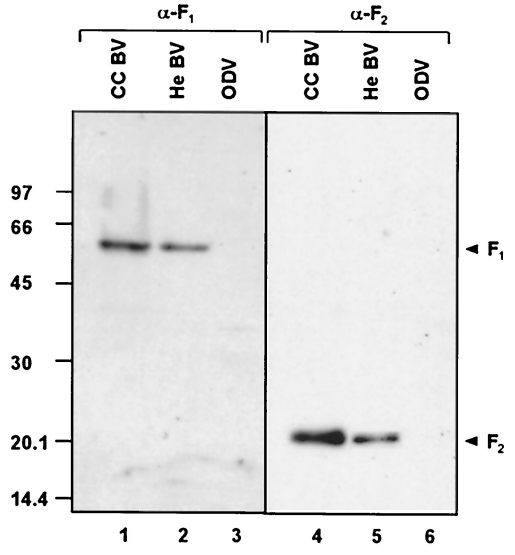

FIG. 5. Western blot analysis of reduced proteins of Se*M*NPV virus preparations. Cell culture-derived (CC) BVs, hemolymph-derived (He) BVs, and polyhedra-derived ODVs were incubated for 10 min at 95°C in Laemmli buffer and subjected to SDS-PAGE (12% polyacrylamide). Proteins were transferred onto an Immobilon-P membrane (Millipore), incubated with either α-F_1 (left) or α-F_2 (right) antibodies, and detected with a chemiluminescent substrate. Size standards in kilodaltons are on the left. F_1, 60-kDa subunit of Se*M*NPV F protein; F_2, 21-kDa subunit of Se*M*NPV F protein.

agents and electrophoresed in a 12% polyacrylamide gel. Western analysis with α-F_1 and α-F_2 detected in both cases a single protein with a molecular mass of 80 kDa ($F_{1,2}$) (Fig. 6A, lanes 1 and 2). The absence of free F_1 and F_2 indicates that these subunits remain covalently linked after cleavage.

To investigate whether F proteins from Se*M*NPV also form disulfide-linked oligomers, a nonreducing SDS-polyacrylamide gel electrophoresis (PAGE) was performed using a 6% polyacrylamide gel. Antibodies α-F_1 and α-F_2 detected a major monomeric form of 80 kDa (Fig. 6B, lanes 1 and 2). Antibody α-F_2 detected also a possible dimeric form as a minor band of 155 kDa (Fig. 6B, lane 2); higher multimeric forms were not detected.

DISCUSSION

Posttranslational cleavage of fusion proteins of enveloped viruses seems to represent a general mechanism used to regulate viral membrane fusion activity. All members of the *Orthomyxoviridae*, *Paramyxoviridae*, *Togoviridae*, *Coronaviridae*, *Retroviridae*, and *Herpesviridae* express their fusion proteins as a precursors, which have to be cleaved by host proteases to render infectious virus (25). The cleavage usually takes place in front of a hydrophobic sequence, the fusion peptide. Exposure to low pH or binding to a receptor triggers a conformational change in which the fusion peptide is believed to translocate and insert itself into the target membrane (25).

In this report it is demonstrated that Se*M*NPV, a representative of a group II NPV within the *Baculoviridae*, also expresses its fusion protein as precursor F_0. This precursor is posttranslationally cleaved by insect cell furin into two disulfide-linked subunits, F_1 and F_2, to become active in tissue culture and in insects. The major envelope protein of HaSNPV BV, the product of *Ha133*, has also been identified and characterized (7; M. Westenberg and H. Z. Wang, personal communication). This protein turned out to be also a C-terminal cleavage product of the Se*M*NPV F protein homolog. In contrast to the Se*M*NPV and HaSNPV F proteins, the Ld*M*NPV BV F protein was reported not to be cleaved (37), despite the fact that it possesses a similar furin cleavage site at a position analogous to those in Se*M*NPV and HaSNPV. An 83-kDa fusion protein was detected in Ld*M*NPV BV purified from cell culture supernatants. Tunicamycin treatment of the cells resulted in an Ld*M*NPV fusion protein with a molecular mass of 72 kDa. The latter value is consistent with a predicted molecular mass of 75 kDa for the product of the whole open reading frame minus the signal sequence (residues 1 to 16). Therefore it is most likely that Ld625Y cells are unable to properly cleave the fusion protein from Ld*M*NPV BV. However the titers of progeny BVs from Ld625Y cells are even higher (41) than that of Se*M*NPV in Se301 cells. So it is very possible that the activation of the fusion protein of Ld*M*NPV during viral uptake by Ld625Y cells is similar to the activation of the influenza virus A/WSN/33 (H1N1) fusion protein in vertebrate cells. This virus possesses only the uncleaved HA, which is cleaved during uptake presumably by an endosomal protease (6).

The cleavage of the F_0 protein by furin was demonstrated by the use of a furin inhibitor at a concentration of 50 μM, which seemed to be the most effective concentration (data not shown). The titers of the progeny BVs produced in the presence of the inhibitor were about 2% of the control titers (Fig. 1). The 2% residual infectious virus is in agreement with similar findings with other viral systems (15, 36, 40) and is probably

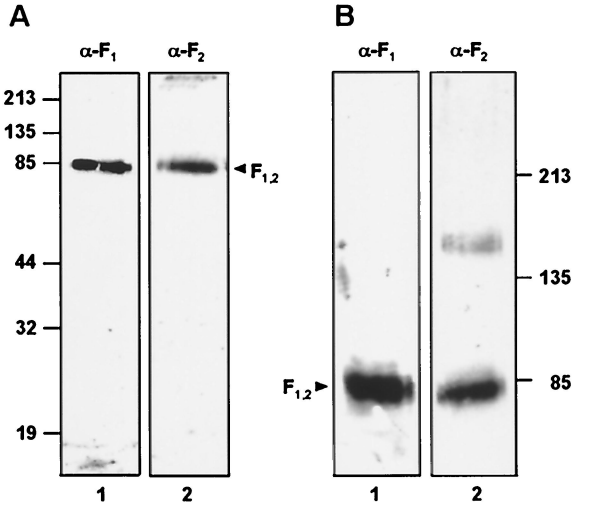

FIG. 6. Western blot analysis of nonreduced proteins of Se*M*NPV BV. Cell culture-derived BVs were incubated for 10 min at 95°C in the presence of 37.5 mM iodoacetamide and in the absence of 2-mercaptoethanol and electrophoresed in a 12% (A) or 6% (B) SDS-polyacrylamide gel. Proteins were transferred onto an Immobilon-P membrane (Millipore), incubated with either α-F_1 or α-F_2, and detected with a chemiluminescent substrate. Size standards (kilodaltons) are indicated. $F_{1,2}$, 60- and 21-kDa disulfide-linked subunits of Se*M*NPV F protein.

the result of the instability of the furin inhibitor in aqueous solutions. However, we cannot exclude the possibility that a small proportion of the F_0 protein is cleaved by another unknown protease. Infected cells were also unable to form syncytia in the presence of the inhibitor (Fig. 3), which is consistent with earlier reports that cell-to-cell fusion after viral infection could be completely blocked by peptidyl-chloroalkylketones (15, 47). BV assembly was not impaired in the presence of the furin inhibitor (Fig. 2). Nevertheless the amount of F_1 protein relative to the amount of F_0 protein is not proportional to the drop in virus titer but is in agreement with similar findings for influenza virus, where the cleavage inhibition of HA_0 was also not proportional to the drop in hemagglutination titer (47). For influenza viruses and group I NPVs it has been shown that at least three HA trimers (5, 8) and approximately six GP64 trimers (38), respectively, cooperate in the formation of the fusion pore. So, the presence of even small amounts of F_0 in BVs can interfere with the mature fusion proteins in the fusion process.

Cleavage products F_1 and F_2 are covalently associated, similar to the fusion proteins of members of the *Orthomomyxoviridae*, *Herpesviridae*, and *Paramyxoviridae* (28, 29, 42). The F_2 subunit of Se*M*NPV contains only a single cysteine residue (C94), which makes this cysteine the only candidate to form the disulfide bridge with F_1. This cysteine residue is conserved among the F protein homologs of HaSNPV (6) and Ld*M*NPV (26). In contrast, the F_1 subunit contains 15 cysteine residues. Ten of these are situated upstream of the predicted transmembrane region, and 9 of the 10 are conserved among the group II NPVs (Se*M*NPV, Ld*M*NPV, HaSNPV). The molecular mass of the F_1 subunit (60 kDa) is in close agreement with the molecular mass of 59 kDa predicted from the DNA sequence. The F_2 subunit was detected by SDS-PAGE as a 21-kDa peptide (Fig. 5). Upon maturation the F_2 subunit might go through some posttranslational modification processes, including N-glycosylation and O-glycosylation (22), which may explain the difference between the theoretical (15 kDa) and actual (21 kDa) sizes of this subunit.

Viral fusion proteins generally form higher-order oligomers (18). In most cases these oligomers are formed by noncovalent interactions. However, envelope fusion protein GP64 of group I NPVs forms a trimer of disulfide-linked monomers (34). The F protein of Se*M*NPV was predominantly present as a monomer (Fig. 6B). A minor band approximately twice the size of the monomer was also detected. Although this band could represent a dimeric form of the protein, most likely this is due to incomplete denaturation, since this band was only detected by α-F_2. Furthermore, treatment of GP64 of Ac*M*NPV under the same conditions resulted in the simultaneous occurrence of trimers together with minor dimers and monomers (data not shown). Therefore, it is concluded that disulfide links are most likely not involved in oligomerization of the F protein. The potential oligomeric structure of the Se*M*NPV F protein remains to be characterized.

The Se*M*NPV F protein is very similar in structure to the paramyxovirus fusion protein. Both consist of a small N-terminal subunit linked by a disulfide bridge to the large C-terminal membrane-anchored subunit. The Se*M*NPV F protein has the highest homology with paramyxovirus human respiratory syncytial virus fusion protein [P03420], with 12% amino acid identity and 36% similarity. Although the overall homology is not high (12%), predicted domains such as α-helices and β-sheets, as well as the furin cleavage sites, the heptad repeats (HRs), and the transmembrane regions, are structurally conserved. Furthermore, the fusion protein of the paramyxoviruses contains a fusion peptide, which is located at the N terminus of the large membrane-anchored subunit. We predict that the Se*M*NPV F protein contains a putative fusion peptide at an analogous position. Amino acids 150 to 166 GLFNFMGHVD KYLFGIM contain nine hydrophobic residues (underlined) with hydrophobicity indices (H.I.) higher than 0.62 according to the normalized consensus scale of Eisenberg (9). These hydrophobic residues can be modeled as a hydrophobic face in an α-helix. The average H.I. of the hydrophobic face is 1.1, and the total amino acid stretch has a H.I. of 0.5, which is consistent with those for other viral fusion proteins (55).

The paramyxovirus fusion proteins contain HRs (HR1 and HR2) immediately adjacent to the fusion peptide and the transmembrane domain (27). Previous studies with other viral fusion proteins suggested that these HRs form trimeric hairpin-like structures, with the HR2 regions packing in an antiparallel manner against the inner coiled-coil formed by the HR1 regions. The likely role of the hairpin structure is to facilitate apposition of the viral and cellular membranes by bringing the fusion peptide, which inserts into the cellular membrane, close to the transmembrane segment, which is anchored in the viral membrane (44). The Se*M*NPV F protein also contains two putative HRs. The first HR is adjacent to the putative fusion peptide and is formed by amino acid residues 174 to 202 (HR1: LhmLantTnsLnsqVkqLndeLivLadyV, where the amino acids in uppercase are the nonpolar, hydrophobic residues). The second putative HR is adjacent to the transmembrane domain and is formed by amino acid residues 521 to 549 (HR2: VddMkinNdlEktnLheLtsrLydLrrrI). Further studies are in progress to confirm whether those motifs indeed are required for the fusion function.

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