Deltabaculoviruses encode a functional type I budded virus envelope fusion protein

Manli Wang,1,2 Shu Shen,2 Hualin Wang,2 Zhihong Hu,2 James Becnel3 and Just M. Vlak1,*

Abstract

Envelope fusion proteins (F proteins) are major constituents of budded viruses (BVs) of alpha- and betabaculoviruses (Baculoviridae) and are essential for the systemic infection of insect larvae and insect cell culture. An F homologue gene is absent in gammabaculoviruses. Here we characterized the putative F-homologue (Cuni-F), encoded by (ORF) 104 of Culex nigripalpus nucleopolyhedrovirus (CuniNPV), the only deltabaculovirus member. When expressed alone, this protein seems to locate on the cell surface and is able to induce cell–cell fusion. When expressed by an alphabaculovirus (Autographa california nucleopolyhedrovirus), it was found to be incorporated into BVs. Western blot analyses detected the uncleaved Cuni-F and the furin-cleaved F forms. Treatment of infected cells with tunicamycin showed that Cuni-F contains N-glycans. Mutagenesis analysis identified the canonical furin cleavage site as being responsible for the cleavage of Cuni-F in insect cells. The collective evidence suggests that CuniNPV encodes a functional F protein.

Baculoviruses (family Baculoviridae) form a group of enveloped, large double-stranded DNA viruses that infect diverse arthropod hosts [1]. The Baculoviridae family is divided into four genera: Alphabaculovirus [lepidopteran nucleopolyhedroviruses (NPVs)], Betabaculovirus [lepidopteran granuloviruses], Gammabaculovirus (hymenopteran NPVs) and Deltabaculovirus (dipteran NPVs) [2]. Phylogenetic analysis suggested that gamma- and deltabaculoviruses are very divergent from lepidopteran baculoviruses and are from an ancient lineage [3]. In a typical replication cycle, lepidopteran baculoviruses produce two morphologically distinct progeny phenotypes, the budded virus (BV) and the occlusion-derived virus (ODV). ODVs infect midgut epithelial cells, while BVs are responsible for systemic infection within the larval body [4, 5].

Envelope fusion proteins (EFPs) of BVs play a pivotal role in mediating baculovirus entry and egress. To date, two distinct types of EFPs, GP64 or F protein, have been found in alpha- and betabaculovirus BVs. GP64 occurs only in Group I alphabaculoviruses, whereas Group II alphabaculoviruses and betabaculoviruses utilize an F protein as their EFP. GP64 and F proteins induce low pH-dependent fusion between baculovirus BVs and host cells, and their mode of action has been well studied [6–12]. F proteins were suggested to be the ancestral EFPs for baculoviruses, while GP64 appeared to be captured by Group I alphabaculoviruses much more recently [8, 13].

However, no homologue of GP64 or F protein has been found in gammabaculoviruses. These viruses occur in hymenopteran insects, where the infection is limited to the midgut [2]. Only the ODV but not the BV phenotype was identified, and EFPs may not be required for short-distance cell-to-cell transport. The only known deltabaculovirus (Culex nigripalpus nucleopolyhedrovirus, CuniNPV) has been completely sequenced, but functional analysis of any ORF is lacking, including a putative EFP gene. Although infection and OBs were observed only in the midgut epithelium cells but not in other tissues within mosquito larvae, it was speculated that the BV may disseminate the infection within the midgut [14, 15]. Interestingly, the presence of BVs is supported by the presence of an F gene homologue (f) in the genome of CuniNPV (Cuni104) [14], but its functionality as a fusion protein has not been experimentally verified.

The ORF of Cuni104 encodes a 567-aa F protein homologue (Cuni-F). Sequence alignment showed that Cuni-F shares a lower similarity (10–16% aa identity) with F proteins from alpha- and betabaculoviruses than homologues to each other (20–40% aa identity) (data not shown). However, on the basis of predicted domain organization, Cuni-F exhibits...
the typical characteristics of a functional baculoviral F protein: an N-terminal signal peptide (1–19 aa), a putative furin cleavage site (126–129 aa) and fusion peptide (130–155 aa), two heptad repeats HR-N (208–236 aa) and HR-C (505–526 aa), and a C-terminal transmembrane (TM) domain (545–567 aa). By convention, the small N-terminal region (1–129 aa) and the large C-terminal region (130–567 aa) of Cuni-F were defined as F2 (with a predicted size of ~15kDa) and F1 (with a predicted size of ~50 kDa), respectively (Fig. 1a). Three potential glycosylation sites (N-X-T), one on the F2 subunit (N54) and two on the F2 subunit (N179 and N435), were predicted by the NetNGlyc 1.0 Server (Fig. 1a, red diamonds).

Since F proteins target the plasma membrane independently of other viral/host proteins for mediating efficient BV budding [16], the intracellular localization of Cuni-F was analysed first of all. The Cuni-f ORF was PCR-amplified using CuniNPV genomic DNA (GenBank accession number: NC_003084.1) as a template and subsequently inserted into the insect cell-specific transient expression vector pIZ/V5-His (Invitrogen) [18]. The resulting expression vector was named p166AcV5-Cuni-f-egfp. Then, p166AcV5-Cuni-f-egfp and the control plasmid p166AcV5-egfp were transfected into insect Sf9 cells, individually, and the fluorescence was observed by confocal microscopy (Olympus IX51 at 60 x objective lens) at 48 h post transfection (h p.t.). As shown in Fig. 1(b), in p166AcV5-egfp transfected cells, EGFP was evenly distributed throughout the cells. In contrast, Cuni-F-EGFP fusion protein was detected at the cell periphery, suggesting that the Cuni-F protein was present on the cell surface.

Membrane fusion is a key step for delivery of viral genomes into host cells. Baculovirus F proteins are low pH-triggered membrane fusion proteins [16–18]. To determine whether Cuni-F is able to mediate low pH-dependent cell-to-cell fusion, Cuni-f was cloned into a transient expression vector pIZ/V5-egfp, in which a phev70-egfp-SV40 cassette was inserted into pIZ/V5-His (Invitrogen) [18]. The resulting expression vector was named pIZ/V5-Cuni-f-egfp (expressing wild-type (WT) Cuni-f under the control of the OpIE2 promoter independently, without egfp fusion). Plasmid pIZ/V5-Cuni-f-egfp, along with the negative control plasmid pIZ/V5-egfp and two previously constructed positive control plasmids with a similar ORF arrangement, pIZ/V5-gp64-egfp (expressing WT AcMNPV gp64) and pIZ/V5-Ha-f-egfp (expressing WT Helicoverpa armigera nucleopolyhedrovirus f) [18], both with an independently expressed GFP construct, were transfected into Sf9 cells, respectively. At 48 h p.t., successful transfections were visualized by fluorescence microscopy through GFP expression (data not shown). Then, the cells were treated with low pH (pH 5.0) or normal pH (pH 6.0) for 5 min and then further cultured with Grace’s medium [17]. At 24 h post-pH shift, multinuclear cells (indicated by arrowheads) could be detected in cells expressing GP64, Ha-F as well as Cuni-F, but not in the cells transfected with empty vector pIZ/V5-egfp (Fig. 1c). Consistent with a previous report, both the number and size of multinuclear cells resulting from GP64 were significantly larger than those from F proteins, again demonstrating that GP64 induces higher fusogenicity than F proteins [9, 18]. The results thus showed that Cuni-F alone is sufficient to mediate low pH-dependent membrane fusion in Sf9 cells.

Fig. 1. Schematic diagram, subcellular localization and fusogenicity of Cuni-F. (a) Schematic representation of the Cuni-F ORF. SP, signal peptide; FP, fusion peptide; HR, heptad repeat; TM, transmembrane domain. The red arrow indicates the putative furin cleavage site. The red diamonds indicate the predicted N-glycosylation sites. (b) Subcellular localization of Cuni-F. Sf9 cells were transiently transfected with p166AcV5-Cuni-f-egfp or control vector p166AcV5-egfp. At 48 h p.t., intracellular localization of Cuni-F was observed with confocal microscopy. Bars represent 5 µm. (c) Low pH-dependent membrane fusion assay. Sf9 cells were transfected with pIZ/V5-Cuni-f-egfp, pIZ/V5-Ha-f-egfp (+ve control), pIZ/V5-gp64-egfp (+ve control) or control plasmid pIZ/V5-egfp (–ve control). At 48 h p.t., cells were treated for 5 min with Grace’s insect medium at pH 6.0 (upper panel) or 5.0 (lower panel) for 5 min. Syncytium formation was observed by fluorescence microscopy 24 h after pH shift. Multinuclear cells are indicated by arrowheads.
F proteins share the common features of Class I viral EFP. They are expressed as a precursor (F₀) and later further cleaved by a cellular furin-like protease during secretion to generate the N-terminal F₂ subunit and the C-terminal F₁ subunit. This cleavage is required for activation of baculovirus F fusogenicity [16], and this seems to be the case for Cuni-F as it shows fusogenicity. To detect the expression and cleavage of Cuni-F, a recombinant AcMNPV bacmid co-expressing Cuni-f under the control of an Op166 promoter and a hsp70-egfp-SV40 cassette (expressing an egfp marker gene under the control of the Drosophila hsp70 promoter and SV40 terminator) [18, 19] was constructed. Western blot analyses using anti-Cuni-F₁ antibody detected an uncleaved F₀ (~72 kDa) and the F₁ subunit (~55 kDa) in the BVs of the Ac-cuni-f-egfp recombinant but not in the BV sample of the control virus Ac-egfp (Fig. 2a). Since the molecular mass of Cuni-F (~72 kDa) is larger than that predicted from its coding sequence (~65 kDa), we deduced that Cuni-F might contain post-translational modifications, for example, N-linked glycosylation. The size difference is compatible with the supposition that three potential glycosylation sites may actually be used.

N-glycosylation is important for viral EFP folding, intracellular trafficking and function. Both GP64 and F proteins are N-glycans-rich, which is crucial for their structure and function [20–23]. For Cuni-F, three N-glycosylation motifs (N-X-S/T) were predicted. When the cells were infected with Ac-cuni-f-egfp in the presence of protein N-glycosylation inhibitor tunicamycin (TUN), a single band of approximately 65 kDa corresponding to the predicted molecular mass of Cuni-F₁ (Cuni-F₁⁰₀⁰) appeared (Fig. 2b, right lane of upper panel). No cleaved band was detected in the TUN-treated cells, as found in the untreated cells (Fig. 2b, middle lane of upper panel). It is probable that the loss of N-glycan inhibited correct folding or intracellular trafficking of Cuni-F for efficient furin cleavage. As a control, the GP64 migration was also faster in the TUN-treated cells than in untreated cells (Fig. 2b, lower panel). To further identify the furin cleavage site, a truncated Cuni-F missing the signal peptide (SP) and the transmembrane (TM) domain (Cuni-fATM, 20–544 aa) was PCR-amplified, and the putative furin cleavage motif RARK₁₁₂₉ was mutated to RARR₁₁₂₉ (Cuni-fATM³¹²₉) using overlap extension PCR [24]. Both Cuni-fATM and Cuni-fATM³¹²₉ were cloned into Drosophila expression vector pMT/BIP/V5-His A (Invitrogen), transfected into Drosophila S2 cells and induced with copper sulfate (500 μmol l⁻¹) for protein expression (Drosophila expression system manual, Invitrogen). As shown in Fig. 2(c), left panel, similar to Sf9 cells, both F₀ and F₁ bands were detected in Cuni-FATM

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**Fig. 2.** Detection of Cuni-F expression, N-glycosylation and cleavage. (a) Western blot analysis of Cuni-F in recombinant AcMNPV. BVs of Ac-Cuni-f-egfp or Ac-egfp were harvested and purified from the supernatants of infected Sf9 cells at 5 days p.i. (days post induction), and the proteins were separated by SDS-PAGE. The blots were probed with antibodies against Cuni-F (upper panel) or GP64 (lower panel). M, the protein molecular weight marker. (b) Inhibition of N-linked glycosylation of Cuni-F by tunicamycin (TUN). Sf9 cells were infected with Ac-Cuni-f-egfp or Ac-egfp (MOI=5) in the presence or absence of 1 μg ml⁻¹ TUN. At 48 h p.i. (h post induction), cells were harvested for Western blot analysis with antibodies against Cuni-F (upper panel) or GP64 as a control (lower panel). Cuni-F₀⁰⁰ or GP64₀⁰⁰ represent the deglycosylated form of Cuni-F or GP64. (c) Analysis of the putative furin cleavage site of Cuni-F. Drosophila mela nogaster S2 cells were transfected with pMT-Cuni-f or pMT-Cuni-f₁₁₂₉ (furin cleavage site mutant) and the protein expression was induced with copper sulfate (500 μmol l⁻¹). At 48 h p.i., the supernatants were harvested for Western blot analysis with antibody against Cuni-F.
expressing S2 cells, further confirming that Cuni-F underwent correct protein cleavage in different insect cells. In contrast, in Cuni-FΔTMR129K expressing S2 cells, only an F0 band could be detected (Fig. 2c, right panel). Taken together, these results confirmed the correct expression, N-glycosylation and furin cleavage of Cuni-F in insect cells.

F proteins and GP64 share no sequence similarity, but F protein from certain Group II alpha- and betabaculoviruses can substitute the functions of AcMNPV GP64 [25, 26]. To test whether Cuni-F is a functional analogue of GP64, a Cuni-f pseudotyped bacmid, AcΔgp64-Cuni-f, was generated (Fig. 3a). However, transfection-infection of the bacmid into Sf9 cells showed that AcΔgp64-Cuni-f failed to produce infectious progeny virions, in contrast with the situation of gp64-rescued bacmid AcΔgp64-gp64 (Fig. 3b). Further quantification PCR (qPCR) analysis on viral genomic DNA copies in transfected cell supernatants indicated that, similar to the gp64-null bacmid AcΔgp64, the DNA replication of AcΔgp64-Cuni-f was as normal (data not shown); however, the efficient production of pseudotyped BVs was blocked (Fig. 3c) [27]. As EFP is also involved in the budding process of baculovirus BVs, and to understand whether Cuni-F could replace GP64 for this case, the transport and budding of nucleocapsids (NCs) were further visualized by EM. In the AcΔgp64-gp64 transfected cells, the budding of NCs through the plasma membrane was readily captured at 60 h p.t., while in the AcΔgp64-cuni-f-transfected cells, assembled NCs were found in the cytoplasm; however,

![Fig. 3.](image-url)

Fig. 3. Construction and characterization of Cuni-f-pseudotyped gp64-null AcMNPV bacmid. (a) Schematic representative genomes of gp64-null AcMNPV bacmid AcΔgp64, gp64 rescued bacmid AcΔgp64-gp64 or Cuni-f pseudotyped bacmid AcΔgp64-Cuni-f. pOp166, OpMNPV fusion protein (ORF126) promoter; SV40, simian virus 40 terminator. (b) Transfection-infection assay. Sf9 cells were transfected with AcΔgp64-gp64 or AcΔgp64-Cuni-f. At 5 d.p.t., supernatants were used to infect healthy Sf9 cells. The cells were observed by fluorescence microscopy at 3 d.p.t. or p.i. (c) Quantification of viral genome copies in transfected cell supernatants. Sf9 cells were transfected with AcΔgp64-Cuni-f or AcΔgp64. At 0, 24, 48 and 72 h p.t., viral DNA in transfected cells was quantified by qPCR. Data are represented as relative frequencies in transfected cell supernatants. Sf9 cells were transfected with AcΔgp64-Cuni-f or AcΔgp64. At 0, 24, 48 and 72 h p.t., viral DNA in transfected cells was quantified by qPCR. Error bars represent standard deviation. (d) Electron microscopy of AcΔgp64-Cuni-f or AcΔgp64 transfected cells. Sf9 cells were transfected with AcΔgp64-Cuni-f or AcΔgp64-gp64, and trafficking of NCs within infected cells (indicated by white arrowheads) or BVs (indicated by black arrowheads) was observed by EM. C, cytoplasm; N, nucleus. Bars represent 1 µm.
budding of NCs was barely detected (Fig. 3d). Therefore, the results of qPCR and EM suggested that Cuni-f can not readily replace the function of gp64 in efficient AcMNPV BV production. The BV titre would have been too low to detect infectivity or these BVs would have been non-infectious. It is possible that the proper assembly of this dip-
teran baculovirus F protein on its own in an AcMNPV con-
text is less compatible with the lepidopteran background of S9 cells.

Taken together, our results show that the F protein of delta-
baculoviruses resembles other baculoviral F proteins in its
second structure, intracellular localization, protein cleavage
and fusogenicity. Although the development of CuniNPV is apparently restricted to the midgut epithelium cells of mosquito larvae, it contains a BV form that spreads the infection quickly within the midgut. Therefore, F pro-
tein (Cuni-F) appears to represent a functional F protein
that likely mediates BV dissemination of CuniNPV in the
midgut epithelium. F proteins of many lepidopteran baculo-
viruses were shown to be functional homologues to GP64,
but there are still some exceptions. For example, the

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