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Bombyx mori nucleopolyhedrovirus FP25K is essential for maintaining a steady-state level of *v-cath* expression throughout the infection

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ABSTRACT

It has been previously reported that the *fp25K* product of *Bombyx mori* nucleopolyhedrovirus (BmNPV) is required for post-mortem host degradation, but the mechanism by which it regulates host degradation is still unknown. This study shows that disruption of BmNPV *fp25K* attenuates the expression of viral cathepsin gene (*v-cath*) at a late stage of infection, and thus reduces the secretion of its product V-CATH. Western blot analysis showed that secretion of V-CATH was severely reduced in BmN cells and *B. mori* larvae infected with Bm25KD, a BmNPV mutant lacking functional *fp25K*, compared to that of wild-type BmNPV. Also, reduced accumulation of pro-V-CATH in Bm25KD-infected cells was observed from 4 days postinfection (dpi), during which V-CATH was first detected in the medium of BmNPV-infected cells. qRT-PCR experiments showed that the expression levels of *v-cath* mRNA in wild-type- and Bm25KD-infected BmN cells were comparable at 3 dpi, but showed a marked decrease in Bm25KD-infected BmN cells at 4 dpi. Collectively, these results suggest that BmNPV FP25K is essential for the proper transcriptional regulation of *v-cath* and efficient secretion of V-CATH, and a steady-state level of *v-cath* expression during the period of V-CATH secretion (after 4 dpi) is required for post-mortem host degradation.

1. Introduction

The *Baculoviridae* is a large family of pathogens that are infectious for arthropods, particularly insects of the Lepidoptera. Nucleopolyhedroviruses (NPVs), a genus of *Baculoviridae*, have a large circular, supercoiled, and double-stranded DNA genome packaged into rod-shaped virions. NPVs produce two types of virions during their infection cycle, to bring about efficient viral replication within infected insect larvae and to spread the virus from insect to insect. Occlusion-derived viruses (ODVs), which are occluded in occlusion bodies (OBs), transmit virus from insect to insect via oral infection, whereas budded viruses (BVs) spread infection to neighboring cells (Granados and Lawler, 1981; Keddie et al., 1989). The end of infection is marked by a dramatic degradation (or melting) of the host cadaver.

Serial passage of NPVs in established cultured cell lines often generates mutants, which are called few polyhedra (FP) mutants, that produce lesser numbers of OBs (polyhedra) in the nuclei of infected cells (Potter et al., 1976). FP mutants frequently acquire host genome fragments (often containing a transposon) and/or lose a portion of the viral genome (Harrison and Summers, 1995). Mapping analysis located a single region involved in the FP phenotype between 37.7 and 38.8 map units of the *Autographa californica* multiple NPV (AcMNPV) genome (Fraser et al., 1983). In this region, a gene, *fp25K*, encoding a 25 kDa protein has been identified and shown to be involved in the FP phenotype (Beames and Summers, 1989). To date, FP mutants have been reported in several NPVs, including AcMNPV (Hink and Vail, 1973), *Trichoplusia ni* MNPV (TnMNPV) (Potter et al., 1976), *Galleria mellonella* MNPV (GmMNPV) (Fraser and Hink, 1982), *Lymantria dispar* MNPV (LdMNPV) (Slavicek et al., 1992), *Bombyx mori* NPV (Katsuma et al., 1999) and *Helicoverpa armigera* NPV (HearNPV) (Chakraborty and Reid, 1999; Wu et al., 2005).

The common characteristics of the FP phenotype are a decrease in the number of OBs produced in each infected cell, an increase in the yield of BVs, and few or no ODVs within OBs in cells infected with FP mutants. Rescue experiments have confirmed that these phenotypes are related solely to functional disruption of *fp25K* (Harrison and Summers, 1995). Previous studies in AcMNPV showed that mutations in *fp25K* result in a decreased amount of polyhedrin (*polh*) mRNA and altered transport of polyhedrin protein into the nucleus (Jarvis et al., 1992; Harrison et al., 1996), decreased amounts of ODV-E66 and impaired transportation of ODV-E66 into the nucleus (Braunagel et al., 1999, 2004), and a marked increase in the synthesis of some structural viral proteins of BVs such as GP64, BV/ODV-E26, and VP39 (Braunagel et al., 1999). Although the effects resulting from mutations in *fp25K* have been reported, the function of this protein is still unknown.

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It has been previously reported that BmNPV FP25K is essential for post-mortem host degradation (Katsuma et al., 1999). Further analyses have shown that the activity of viral cathepsin, V-CATH, is severely reduced in the hemolymph of *B. mori* larvae infected with Bm25KD, an *fp25K*-mutated BmNPV, which probably results in inhibition of host degradation after death (Katsuma et al., 2004a). This study shows that deletion of *fp25K* from the BmNPV genome reduced the secretion of V-CATH in larval hemolymph or in the medium of BmN cells. Also, this reduction is revealed to result from a marked down-regulation of *v-cath* expression at the beginning of V-CATH secretion.

2. Materials and methods

2.1. Insect, cell lines, and viruses

B. mori larvae were reared as described previously (Katsuma et al., 2006). The BmN (BmN-4) cells were cultured at 27 °C in TC-100 medium supplemented with 10% fetal bovine serum. BmNPV T3 was used as a wild-type virus. Three deletion BmNPVs, Bm25KD, BmCysPD and BmChiAD have been reported previously (Katsuma et al., 1999, 2004a; Ohkawa et al., 1994). Virus titers of T3 and recombinant BmNPVs were determined by plaque assay on BmN cells (Katsuma et al., 2006).

2.2. Generation of a recombinant BmNPV expressing His-tagged FP25K

To generate a BmNPV expressing His-tagged FP25K, we constructed a plasmid containing the flanking region of the BmNPV *fp25K*. DNA fragments containing both the upstream (nt 44,299–45,376; GenBank Acc. no. L33180) and downstream (nt 42,640–43,653) regions of the BmNPV *fp25K* were amplified by polymerase chain reaction (PCR) using primers listed in Supplementary Table 1, and cloned into pcDNA3.1(−) (Invitrogen), and designated as fp25Knull/pcDNA. The nucleotide sequence was confirmed using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and the ABI Prism 3100 DNA sequencer (Applied Biosystems). We next amplified and cloned a His-tagged *fp25K* fragment into fp25Knull/pcDNA. The resultant plasmid was cotransfected with the *Bsu*36I-digested Bm25KD DNA into BmN cells using Cellfectin (Invitrogen). Five days after transfection, the medium was collected and stored at 4 °C until use. A BmNPV expressing His-tagged *fp25K* (Bm25KD-Bm) was isolated by identification of plaques that did not express β-galactosidase (Katsuma et al., 2006). Insertion of His-tagged *fp25K* was confirmed by PCR using primers shown in Supplementary Table 1 and Western blot analysis with anti-His antibody (Qiagen, Fig. 3A).

2.3. Western blot analysis of V-CATH and GP64

BmN cells were infected with T3 or recombinant BmNPVs at a multiplicity of infection (MOI) of 5. At each dpi, the cells and medium were collected and subjected to Western blot analysis with an antibody against BmNPV V-CATH (Daimon et al., 2007). Fifth instar *B. mori* larvae were starved for several hours and then injected with 50 μl of a viral suspension containing 1×10^5 plaque forming units, returned to the artificial diet at 27 °C. At 2 or 4 dpi, the hemolymph was collected and subjected to Western blot analysis using antibodies against BmNPV V-CATH. BVs and ODVs were purified from culture medium of BmNPV-infected BmN cells and OBs, respectively (Iwanaga et al., 2002). Western blot analysis of BVs was performed with anti-GP64 antibody (Santa Cruz).

2.4. Cysteine protease assay

Cysteine protease activity was examined as described previously (Katsuma et al., 2004a). Hemolymph was collected from virus-infected *B. mori* larvae at 4 dpi, centrifuged at 20,000 × *g* for 10 min at 4 °C, and the supernatants were used immediately for cysteine protease assays (30 μl/assay). Protease activity was measured in an azocasein assay. The medium of BmNPV-infected BmN cells was collected at 5 dpi. Each 83 μl aliquot was used immediately to measure proteolytic activity in an azocasein assay.

2.5. Aspartic proteinase activity in BmNPV-infected BmN cells

Aspartic proteinase activities were measured as described previously (Rabossi et al., 2004) with few modifications. The substrate used was 2% hemoglobin, prepared in distilled water, and denatured by adjusting the pH to 3.5 with 272 mM acetic acid and 4 mM $(NH_4)_2SO_4$. The reaction mixture containing cell extract and acid-denatured hemoglobin was incubated for 30 min at 37 °C. The reaction was stopped by addition of trichloroacetic acid. After mixing and 30 min precipitation at room temperature, the liberated peptides in the soluble phase were recovered. The solution was assayed by adding NaOH and Folin-Ciocalteau reagent. After 5 min stabilization, absorbance at 750 nm was measured.

2.6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was prepared from BmN cells infected with T3, Bm25KD, or Bm25KD-Bm using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized from 0.2 μg of total RNA, and real-time quantitative RT-PCR was carried out using Power SYBR Green PCR master mix (Applied Biosystems) and the primers listed in Supplementary Table 1. Amplification was detected with the ABI PRISM 7000 Sequence Detection system (Applied Biosystems) as reported previously (Katsuma et al., 2006).

3. Results

3.1. Secretion of V-CATH in BmN cells and B. mori larvae infected with Bm25KD

Previous studies on BmNPV FP mutants have shown that mutations in *fp25K* result in inhibition of host degradation after death (Katsuma et al., 1999, 2004a). Further experiments revealed that the activity of viral cathepsin L-like protease, V-CATH, is heavily lessened in the hemolymph of *B. mori* larvae infected with Bm25KD, an *fp25K*-disrupted mutant of BmNPV (Katsuma et al., 2004a; Fig. 1A). Combined with the observation that BmNPV V-CATH is essential for post-mortem host degradation (Ohkawa et al., 1994), we speculated that some pathways involved in V-CATH secretion or activation might be impaired in Bm25KD-infected *B. mori* larvae, and this might lead to a lack of host degradation. To investigate whether this reduced activity of V-CATH in the hemolymph is due to a decrease in V-CATH secretion, we first performed Western blot experiments of hemolymph proteins from wild-type T3- and Bm25KD-infected larvae using anti-V-CATH antibody. As shown in Fig. 1C, V-CATH was detected at 4 dpi in hemolymph of either T3- or Bm25KD-infected larvae, but its amount in Bm25KD-infected larvae was markedly reduced. V-CATH secretion was not observed in the hemolymph of larvae infected with BmCysPD, a *v-cath* deleted BmNPV (Ohkawa et al., 1994). Previously, we also observed that V-CATH activity in the medium of Bm25KD-infected BmN cells was reduced (Katsuma et al., 2004a; Fig. 1B). As shown in Fig. 1D, we found that its secretion was severely reduced in Bm25KD-infected