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The fibroblast growth factor homolog of *Bombyx mori* nucleopolyhedrovirus enhances systemic virus propagation in *B. mori* larvae

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ABSTRACT

All lepidopteran baculovirus genomes sequenced to date encode a viral fibroblast growth factor homolog (vFGF). Recently, we generated a *Bombyx mori* nucleopolyhedrovirus (BmNPV) mutant lacking functional *vfgf* and found that BmNPV *vfgf* contributes to virus virulence in *B. mori* larvae. However, the steps at which BmNPV vFGF works during *in vivo* virus infection were unclear. To uncover the role of vFGF during systemic infection of silkworm larvae, we generated a BmNPV mutant, BmIEGFP, possessing an *ie-1* promoter-driven green fluorescent protein gene, and its derivative BmIEGFP/FGFD, in which *vfgf* was partially deleted from the genome of BmIEGFP. Intrahemocoelic and oral infection experiments using these viruses revealed that the loss of functional vFGF reduces viral infectivity in *B. mori* hemocytes. Our results suggest that BmNPV vFGF is required for efficient systemic infection, presumably by a chemotactic effect that allows budded virus to infect hemocytes efficiently.

1. Introduction

The *Baculoviridae* is a diverse family of pathogens that are infectious for arthropods, particularly insects of the order Lepidoptera. Nucleopolyhedroviruses (NPVs), the larger of the two genera of *Baculoviridae*, possess a large circular and double-stranded DNA genome within a rod-shaped virion. NPVs produce two types of virions during the infection cycle: occlusion-derived virus (ODV) transmits infection from insect to insect (oral infection), whereas budded virus (BV) spreads infection to neighboring cells.

All lepidopteran baculovirus genomes sequenced to date encode a viral fibroblast growth factor homolog (vFGF) (Ikeda et al., 2006). Detvisitsakun et al. (2005) reported several properties of *Autographa californica* NPV (AcMNPV) vFGF, demonstrating that it was secreted into the extracellular fluid, and was able to stimulate migration of *Spodptera frugiperda* SF-21 cells, *Trichoplusia ni* TN-368 cells, and *T. ni* hemocytes. Recently, Detvisitsakun et al. (2006, 2007) characterized an AcMNPV mutant lacking functional *vfgf*. Although no obvious differences were observed in cultured cells between the mutant and parental AcMNPVs, deletion of *vfgf* delayed the time to death in *S. frugiperda* and *T. ni* larvae when the virus was delivered by feeding but not by intrahemocoelic injection. These results suggest that AcMNPV *vfgf* may play a role in dissemination of virus from the midgut in an infected larva.

We recently constructed a *vfgf* deletion mutant of *Bombyx mori* NPV (BmNPV) and characterized its infectivity in *B. mori* larvae (Katsuma et al., 2006b). Unlike AcMNPV, we observed that the mutant virus took 20 h longer than wild-type BmNPV to kill *B. mori* larvae by both per os and intrahemocoelic infection. In this study, to clarify the role of vFGF during systemic infection of BmNPV, we constructed a BmNPV mutant possessing a green fluorescent protein gene, and its derivative in which *vfgf* was deleted from the genome. Larval bioassays suggest that BmNPV vFGF is required for efficient systemic infection in *B. mori* larvae, presumably by a chemotactic effect that allows budded virus to infect hemocytes efficiently.

2. Materials and methods

2.1. Insects, cells, and viruses

B. mori larvae (F1 hybrid Kinshu × Showa) were reared as described previously (Katsuma et al., 2006b). The BmN cell line was maintained in TC-100 with 10% fetal bovine serum as described previously (Katsuma et al., 2006b). BmNPV-abb (Katsuma et al.,

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2004) was propagated in BmN cells. Virus titers were determined by plaque assay as described previously (Katsuma et al., 2006b).

2.2. Generation of a recombinant BmNPV expressing GFP

An enhanced green fluorescent protein (*gfp*) gene was amplified by polymerase chain reaction (PCR), using the primers EF (5′-tcaagatctgatggtgagcaagggcgagga-3′) and ER (5′-tcaggtaccttacttgtacagctcgtccat-3′), and cloned into the vector pIEx-4 (Novagen), in which the *ie-1* promoter of AcMNPV drives constitutive expression of the gene of interest. A DNA fragment containing *ie-1* promoter/*gfp*/*ie-1* terminator was purified from the resulting plasmid, and inserted into the transfer vector pBmEPS1 (Kang et al., 1998). The transfer plasmid was cotransfected with *Bsu*36I-digested BmNPV-abb genomic DNA into BmN cells using Cellfectin (Invitrogen) (Katsuma et al., 2004). A recombinant BmNPV expressing *gfp* under the control of the AcMNPV *ie-1* promoter (BmIEGFP: Fig. 1A) was isolated by two rounds of plaque assay as described previously (Katsuma et al., 2006b).

2.3. Construction of a vfgf-deleted BmNPV

A *vfgf*-deleted BmIEGFP, named BmIEGFP/FGFD, was generated as described previously (Katsuma et al., 2006b). We used a plasmid with the *β-galactosidase* gene under the control of the *Drosophila hsp70* promoter inserted within the *vfgf* coding region such that a large portion of the *vfgf* coding region (approximately 420 bp) is deleted (Fig. 1A). This plasmid was cotransfected with BmIEGFP DNA into BmN cells. Recombinant BmNPV was isolated by identification of plaques expressing β-galactosidase (Katsuma et al., 2006b). Deletion of *vfgf* was confirmed by PCR using the primers CF1 (5′-gtatcgtttgctggcacttg-3′) and CR1 (5′-ttatattaggacacgacgtg-3′) (Fig. 1B).

2.4. Assays for GFP expression in B. mori larvae

In GFP expression assay, we used *B. mori* larvae in the first day of 5th or 4th instar (50–100 larvae for each experiment and 5 larvae were dissected for each virus at every time point to analyze the tissues). For intrahemocoelic inoculation with BV, BVs (10^5 pfu/larva) were injected into the body cavity of 5th-instar *B. mori* larvae (Katsuma et al., 2006b). For oral inoculation with ODV, 4th-instar *B. mori* larvae were fed with 10^7 occlusion bodies (OBs) per larva (Katsuma et al., 2006b). In all cases, inoculation of 4th-instar larvae with 10^7 OBs of BmIEGFP or BmIEGFP/FGFD led to 100% mortality. OBs were produced in 5th-instar *B. mori* larvae, purified by centrifugation and resuspended in double-distilled H_2O as described previously (O'Reilly et al., 1992). Purified OBs were quantified using a hemocytometer. OBs were applied to a small plug of artificial diet in individual containers, and only larvae that completely ingested the contaminated diet within 8 h were further reared.

Tissues of the infected larvae were excised, washed in ice-cold phosphate-buffered saline, mounted on glass slides and observed under a fluorescence microscope (Olympus BX-URA2). Hemolymph was collected from the infected larvae at 6, 12, 24, 48, 72, 96, and 120 h postinfection (hpi), and examined by fluorescence microscopy using a hemocytometer. The number of GFP-positive and GFP-negative hemocytes (approximately 200 hemocytes per field) was counted at two fields per larva in three individuals. The data were reported as the % hemocytes showing GFP expres-

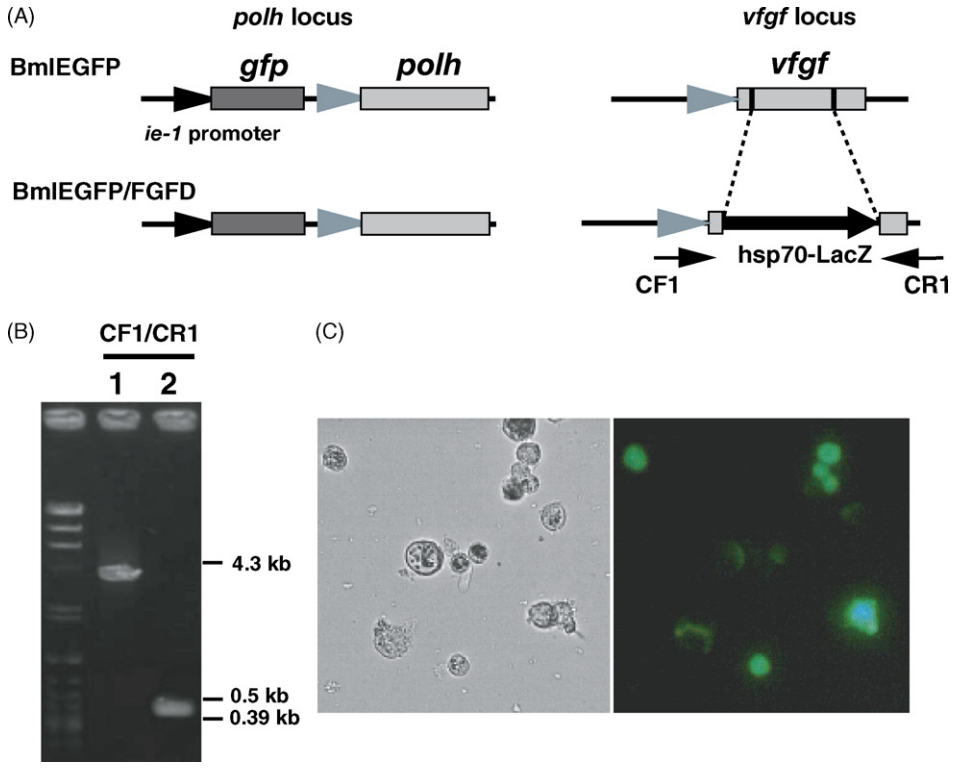

Fig. 1. Generation of BmIEGFP and BmIEGFP/FGFD. (A) The *polh* and *vfgf* loci of BmIEGFP and BmIEGFP/FGFD. BmIEGFP expresses *gfp* under the control of the AcMNPV *ie-1* promoter. BmIEGFP/FGFD, a *vfgf*-deleted BmIEGFP, was generated by homologous recombination in BmN cells. (B) PCR analysis of the genome of BmIEGFP/FGFD (lane 1) and BmIEGFP (lane 2). Deletion of *vfgf* was confirmed by PCR using the primers CF1/CR1. The left lane contains size markers. (C) GFP expression in BmIEGFP-infected BmN cells. BmN cells were infected with BmIEGFP at an MOI of 0.5, and examined by fluorescence microscopy at 72 hpi. The left panel shows bright field image and the right panel shows GFP expression. Note that GFP expression was rarely detected in BmNPV-infected cells showing OBs.