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Transient expression of a polydnaviral gene, CpBV15β, induces immune and developmental alterations of the diamondback moth, *Plutella xylostella*

Madanagopal Nalini, Yonggyun Kim *

Department of Bioresource Sciences, Andong National University, Andong 760-749, Republic of Korea

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ABSTRACT

The diamondback moth, *Plutella xylostella*, parasitized by its endoparasitoid wasp, *Cotesia plutellae*, undergoes various physiological alterations which include immunosuppression and an extended larval development. Its symbiotic virus, *C. plutellae* bracovirus (CpBV), is essential for their successful parasitization with more than 136 putative genes encoded in the viral genome. CpBV15β, a CpBV gene, has been known to play significant role in altering host physiological processes including hemocyte-spreading behavior through inhibition of protein synthesis under *in vitro* conditions. In the current study, we investigated its specific involvement in physiological processes of the host by transient expression and RNA interference techniques. The open reading frame of CpBV15β was cloned into a eukaryotic expression vector and this recombinant CpBV15β was transfected into nonparasitized 3rd instar *P. xylostella* by microinjection. CpBV15β was expressed as early as 24 h and was consistent up to 72 h. Due to the expression of this gene, plasma protein levels were significantly reduced and the ability of the hemocytes to adhere and spread on extracellular matrix was inhibited, wherein CpBV15β was detectable in the cytoplasm of hemocytes based on an indirect immunofluorescence assay. To confirm the role of CpBV15β, its double stranded RNA could efficiently recover the hemocyte-spreading behavior and synthesis of plasma proteins suppressed by the transient expression of CpBV15β. In addition, the larvae transfected with CpBV15β significantly suffered poor adult development probably due to lack of storage proteins. Thus these results demonstrate the role of CpBV15β in altering the host physiological processes involving cellular immune response and metamorphic development, which are usually induced by wasp parasitization.

1. Introduction

Koinobiotic endoparasitoids, which allow host development during parasitization, have evolved successful parasitization by modulating host immune and developmental processes, usually resulting in immunosuppression and extension of larval period (Quicke, 1997; Pennachio and Strand, 2006). These physiological alterations would be induced by maternal factors including polydnavirus, ovarian protein or venom (Webb and Luckhart, 1996; Asgari, 2006) or by embryonic factors including teratocyte or parasitoid larva (Dahlman, 1991; Lanzrein et al., 2001). To identify the parasitic factor(s) and target molecule(s) corresponding to a specific physiological alteration has been a main interest in this science field.

Polydnaviruses are an insect DNA virus group exhibiting a mutual symbiosis with its parasitoid wasp found in two families, Braconidae and Ichneumonidae (Stoltz and Vinson, 1979). They are divided into two viral families, bracovirus (BV) and Ichnovirus (IV) according to their symbiotic host families and viral morphology (Webb et al., 2000). A polydnavirus resides in a proviral state on its host chromosome and is vertically transmitted to successive generations (Fleming and Summers, 1986). It replicates in the female ovarian calyx to form viral particles during parasitoid pupal stage and is delivered into host hemocoel along with the parasitoid egg during oviposition (Theilmann and Summers, 1986). By exploiting host transcriptional and translational machineries, these polydnaviruses express their own genes, and the resulting gene products play critical roles in manipulating host physiological processes by interrupting their effective immunological responses and developmental program (Webb and Strand, 2005; Kim, 2005). There are several characteristic gene families reported in the polydnaviral genomes (Kroemer and Webb, 2004) with numerous hypothetical putative genes (Espagne et al., 2004; Webb et al., 2006; Tanaka et al., 2007). These genome studies support a hypothesis that BV and IV independently originated and have been evolved with the host wasp by successive processes of gene selection, duplication or mutation (Friedman and Hughes, 2006; Dupuy et al., 2006). Among known polydnaviral genomes, *Cotesia*-associated BVs comprise relatively large genome sizes (470–567 kb) compared to 187–248 kb of other polydnaviral genomes and possess several unique genes (Kim et al., 2007).

* Corresponding author. Fax: +850 82 54 823 1628.
E-mail address: hosanna@andong.ac.kr (Y. Kim).

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A koinobiotic endoparasitoid, *Cotesia plutellae*, is a solitary braconid wasp that parasitizes young larvae of the diamondback moth, *Plutella xylostella*, and possesses a symbiotic polydnavirus, CpBV (Bae and Kim, 2004). Its genome is estimated to be about 470 kb scattered on at least 27 genome segments (Kim, 2006). Its partial genome sequence encompassing 351,229 bp predicts several bracoviral gene families including CpBV-PTP, CpBV-IκB, and CpBV-ELP (Choi et al., 2005; Kim et al., 2007), which are expressed in *P. xylostella* parasitized by *C. plutellae* (Ibrahim et al., 2007; Kim et al., 2006; Kwon and Kim, 2008). The genome study also introduces several unique genes to *Cotesia*-associated BVs including CpBV-H4, CpBV-E94, and CpBV-lectin, which are also expressed in the parasitized *P. xylostella* (Gad and Kim, 2008; Ibrahim et al., 2005; Lee et al., 2008). Currently, two homologous viral genes (CpBV15α and CpBV15β) are identified from the cDNA library of the parasitized *P. xylostella* and these do not match with any known polydnaviral genes (Lee and Kim, 2008). A recombinant protein of CpBV15β prepared from a baculovirus expression system significantly impairs hemocyte-spreading behavior under *in vitro* conditions and has been considered as an immunosuppressant (Nalini and Kim, 2007). However, its late expression during parasitization leads to hypothesize its significant physiological role(s) in the parasitism other than inducing immunosuppression. To address this speculation, in the present study, we used the transient expression technique to monitor the parasitic role of CpBV15β in intact insect conditions during late larval instar. The physiological alterations induced by the transient expression were confirmed by silencing the transiently expressed CpBV15β by double stranded RNA to demonstrate the specific role of CpBV15β.

2. Materials and methods

2.1. Insect rearing

P. xylostella larvae were reared on cabbage leaves at 25 ± 1 °C under a photoperiod of 16:8 (L:D) h. Adults were fed 10% sucrose solution. Early third instar larvae (5 days from egg stage) were used for microinjection experiment.

2.2. Reagents

L-Cysteine···HCl, glutaraldehyde, Triton X-100, *t*-octylphenoxypolyethoxyethanol, glycerol, and fluorescein isothiocyanate (FITC)-labeled or unlabeled anti mouse IgG antibody were purchased from Sigma (St. Louis, MO, USA). Tris-buffered saline (TBS) was composed of 50 mM Tris–HCl, 100 mM dextrose, 5 mM KCl, 2.5 mM $MgCl_2$, and 50 mM NaCl, and then adjusted to pH 7.5. Phosphate-buffered saline (PBS) was composed of 100 mM $Na_2HPO_4{\cdot}12H_2O$, 18 mM KH_2PO_4, 138 mM NaCl, and 28 mM KCl, and then adjusted to pH 7.4. For preparing anticoagulant buffer (ACB), 4 mg of L-cysteine···HCl was dissolved in 5 ml of TBS and the pH was adjusted to 7.5 using 0.1 N NaOH.

2.3. Transient expression of CpBV15β

In order to analyze and confirm the role of CpBV15β on the functional efficacy of hemocytes in mediating cellular functions, transient expression was performed according to method described by Ibrahim and Kim (2008). Briefly, recombinant vector pBACPAK9-CpBV15β was prepared according to the method described by Jung et al. (2006). For *in vivo* transfection, 2 μl of recombinant vector, pBACPAK9-CpBV15β (≈400 ng) was mixed with 3 μl of Metafectene PRO transfection reagent (Biontex, Planegg, Germany) and incubated for 20 min at room temperature to form DNA-lipid complexes. Four hundred nanoliters of this mixture was injected into hemocoel of early 3rd instar larvae of *P. xylostella* using a micro injector (Micro 4™, WPI, Sarasota, FL, USA). The plasmid pBACPAK9 vector alone mixed with Metafectene PRO served as the control.

2.4. RT-PCR analysis of CpBV15β

Larvae injected with vector and recombinant vector were maintained separately and fed with cabbage leaves at 25 ± 1 °C under a photoperiod of 16:8 (L:D) h. Total RNA was extracted from vector or recombinant vector-injected larvae at 24, 48 or 72 h using Trizol reagent (MRC, OH, USA). After DNase treatment at 37 °C for 30 min with RNase-free DNase (Qiagen, LRS Laboratories, Seoul, Korea), the RNA extract was reverse-transcribed with RT-premix (Bioneer, Daejon, Korea) using oligo-dT primer (5′-CCAGT GAGCA GAGTG CGAGG ACTCG AGCTC AAGCT TTTTT TTTTT TTTTT-3′) followed by RNase H treatment. The resulting cDNA strand was used as template for RT-PCR using open reading frame (ORF) primers of CpBV15β (ORF-FP: 5′-ATGAA TACTT TCTTG TTTTG-3′ and ORF-RP: 5′-ATTAC G TTTA GGCTC AGTGA-3′) under the following conditions: 94 °C for 1 min, 50 °C for 45 sec and 72 °C for 1 min with 35 cycles.

2.5. Plasma protein analysis

Vector or recombinant vector 72 h post-injected larvae were surface sterilized by holding them in 70% ethanol for 30 s, gently pricked using sharp needle, and the hemolymph oozing out was collected in a graduated micro-capillary tube and transferred to sterile Eppendorf tube. These hemolymph samples were diluted to 1:1 ratio with PBS (pH 7.4) and were briefly centrifuged (3000*g*, 3 min, 4 °C). The separated plasma samples were quantified in their total proteins per unit volume by Bradford (1976) method and compared among treatments. The plasma proteins were also separated on 10% SDS-PAGE and immunoblotted against antibodies of CpBV15β (Lee and Kim, 2008) and larval storage protein (Ibrahim et al., 2006).

2.6. Analysis of hemocyte-spreading behavior

After microinjection of CpBV15β recombinant vector or vector alone, larvae of *P. xylostella* were collected at different times (24, 48 or 72 h) and were used for collecting hemolymph as described above. A ratio of hemolymph from 10 larvae in 100 μl ACB was used as hemocyte suspension. Hemocyte monolayers were made using 50 μl of hemocyte suspension and left in the moist chamber at 23 °C for 45 min. Hemocyte spreading was determined by counting the presence of pseudopodial extensions on 250 cells chosen randomly under a phase contrast microscope (BX41, Olympus, Japan) at 40× magnification.

2.7. Indirect immunofluorescence assay of CpBV15β

Hemocyte monolayers prepared as described above from larvae with CpBV15β recombinant vector, dsRNA + CpBV15β recombinant vector or vector alone were fixed with 1% glutaraldehyde for 15 min, washed with PBS, and overlaid with PBS containing 0.2% Triton X-100 for permeabilization for 5 min. After rinsing thoroughly with PBS, these monolayers were blocked with 20-fold diluted normal goat serum for 15 min, washed thoroughly, and overlaid with 500-fold diluted primary antibody for 45 min. After washing with PBS, 80-fold diluted FITC-conjugated antimouse IgG was added and incubated in darkness for 45 min. The monolayers were overlaid with 25 μl of PBS: glycerol (1:1) and observed under a confocal microscope (Olympus IX70, Tokyo, Japan) at 400×.