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Identification of three host translation inhibitory factors encoded in *Cotesia glomerata* bracovirus

Karen P. Barandoc, Yonggyun Kim *

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

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ABSTRACT

An endoparasitoid wasp, *Cotesia glomerata* (Braconidae: Hymenoptera), parasitizes larvae of the diamondback moth, *Plutella xylostella* (Yponomeutidae: Lepidoptera). Parasitized *P. xylostella* exhibits immunosuppression and developmental alteration. In this study, the parasitized larvae exhibited a marked suppression in storage proteins (SPs) especially at SP1 out of three SPs, although there was significant mRNA level of SP1. In addition, the parasitization significantly inhibited spreading behavior of hemocytes, in which a host translation inhibitory factor (HTIF)-like protein was detected in cytoplasm. A bracovirus is a symbiotic polydnavirus of *C. glomerata* (CgBV) and has been suspected as a main parasitic factor manipulating host physiological processes. Here, we cloned three putative HTIFs derived from CgBV: Cg-HTIF(I), Cg-HTIF(II) and Cg-HTIF(III). All Cg-HTIFs share sequence homologies with eukaryotic initiation factors for translation as well as with two other known HTIFs of *Cotesia plutellae* bracovirus. Quantitative RT-PCR and immunoblotting assays indicated that these genes were mostly expressed at late developmental stage of *P. xylostella* parasitized by *C. glomerata*. Transfection of a recombinant Cg-HTIF(I) in *P. xylostella* by microinjection expressed the gene as early as 12 h and maintained until 72 h, at which hemocyte behavior was impaired and the SP levels were significantly reduced. However, the expression of Cg-HTIF(I) did not inhibit transcription of SP1. Co-injection of the recombinant vector along with double-stranded RNA specific to Cg-HTIF(I) notably recovered the hemocyte-spreading behavior and SP1 translation.

1. Introduction

Polydnaviruses (PDVs) are insect DNA viruses with segmented genome residing in host chromosome as a proviral form (Krell et al., 1982). These viruses have been found from some endoparasitic wasps belonging to Braconidae and Ichneumonidae families which give rise to two PDV families: bracovirus (BV) and ichnovirus (IV) (Webb et al., 2000). PDVs and their gene products have been implicated in host immune and developmental perturbations to facilitate wasp host survival and development (Webb et al., 2006). These alterations include specific inhibition of host protein synthesis (Beckage and Kanost, 1993; Shelby and Webb, 1994), regulation of host developmental program (Pennacchio et al., 1998; Vinson et al., 1998), and host immunosuppression (Lawrence and Lanzrein, 1993; Stoltz, 1993; Strand and Pech, 1995).

Cotesia glomerata (Braconidae: Hymenoptera) is a gregarious endoparasitoid that has been known to parasitize the diamondback moth, *Plutella xylostella* (Jung et al., 2006) in addition to its main natural hosts, *Pieris brassicae* and *P. rapae* (Laing and Levin, 1982). It contains PDV called *C. glomerata* bracovirus (CgBV) and the viral genome is segmented from 0.6 to 30 kb sizes with different molar ratios (Madanagopal and Kim, 2007). Host parasitized by *C. glomerata* exhibits immunosuppression and developmental arrest (Kim et al., 2006). These physiological alterations in *P. xylostella* have been well documented in the parasitization of *Cotesia plutellae*, a closely related endoparasitoid of *C. glomerata*. For example, parasitism of *C. plutellae* reduces total hemocyte population number of *P. xylostella* and inhibits hemocyte encapsulation in response to parasitoid egg (Ibrahim and Kim, 2006), in which CpBV has been regarded as a main parasitic factor (Kim et al., 2007). Products of CpBV genes modulate host molecular targets and control gene expression at transcription (Gad and Kim, 2008) and at post-transcription (Lee and Kim, 2008; Nalini and Kim, 2009).

Host translation inhibitory factor (HTIF) has been first reported from *Heliothis virescens* larvae parasitized by *Campoletis sonorensis* (Shelby and Webb, 1994). HTIF selectively inhibits host gene expression at post-transcriptional level to suppress host immune response and nutrient usage, but does not inhibit host housekeeping genes to maintain koinobiont life form of the host parasitoid (Shelby et al., 1998). Later, HTIF was identified as VHv1.4 and VHv1.1 that are viral genes encoded in *C. sonorensis* IV (Kim, 2005).

A recent study using *in vitro* analysis through hemocyte culture of *P. xylostella* showed that CpBV15β gave selective inhibition on host mRNAs such as storage proteins at post-transcriptional level

* Corresponding author. Tel.: +82 54 820 5638; fax: +82 54 820 6320.
E-mail addresses: karenbarandoc@yahoo.com (K.P. Barandoc), hosanna@andong.ac.kr (Y. Kim).

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(Madanagopal and Kim, 2007). This suggests that HTIF encoded by CpBV manipulates host protein synthesis to transform the parasitized host to be favorable for wasp development. Based on the fact that *C. glomerata* and *C. plutellae* share similar parasitic capacity on *P. xylostella* (Kim et al., 2006), we hypothesized that the symbiotic polydnavirus, CgBV, may encode similar genes which may be implicated in manipulating host translational processes in favor of the wasp survival.

This study shows a specific inhibition of a storage protein at post-transcriptional level in *P. xylostella* parasitized by *C. glomerata*. It also shows that using an immunoblotting and indirect immunofluorescence assay, HTIF-like protein(s) called Cg-HTIF is present in parasitized plasma and hemocytes of *P. xylostella*, which undergo significant impairment in cellular immunity. Then we cloned three highly homologous Cg-HTIF genes from the parasitized *P. xylostella*. We also investigated their involvement in host's physiological alterations by transient expression and RNA interference techniques.

2. Materials and methods

2.1. Insect rearing and parasitization

P. xylostella larvae were reared under 25 ± 1 °C and 16:8 h (L:D) photoperiod with cabbage leaves. Adults were fed 10% sucrose solution. Late second-instar larvae were parasitized by *C. glomerata* at 1:2 (wasp:host) density for 24 h under the rearing condition. Then the parasitized larvae were fed cabbage leaves and incubated at the rearing environment. After emergence, adult wasps were allowed to mate for 24 h and then used for parasitization (Kim et al., 2006).

2.2. Chemicals

Trizol (Invitrogen, Carlsbad, CA, USA); 70% ethanol; diethylpyrocarbonate (DEPC)-treated water; ethidium bromide; phosphate buffered saline (PBS): 50 mM NaH_2PO_4, 150 mM NaCl pH 7.2; agarose; sodium dodecyl sulfate (SDS); anticoagulant buffer: 5 mM L-cysteine hydrochloride in Tris-buffered saline (TBS) containing 50 mM Tris, 100 mM dextrose, 2.5 mM $MgCl_2$, 5 mM KCl, 50 mM NaCl, pH 7.5; Triton X-100, glutaraldehyde, normal goat serum; fluorescein isothiocyanate (FITC), FITC-conjugated anti-mouse IgG antiserum; glycerol. All these chemicals were obtained from Sigma-Aldrich Korea (Seoul, Korea).

2.3. RNA extraction and cloning Cg-HTIF

Total RNAs were extracted from parasitized *P. xylostella* (6 days after parasitization) using Trizol reagent followed by isopropanol precipitation. The resulting RNA pellet was washed with 70% ethanol and resuspended in DEPC-treated water. Total RNA (1 μg) was reverse-transcribed with RT-PCR premix (Intron, Daejeon, Korea). The resulting cDNA was used as template to amplify an HTIF gene encoded in CgBV using primers (5′-ATG AAT ACT TTC TTG TTT TG-3′, 5′-ATT ACG TTT AGG CTC AGT GA-3′) under PCR conditions: 94 °C for 50 s, 50 °C for 50 s and 72 °C for 1 min with 35 cycles. The PCR product was cloned into pCR2.1-TOPO TA cloning vector (Invitrogen) and sequenced (Macrogen, Daejeon, Korea). The identified three Cg-HTIF genes (Cg-HTIF(I), Cg-HTIF(II) and Cg-HTIF(III)) were aligned with sequences of known HTIFs and eukaryotic initiation factors using Clustal W of the DNAstar program (Version 5.01, DNAstar Inc., Madison, WI, USA).

To obtain 5′ untranslated region sequence (UTR) of Cg-HTIF(I), we conducted rapid amplification of cDNA ends (RACE) (Invitrogen) according to the manufacturer's instructions. Gene-specific primers were designed on the basis of predicted open reading frame (ORF) sequences of Cg-HTIF(I): 5′-GCA TCG CCA GTA TTC GTG TT-3′ and 5′-GCG ATT TTT GCA AAC TCA ATC-3′. With these two primers, nested PCRs were performed under conditions described above except for 52 °C annealing temperature. The resulting PCR product was sequenced as described above. Its 3′ UTR sequence was obtained by 3′ RACE with forward primer (5′-ATG AAT ACT TTC TTG TTT TG-3′) and reverse primer (5′-GAG CTC AAG CTT TTT TTT TTT TTT TTT-3′) under PCR conditions described above.

2.4. RT-PCR analyses of Cg-HTIFs

Total RNAs were extracted from parasitized *P. xylostella* larvae, either from whole insects or individual tissues at various time points following parasitization by *C. glomerata*. Total RNA was reverse-transcribed as described above. The resulting cDNAs were used as a template for PCR amplification of Cg-HTIF gene with a common forward primer (5′-TCG CTG TTG GCT TAG TGG GC-3′) and gene-specific reverse primers: 5′-GCG TGA CTT CGA ACT GCA GC-3′ for Cg-HTIF(I), 5′-GGC TTA GTG ACT TGC TTT AC-3′ for Cg-HTIF(II), and 5′-GTT AAC CTG TGC TCC AGC GT-3′ for Cg-HTIF(III). Amplification was performed in 35 cycles using a program of 1 min at 94 °C, 45 s at 50 °C, and 2 min at 72 °C.

Using the same primer sets in each of Cg-HTIFs, quantitative real-time PCR (qRT-PCR) was performed with an Exicycler™ Quantitative Thermal Block (Bioneer, Daejeon, Korea) using SYBR green chemistry and real-time fluorescence measurements. This technique was carried out using Accupower Greenstar™ PCR premix (Bioneer). The 20 μL reaction mixture consisted of 1× Greenstar™ PCR Master Mix, 10 mM $MgCl_2$, 0.5 μM of primers, and 250 ng of cDNA. Initial incubation at 95 °C for 15 min was done to activate Hotstart *Taq* DNA polymerase. qRT-PCR was performed with 40 cycles of an array of reactions of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C. β-Actin gene was used as a control with primers: 5′-TGG CAC CAC ACC TTC TAC-3′ and 5′-CAT GAT CTG GGT CAT CTT CT-3′. Each cycle was scanned to quantify the PCR products of each treatment with three replicates. Amplification plots in real-time were constructed using Exicycler™ program. Quantitative analysis of Cg-HTIF expression was done using the comparative C_T (ΔC_T) method (Livak and Schmittgen, 2001).

2.5. Immunoblotting assays of Cg-HTIF and larval storage protein

Proteins extracted from whole body of both parasitized and non-parasitized larvae were separated on 15% SDS-PAGE and electro-transferred onto Immun-blot™ PVDF membrane (Bio-Rad, Hercules, CA, USA) by the method of Towbin et al. (1979). Non-specific sites were blocked with 5% skim milk for 1 h at room temperature. The membrane was washed thrice with PBS and incubated for 1 h at room temperature with the primary antibody raised against *C. plutellae*-HTIF (Cp-HTIF) known as CpBV15α/β (Lee and Kim, 2008) or storage protein of *P. xylostella* (Ibrahim et al., 2006). Further washing thrice with PBS, the membrane was incubated with an antimouse-IgG secondary antibody (1/2000 dilution) conjugated with alkaline phosphatase for 1 h at room temperature. Finally, again after three washes with PBS, the membrane was reacted with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich Korea).

2.6. Indirect immunofluorescence assay

Hemocyte monolayers were prepared by collecting hemolymph as described above. Each monolayer was incubated in a moist chamber at 25 °C for 45 min. Following 45 min spreading period, cells were fixed with 1% glutaraldehyde for 15 min, washed with several volumes of PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. 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-labeled antimouse IgG antibody was added and incubated for 45 min under darkness. Cells were washed with PBS,