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A SERI technique reveals an immunosuppressive activity of a serine-rich protein encoded in *Cotesia plutellae* bracovirus

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Polydnavirus genome is segmented and dispersed on host wasp chromosome. After replication, the segments form double-stranded circular DNAs and embedded in viral coat proteins. These viral particles are delivered into a parasitized host along with parasitoid eggs. A serine-rich protein (SRP) is predicted in a polydnavirus, *Cotesia plutellae* bracovirus (CpBV), genome in its segment no. 33 (CpBV-S33), creating CpBV-SRP1. This study explored its expression and physiological function in the diamondback moth, *Plutella xylostella*, larvae parasitized by *C. plutellae*. CpBV-SRP1 encodes 122 amino acids with 26 serines and several predicted phosphorylation sites. It is persistently expressed in all tested tissues of parasitized *P. xylostella* including hemocyte, fat body, and gut. Its physiological function was analyzed by injecting CpBV-S33 and inducing its expression in nonparasitized *P. xylostella* by a technique called SERI (segment expression and RNA interference). The expression of CpBV-SRP1 significantly impaired the spreading behavior and total cell count of hemocytes of treated larvae. Subsequent RNA interference of CpBV-SRP1 rescued the immunosuppressive response. This study reports the persistent expression of CpBV-SRP1 in a parasitized host and its parasitic role in suppressing the host immune response by altering hemocyte behavior and survival. [BMB reports 2010; 43(4): 279-283]

INTRODUCTION

Polydnaviruses (PDVs) are a group of insect DNA viruses that exhibit unique symbiosis with specific insect hosts, specifically those in the subfamilies of Braconidae and Ichneumonidae (1). PDVs have their segmented genomes on the host wasp chromosomes in a proviral form and are transmitted vertically to offspring (2). However, they exhibit viral replication in the ovarian calyx of female endoparasitic wasps and are delivered into lepidopteran host hemocoel along with the wasp's eggs during parasitization (3). Inside the lepidopteran host, PDVs express their own genes using host transcription/translation machineries (4) and the gene products manipulate host immune and development systems for successful parasitization (5).

An endoparasitoid wasp, *Cotesia plutellae*, parasitizes larvae of the diamondback moth, *Plutella xylostella* (6). Its symbiotic PDV is the *C. plutellae* bracovirus (CpBV). Consisting of at least 27 segments, the genome of CpBV is estimated to be at least 470 kb (7). Protein tyrosine phosphatases (PTPs) are present in the largest CpBV gene family, which consists of at least 39 putative genes (8). Viral PTPs play a role in altering host PTP activities and in the suppression of the cellular immune response (9). Another abundant gene family, vankyrins, significantly suppress the antiviral response of *P. xylostella* (10). Other genes were also identified in the CpBV genome, such as EP1-like proteins similar to those found in *C. congregata* and *C. kariyai* bracoviruses (11). Viral lectin, histone H4 and Duffy-binding-like domain genes are also encoded in CpBV and are known to contribute to the immunosuppression and developmental alteration of the parasitized host (12, 13). Two homologous genes, CpBV15α and CpBV15β, were also identified in the CpBV genome. CpBV15β is known to inhibit spreading behavior and F-actin growth in hemocytes of the parasitized host (14). A putative actin destabilizer (CpBV-RTX) and cysteine-rich protein of CpBV are also expressed in parasitized larvae of *P. xylostella*, and impair the hemocyte immune response by inhibiting actin polymerization and delaying host larval development (15, 16). Despite current efforts to verify the physiological functions of predicted CpBV genes, more than 50% of the putative genes are yet to be unknown in their roles in the parasitism. Here, we identified a physiological function of CpBV-SRP1 in the parasitized *P. xylostella* as an immunosuppressant using a technique of SERI (segment expression and RNA interference).

RESULTS

Molecular characteristics of CpBV-SRP1

CpBV-SRP1 (Genbank accession number: EU127911) was predicted in segment 33 of CpBV (Fig. 1A). This segment encodes

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a sequence of 122 amino acids with 26 serine residues, a signal peptide and four phosphorylation sites. CpBV-SRP1 is similar to serine-rich proteins identified in other organisms (Fig. 1B). CcBV-SRP in particular showed 82% similarity in amino acid sequence to CpBV-SRP1 with an e-value of 1e-27. MdBV-SRP2 showed 39% similarity with CpBV-SRP1.

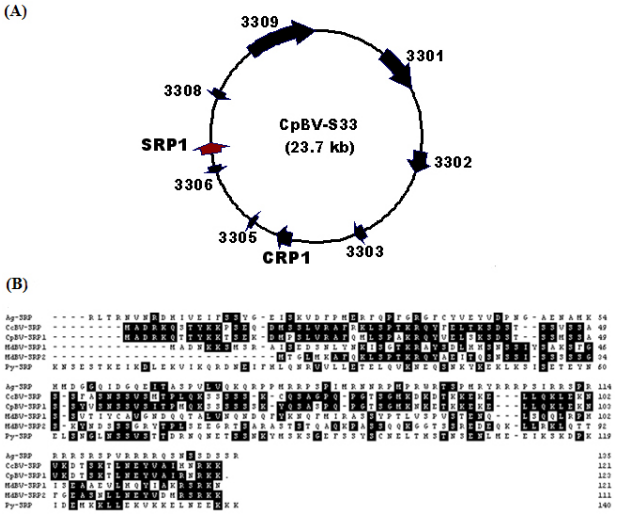

Fig. 1. Segment map of CpBV-S33 and sequence alignment of CpBV-SRP1 with proteins showing sequence homology. (A) CpBV-S33 has nine predicted ORFs shown in by arrow with a total size of 23,697 bp. (B) Amino acid sequence alignment of CpBV-SRP1 with other serine-rich protein genes. Accession numbers are the following: Ag-SRP (*Anopheles gambiae*, XP_318582), CcBV-SRP (*Cotesia congregata*, YP_184888), MdBV-SRP1 and MsBV-SRP2 (*Microplitis demolitor*, YP_ 239375 and YP_2393993), Py-SRP (*Plasmodium yoelii yoelii*, XP-725576) and CpBV-SRP1 (*C. plutellae*, ABX27952).

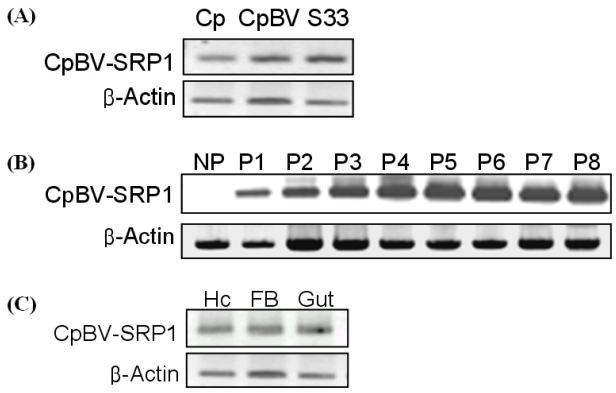

Fig. 2. Expression of CpBV-SRP1 localized to segment 33 of the CpBV genome in *Plutella xylostella* larvae parasitized by *Cotesia plutellae*. (A) Localization of the CpBV-SRP1 gene by PCR analysis using genomic DNAs of adult *C. plutellae* (CP), the symbiotic virus (CpBV), and viral segment 33 (S33) (B) Expression of CpBV-SRP1 in different ages of parasitized (P) and nonparasitized (NP) *P. xylostella* (C) Expression of CpBV-SRP1 in different tissues including hemocyte (Hc), fat body (FB), and gut of P7 larvae. The numbering of P1-P8 indicates age (days) of larvae after the parasitization.

Expression of CpBV-SRP1 in *P. xylostella* larvae parasitized by *C. plutellae*

PCR amplification of wasp and viral genomic DNAs as templates showed that CpBV-SRP1 is encoded as a provirus in the *C. plutellae* and CpBV genomes (Fig. 2A) owing to its proviral

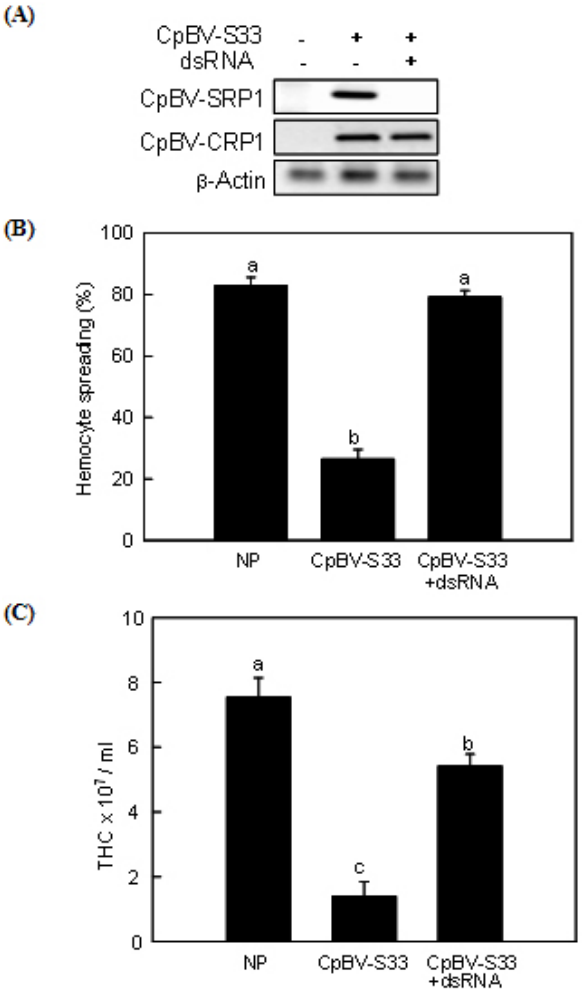

Fig. 3. Analysis of immunosuppressive activity of CpBV-SRP1 in *Plutella xylostella* by SERI. (A) Expression levels of CpBV-SRP1 and CpBV-CRP1 were checked 24 h post-injection of CpBV-S33 into nonparasitized (NP) *P. xylostella* with or without CpBV-SRP1 dsRNA to validate the success of transient expression. (B) Inhibition of hemocyte spreading was analyzed 24 h after microinjection of CpBV-S33 with or without CpBV-SRP1 dsRNA. Each treatment was replicated ten times. (C) Total hemocyte counts (THCs) used diluted hemolymph of treated larvae with 10 replications. Different letters above standard deviation bars indicate a significant difference among their means at Type I error = 0.05 (LSD test).

nature. CpBV-SRP1 was expressed early at day 1 after parasitization of *P. xylostella* and was increased in expression in all larval stages (Fig. 2B). Furthermore, CpBV-SRP1 was expressed in all tested tissues, such as hemocyte, fat body, and gut (Fig. 2C).

CpBV-SRP1 suppresses a cellular immune response of *P. xylostella*

To determine the effect of CpBV-SRP1 expression on *P. xylostella*, we utilized SERI, a technique previously shown to be effective for studying the physiological functions of CpBV-encoded genes (16). Microinjection of CpBV-S33 into the nonparasitized larvae of *P. xylostella* resulted in transient expression of the encoding genes CpBV-SRP1 and CpBV-CRP1 24 h post-injection. Co-injection of double stranded RNA (dsRNA) specific to CpBV-SRP1 was able to knockdown expression (Fig. 3A). However, this dsRNA did not affect the expression of other encoding genes of CpBV-S33 like CpBV-CRP1. The transient expression of CpBV-SRP1 resulting from microinjection of CpBV-S33 significantly inhibited the spreading behavior of hemocytes in treated larvae 24 h post-injection (Fig. 3B). To determine whether this inhibition was caused by CpBV-SRP1, dsRNA specific to SRP1 was co-injected with CpBV-S33, and the spreading behavior of hemocytes was recovered up to 63% compared to untreated control.

Similar analysis of total hemocyte count was performed 24 h post injection (Fig. 3C). The larvae injected with CpBV-S33 showed a significant decrease in total hemocyte count. The hemocyte populations were recovered by dsRNA treatment up to about 67% compared to untreated control.

DISCUSSION

PDV infections, along with other parasitic factors such as ovarian proteins, venom and teratocytes, modify host immune and developmental processes (4). PDVs harbor large multigene families that are often implicated in immunosuppression and immunoevasion in parasitized hosts (17). CpBV is regarded as a major factor in reducing cellular immune capacity in parasitized *P. xylostella* (18, 19).

There are nine open reading frames (ORFs) identified in CpBV-S33. Transcriptional analysis of several of these ORFs indicates that they differ in expression pattern. For example, the expression of CpBV-CRP1, a cysteine-rich protein, was abundant 24 h after parasitization (16) but then decreased thereafter. ORF3303 showed a relatively constitutive expression pattern (data not shown). CpBV-SRP1 analyzed in this study showed the opposite expression pattern from that of CpBV-CRP1 in *P. xylostella*, which showed persistent and increasing expression during parasitization. These results suggest that genes in a segment of CpBV can be differentially expressed under their own promoters.

In a previous study, CpBV-SRP1 identified in *P. xylostella* parasitized by *C. plutellae* (20) was expressed the brain and hemocytes during the early stages of parasitization. However, the physiological role of CpBV-SRP1 was not addressed. Therefore, this study attempted to predict the physiological funciton of CpBV-SRP1 by sequence analysis. The fact that other PDVs also have SRP genes suggests this is critically important to the parasitism in our study. In detail, the similarity of CpBV-SRP1 to SRPs from other insects like *Anopheles gambiae*, an important vector of the malaria strain *Plasmodium yoleii yoleii*, suggests that CpBV-SRP1 plays a role in altering host physiology by targeting the immune response. *In situ* hybridization of CpBV-SRP1 found that it was expressed only in granular cells of parasitized *P. xylostella*, further suggesting its involvement in immunosuppression (20). In addition, the persistent expression of CpBV-SRP1 in this study suggests its critical role(s) in the parasitism of *P. xylostella* by *C. plutellae*.

SERI technique has been used previously to study the physiological function of CpBV-CRP1 (16). Here, we used the same technique to validate our hypothesis that CpBV-SRP1 may play a role in the immunosuppression of a parasitized host. Microinjection of CpBV-S33 into nonparasitized *P. xylostella* showed clear expression of CpBV-SRP1 as well as other encoded genes like CpBV-CRP1 and ORF3303. Transient expression experiments were also performed to address the physiological functions of other CpBV-encoded genes like CpBV-PTP, CpBV-ELP1 and CpBV-H4 (8, 11, 12). Segment injection allows the expression of other genes in the same segment and may cause a compounding effect on the expression and physiological function of CpBV-SRP1. This problem was avoided by selectively suppressing a target gene using dsRNA specific to SRP1 along with injection of CpBV-S33. Our results showed the clear suppression of CpBV-SRP1 while other ORFs found in the same segment were still expressed. Under this condition, the spreading behavior of hemocytes showed significant recovery compared to the untreated host. These findings suggest that CpBV-SRP inhibits cellular activity of the host through molecular interaction, causing impaired host cellular immune response (9).

The decrease in total hemocyte count after micro-injection with CpBV-S33 was reversed by co-injection of SRP1 dsRNA. Hemocytes are originally derived from stem cells from hematopoietic organs and subsequently multiply upon the division of hemocytes already in circulation (21). Thus, hematopoietic organs of lepidopteran species provide prohemocytes and plasmatocytes which are released into the hemolymph where they are differentiated into granular cells, oenocytoids and spherulocytes (22, 23). Maintaining hemocyte populations in circulation mostly depends on their combined divisions after differentiation (23). In several endoparasitoids, parasitism results in the significant reduction of host hemocyte populations, leading to host immunosuppression (24). Reduction in hemocyte levels due to parasitism of *C. kariyai* was caused by cell death of circulating hemocytes and by histolysis of hematopoietic organs (25). The molecular mechanism of how CpBV-SRP1 affects the spreading behavior of hemocytes and the total

hemocyte count was not determined in this study.

In summary, CpBV-SRP1 is a virulence factor in the parasitism of *C. plutellae* that suppresses the immune response of *P. xylostella*. The persistent expression of CpBV-SRP1 in parasitized larvae suggests that it plays a role in maintaining host immunosuppression.

MATERIALS AND METHODS

Insect rearing and parasitization

Larvae of *P. xylostella* were reared under 25 ± 1^{o}C and a 16 : 8 h (L : D) photoperiod on cabbage leaves. Adults were fed 10% sucrose. Late second instar larvae were parasitized by *C. plutellae* at a 1 : 2 (wasp : host) density for 24 h and reared on cabbage leaves in the proper environment. After emergence, adult wasps were allowed to mate for 24 h and then used for parasitization.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were extracted from parasitized and nonparasitized larvae of *P. xylostella* using Trizol reagent (MRC, OH, USA) and then precipitated with isopropanol. The RNA pellet was washed with 70% ethanol and resuspended in diethylpyrocarbonate-treated water. Total RNA (1 μg) was reverse-transcribed with RT-PCR premix (Intron, Daejeon, Korea). The resulting cDNA acted as a template to amplify CpBV-SRP1 using primers (5′-ATG GCT GAC CGC AAG CAA ACG AC-3′, 5′-CTT CTT ACG GTT ACG GAT AGC-3′). The PCR reaction was performed in a total volume of 20 μL and denaturation was run for 30 cycles at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min.

Bioinformatic analysis

The ORF sequence of CpBV-SRP1 was predicted using the DNAstar program (Version 5.02, DNAstar Inc, Madison, USA). Homologous genes were identified through a NCBI BLASTp search of NCBI (http://www.ncbi.nlm.nih.gov/). Alignment with different SRP genes was performed using the Clustal W method of the DNAstar program with the following parameters: gap penalty, 10; gap length penalty, 0.20; delay divergent seq, 30%; DNA transition width, 0.50. Post-translational modifications of CpBV-SRP1 were predicted using the SignalP3.0 and PPSearch programs of expasy (http://us.expasy.org/).

Microinjection of CpBV-S33 segment into larvae

A segment of CpBV-S33 was cloned using transposon encoded in pCS-S vector (26). Samples for microinjection were prepared by mixing CpBV-S33 segment DNA with Metafectene PRO transfection reagent (Biontex, Planneg, Germany). Briefly, 0.5 μg of CpBV-S33 were mixed with 3 μl of transfection reagent followed by incubation for 20 min at room temperature to allow DNA-lipid complexes to form before injection into the hemocoel of second instar *P. xylostella*. Glass capillary (World Precision Instruments, Sarasota, FL, USA) injection needles were made using a micropipette puller PN-30 (Narishige, Japan). The DNA-transfection reagent complex (60 nL) was injected into each larva at a rate of 10 nL/sec using an UltraMicroPumpIV with SYS-microcontroller (World Precision Instruments, Sarasota, FL, USA). Microinjection was performed under a stereomicroscope (S730 Olympus, Tokyo, Japan).

RNA interference (RNAi) against CpBV-SRP1

RNAi was performed using dsRNA prepared using T7 RiboMAX^{TM} Large Scale RNA Production System (Promega, Madison, WI, USA) according to the manufacturer's instructions. dsRNA 366 bp in size was prepared using two gene-specific primers as in RT-PCR. Two separate clones with the same target region, i.e. CpBV-SRP1, but different orientations were confirmed and analyzed before linearization by restriction enzyme digestion using *Hind*III. After digestion, the two linearized DNA templates were used for in vitro transcription reactions. Sense and anti-sense strands were synthesized using T7 RNA polymerase. Annealing of both strands into dsRNA was performed by heating the reaction to 75°C for 5 min followed by mixing and cooling to room temperature. DNA and ssRNA were digested using DNaseI and RNase, respectively. The resulting dsRNA was purified and eluted in elution buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0).

Hemocyte spreading assay and total hemocyte counts

Larvae were surface-sterilized with 70% ethanol and hemolymph was collected in 150 μL of anticoagulant buffer (ACB) that was freshly prepared by dissolving 4 mg of L-cysteine hydrochloride (Sigma, MO, USA) in 5 mL of Tris-buffered saline [50 mM Tris/HCl (pH 7.5), 100 mM glucose, 5 mM KCl, 2.5 mM $MgCl_2$ and 50 mM NaCl]. Fifty μL of this suspension was used to prepare hemolymph monolayer, which was left in a moist chamber at 25°C for 45 min. The spreading behavior of hemocytes was characterized by the presence of filopodial extension. The percentage of spreading hemocytes was scored by randomly counting 100 hemocytes from 10 selected microscopic fields at 400x magnification under a phase-contrast microscope (BX41, Olympus, Japan). For hemocyte quantification, 1 μl of hemolymph was diluted and hemocytes were visually counted on a hemocytometer grid under the microscope.

Statistical analysis

Treatment means and variances were analyzed in a one-way ANOVA using PROC GLM of the SAS program (27). All means were compared by least-squared difference (LSD) test using Type I error = 0.05.

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