Journal of Invertebrate Pathology 106 (2011) 217–222

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Immunosuppression induced by entomopathogens is rescued by addition of apolipophorin III in the diamondback moth, *Plutella xylostella*

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ARTICLE INFO

Article history:
Received 1 July 2010
Accepted 30 September 2010
Available online 16 October 2010

Keywords:
Apolipophorin III
Immunosuppression
Cotesia plutellae
Xenorhabdus nematophila
Plutella xylostella

ABSTRACT

Apolipophorin III (ApoLpIII) has been known to play critical roles in lipid transport and immune activation in insects. This study reports a partial ApoLpIII gene cloned from the diamondback moth, *Plutella xylostella*. It showed that the gene was expressed in all developmental stages of *P. xylostella*. In larval stage, it was expressed in all tested tissues of hemocyte, fat body, gut, and epidermis. In response to bacterial challenge, the larvae showed an enhanced level of ApoLpIII expression by a quantitative real-time RT-PCR. RNA interference of ApoLpIII by its specific double stranded RNA (dsRNA) caused significant knockdown of its expression level and resulted in significant suppression in hemocyte nodule formation in response to bacterial challenge. However, larvae treated with the dsRNA exhibited a significant recovery in the cellular immune response by addition of a recombinant ApoLpIII. Parasitization by an endoparasitoid wasp, *Cotesia plutellae*, suppressed expression of ApoLpIII and resulted in a significant suppression in the hemocyte nodule formation. The addition of the recombinant ApoLpIII to the parasitized larvae significantly restored the hemocyte activity. Infection of an entomopathogenic bacterium, *Xenorhabdus nematophila*, caused potent pathogenicity of *P. xylostella*. However, the addition of the recombinant ApoLpIII to the infected larvae significantly prevented the lethal pathogenicity. This study suggests that ApoLpIII limits pathogenicity induced by parasitization or bacterial infection in *P. xylostella*.

1. Introduction

Apolipophorin (ApoLpIII), a component of lipid transport molecule in insect hemolymph by stabilizing low-density lipophorin particles, has been known to play a crucial role in innate immune response in several insect species by acting in pattern recognition (Weers and Ryan, 2006). ApoLpIII is homologous to a mammalian lipoprotein, apolipoprotein E, which binds to lipopolysaccharide (LPS) and detoxifies its endotoxic shock (Feingold et al., 1995). Indeed, ApoLpIII binds to both Gram-positive and –negative bacteria by recognizing lipoteichoic acid (Halwani and Dunphy, 1999; Halwani et al., 2000) and LPS (Kato et al., 1994; Dunphy and Halwani, 1997). The recognition and binding of ApoLpIII leads to promoting phagocytosis and enhancing antibacterial activity (Niere et al., 1999; Kim et al., 2004). Against fungal infection, ApoLpIII is also able to recognize β-1,3-glucan and has a protective role by enhancing encapsulation response (Whitten et al., 2004).

Two entomopathogens are well known to inhibit insect immune responses. An entomopathogenic bacterium, *Xenorhabdus nematophila*, is symbiotic to a nematode, *Steinernema carpocapsae*, and kills insects by inducing septicemia (Park and Kim, 2000). The host larvae infected by the bacteria fail to express their effective cellular and humoral immune responses and are in a state of immunosuppression, in which *X. nematophila* produces LPS and proteases to give cytotoxic effects on hemocytes and other immune-associated cells (Dunphy and Webster, 1988; Caldas et al., 2002), and secretes inhibitors of eicosanoid biosynthesis to inhibit signal mediation from nonself recognition to immune effectors (Park and Kim, 2003; Kim et al., 2004). An endoparasitoid wasp, *Cotesia plutellae*, parasitizes larvae of the diamondback moth, *Plutella xylostella*. With its parasitic factors, the wasp induces significant immunosuppression of the parasitized host (Ibrahim and Kim, 2006). However, it has been not clearly understood whether both bacterial and parasitoid pathogens suppress immune recognition by the infected host.

A current expressed sequence tag (EST) analysis annotates several immune candidate genes of *P. xylostella* including ApoLpIII (Eum et al., 2007). This study analyzed sequence and expression patterns of ApoLpIII in naïve and infected larvae of *P. xylostella*. To determine an effect on immune activation, expression level of ApoLpIII was manipulated by RNA interference (RNAi). Finally, any rescue effect of ApoLpIII on the larvae infected by the pathogens was tested by injecting a recombinant ApoLpIII protein into the hemocoel.

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doi:10.1016/j.jip.2010.09.023

2. Materials and methods

2.1. Insects 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. plutellae* for 12 h under the rearing condition. Then, the parasitized larvae were fed the cabbage leaves and incubated at the rearing environment. Wasp cocoons were collected and held in plastic cage until their emergence at the rearing environment. The emerged wasp adults were collected every day and allowed to mate each other for 24 h before use for parasitization. Adult wasps were fed on 10% sucrose solution.

2.2. RNA extraction and cDNA

Total RNA was extracted from nonparasitized and parasitized *P. xylostella*, in which an extraction was performed in different developmental stages using 200 eggs, four larvae (4th instar), four pupae or four adults with trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). For RNA extraction from different tissues, hemocyte, fat body, whole gut, and epidermis were isolated from the fourth instar larvae. For preparation of hemocyte sample, hemolymph was collected by cutting prologs of about 50 larvae and centrifuged at 400*g* for 5 min. For preparation of other tissue samples, 10 larvae were dissected to obtain individual tissues. First-strand cDNA was synthesized by RT premix (Intron Biotechnology, Seoul, Korea) containing oligo$(dT)_{15}$ primer in reaction volume of 20 μL.

2.3. RT-PCR

The synthesized single strand cDNA was used as a template for PCR amplification with specific primers for ApoLpIII gene (5′-ATG GTC CGC CGC GAG GCG CC-3′ and 5′-AGG CCT TCG GAG TTT CGG-3′) with 35 cycles under conditions of 1 min at 94 °C for denaturation, 40 s at 53 °C for annealing, and 1 min 30 s at 72 °C for extension. To identify the remaining 3′ORF region, a reverse degenerative primer (5′-TYA CTG YTT GSW RGC GGC STC-3′) was designed, in which the codes represent Y for C and T, S for G and C, W and for A and T, and R for A and G. A PCR product using the gene-specific forward primer and the reverse degenerative primer was sequenced.

With the same gene-specific primer set as described for RT-PCR, quantitative real-time PCR (qRT-PCR) was performed with 7500 Fast Real-Time PCR (Applied Biosystems, ABI 7500, Foster City, CA, USA) using SYBR® Advantage® qPCR Premix (Clontech, Mountain View, Canada). qRT-PCR was performed with 40 cycles of an array of reactions of 15 s at 95 °C, 30 s at 53 °C, and 33 s 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. Each treatment was independently replicated three times. Quantitative analysis of ApoLpIII expression was done using the comparative C_T ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001).

2.4. Production of recombinant ApoLpIII protein

For bacterial expression, open reading frame (ORF) of ApoLpIII with a partial signal peptide (see Fig. 1A) was cloned from cDNA with primers (5′-ATG GTC CGC CGC GAG GCG CC-3′ and 5′-CTG TTT GCT GGC GGC GTC GG-3′) using the following PCR conditions: 35 cycles of 94 °C for 1 min, 53 °C for 40 s, and 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min. PCR products (510 bp) were cloned into the expression vector pBAD-TOPO (Invitrogen) containing a polyhistidine tail and a V5 epitope tag at C-terminus according to the manufacturer's instruction. After transfection into electrocompetent *Escherichia coli* Top10 cells, colonies harboring in-frame expressed constructs were selected. Recombinant bacteria were cultured at 37 °C to 0.5–0.7 optical density at 600 nm. Over-expression of the target gene was induced by adding L-arabinose at a final concentration of 0.002%. Bacterial cells were harvested by centrifugation at 10,000*g* (Kontron Instruments, Milan, Italy), and then re-suspended in a double detergent buffer (50 mM Tris, pH 7.0, 30 mM NaCl, 0.1% SDS, 0.02% NaN_3, 0.01% Igepal, 0.005% phenylmethanesulfonyl fluoride, 0.005% protein inhibitor cocktail (Sigma–Aldrich Korea, Seoul, Korea). The bacterial cell suspension was lysed on ice using an ultra-sonicator (Bandelin Sonopuls, Berlin, Germany) at 95% intensity with 15 cycles of 1 min burst separated by 30 s gaps. The cell lysate was centrifuged at 24,000*g* and the supernatant was collected. The recombinant ApoLpIII produced by the transfected *E. coli* was purified by affinity chromatography. Supernatant was mixed with 10× binding buffer (100 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0) and the mixture was added to 1 mL of prewashed nickel–nitrilotriacetic acid resin (Qiagen, Valencia, CA, USA) and incubated for 2 h at 4 °C while gently rocking. The incubated slurry was packed into a polypropylene column (1.5 cm diameter, 14 cm height, Bio-Rad Korea Ltd., Seoul, Korea) and washed with 200 mL of a washing buffer (100 mM NaH_2PO_4, 0.5 M NaCl, pH 6.3). Protein was eluted in 5 mL fractions of an imidazole gradient (10, 100, 200, and 400 mM). The recombinant protein (approximately 20 kDa) was confirmed by SDS–PAGE and immunoblotting against the V5 tag.

2.5. RNA interference of ApoLpIII expression

Double stranded RNA (dsRNA) specific to ApoLpIII was prepared with gene-specific primers used in RT-PCR according to the Megascript RNAi kit instruction (Ambion, TX, USA). Briefly, PCR product was cloned into the pCR2.1 cloning vector (Invitrogen) and the insert orientations were analyzed by PCR using a combination of vector and insert gene-specific primers. Sense and antisense strands were *in vitro* transcribed with T7 RNA polymerase against two opposite insert clones. The sense and antisense strands were annealed at 37 °C for 4 h and then 75 °C for 5 min. Injection of dsRNA was carried out with Metafectene PRO transfection reagent (Biontex, Plannegg, Germany) in a 1:1 volume ratio and incubated for 20 min at 25 ± 1 °C. Fifty nanolitre of the dsRNA (50 ng) solution was injected into larval hemocoel. For the microinjection, glass capillary injection needles were made using a Micropipette puller PN-30 (Narishige, Tokyo, Japan). The DNA-transfection reagent complex was injected to each larva using an Ultra Micropump (Four) with SYS-microcontroller (World Precision Instruments, Sarasota, FL, USA). Microinjection was performed under a microscope (Olympus S730, Tokyo, Japan). Knockdown of the target gene expression was evaluated by RT-PCR at selected times up to 48 h post-injection.

2.6. Nodulation assay

To assess the effect of bacterial dose on hemocyte nodulation, different concentrations of *E. coli* (5×10^5 cells per larva) were injected to the third instar *P. xylostella* larvae. To prepare the bacterial suspension, *E. coli* were cultured in Luria–Bertani (LB) broth for 16 h at 37 °C and the bacterial cells were harvested and suspended in a phosphate buffered saline (PBS, 50 mM sodium phosphate, 0.7% NaCl, pH 7.4). The cells were killed by exposure to 95 °C for 30 min. After 8 h post-injection at 25 °C, the nodules counted were dark brown to black in color, located on the hemocoel, and are usually attached to gut or fat body. The melanized nodules within the hemocoel were observed using a stereomicroscope and nodule