Molecular Immunology 65 (2015) 123–132

Contents lists available at ScienceDirect

Molecular Immunology

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

Molecular characterization of a peptidoglycan recognition protein from the cotton bollworm, *Helicoverpa armigera* and its role in the prophenoloxidase activation pathway

Yu-Ping Li, Min Xiao, Li Li, Cai-Xia Song, Jia-Lin Wang, Xu-Sheng Liu *

Hubei Key Laboratory of Genetic Regulation and Integrative Biology, School of Life Sciences, Central China Normal University, 152 Luoyu Road, Wuhan 430079, China

ARTICLE INFO

Article history:
Received 19 October 2014
Received in revised form 14 January 2015
Accepted 15 January 2015
Available online 3 February 2015

Keywords:
Peptidoglycan recognition protein
Helicoverpa armigera
Pattern recognition receptor
Prophenoloxidase activation
Melanization

ABSTRACT

Peptidoglycan recognition proteins (PGRPs), which are evolutionarily conserved from invertebrates to vertebrates, function as pattern-recognition and effector molecules in innate immunity. In this study, a PGRP (HaPGRP-A) from the cotton bollworm, *Helicoverpa armigera* was identified and characterized. Sequence analysis indicated that HaPGRP-A is not an amidase-type PGRP. Increased levels of *HaPGRP-A* mRNA were observed in the fat body and hemocytes of *H. armigera* larvae following the injection of microbes or Sephadex beads. Analysis using purified recombinant HaPGRP-A showed that it (i) could bind and agglutinate Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*, (ii) enhanced prophenoloxidase activation in the presence of microbes, (iii) promoted the formation of melanotic nodules *in vivo*, and (iv) enhanced the melanization of Sephadex beads *in vivo*. RNA interference assays were performed to further confirm the function of *HaPGRP-A*. When the expression of *HaPGRP-A* in *H. armigera* larvae was inhibited by dsHaPGRP-A injection, the phenoloxidase activity in larval hemolymph was significantly decreased and RNAi-treated insects infected with bacteria showed higher bacterial growth in hemolymph compared with infected control larvae. These results indicated that HaPGRP-A acts as a pattern recognition receptor and binds to the invading organism to trigger the prophenoloxidase activation pathway of *H. armigera*, and the activated phenoloxidase may participate in the melanization process of nodulation and encapsulation responses.

1. Introduction

Insects lack an adaptive immune system and rely solely on innate immune reactions for their defense. In response to various infections, insects apply both cellular and humoral immune responses (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007). These responses include hemocyte-mediated phagocytosis, nodulation, and encapsulation (Lavine and Strand, 2002), synthesis of antimicrobial peptides (Lemaitre and Hoffmann, 2007), and activation of proteinase cascades such as the blood clotting system (Karlsson et al., 2004) and the prophenoloxidase (PPO) activation pathway (Kanost and Gorman, 2008).

The recognition of pathogens in insects is accomplished by a set of pattern recognition receptors (PRRs). Several families of proteins involved in the recognition of surface characteristics of microbes have been identified, including peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBPs), and β-1,3-glucan recognition proteins (βGRPs) (Lemaitre and Hoffmann, 2007). PGRPs are a type of PRRs that recognize peptidoglycan (PGN) which is an essential and unique cell wall component of virtually all bacteria, but not present in eukaryotic cells (Schleifer and Kandler, 1972; Rosenthal and Dziarski, 1994). PGRPs are immunity-related molecules conserved from insects to humans (Kang et al., 1998).

PGRPs are found in most animals, including insects, echinoderms, molluscs and vertebrates, but not in nematodes or plants (Dziarski and Gupta, 2006). These highly conserved molecules that play a role in innate immunity were first discovered in the silkworm, *Bombyx mori* as 20 kDa proteins with the ability to trigger the PPO cascade upon binding to PGN (Yoshida et al., 1996). The PGRPs of insects are categorized into two types: short (S), with a molecular weight of nearly 20 kDa, and long (L), of up to 90 kDa (Dziarski and Gupta, 2006). *Drosophila melanogaster* has 13 *PGRP* genes, which are transcribed and translated into at least 17 PGRP proteins through alternative splicing (Werner et al., 2000). Insect PGRPs play diverse roles in immune responses such as recognizing and binding bacterial PGN to activate Toll pathway (Michel et al., 2001; Park et al.,

* Corresponding author. Tel.: +86 27 67867704.
E-mail address: xsliu@mail.ccnu.edu.cn (X.-S. Liu).

http://dx.doi.org/10.1016/j.molimm.2015.01.016
0161-5890/

2007; Yu et al., 2010), IMD pathway (Choe et al., 2002; Kaneko et al., 2006), and PPO activation system (Yoshida et al., 1996; Takehana et al., 2002; Lee et al., 2004; Park et al., 2007; Sumathipala and Jiang, 2010; Sun et al., 2014; Chen et al., 2014); hydrolyzing bacterial PGN to exhibit bactericidal activity (Mellroth et al., 2003); and induction of phagocytosis (Rämet et al., 2002).

A recent transcriptome analysis of the cotton bollworm, *Helicoverpa armigera* hemocytes identified a *PGRP* gene (*HaPGRP-A*) (Yang et al., 2013). In the current study, we investigated the role of HaPGRP-A in the immune responses of *H. armigera* and found that HaPGRP-A plays an important role in the PPO activation pathway.

2. Materials and methods

2.1. Insect rearing

H. armigera larvae were reared on an artificial diet at 28 ± 1 °C under a 14-h light/10-h dark photoperiod, as described by Li et al. (2009).

2.2. Identification and sequence analysis of HaPGRP-A gene

The immune transcriptome of *H. armigera* hemocytes was sequenced as described previously (Yang et al., 2013). A cDNA encoding a protein with homology to PGRP was identified and termed *HaPGRP-A*. The physical and chemical properties of the deduced protein were analyzed using ExPASy (http://web.expasy.org/protparam/). Signal peptide and domain predictions were performed using Signal P (http://www.cbs.dtu.dk/services/SignalP/) and SMART (http://smart.embl-heidelberg.de/), respectively. ClustalW (Thompson et al., 1994; Larkin et al., 2007) and MEGA4 (Tamura et al., 2007) software were employed for multiple sequence alignments and construction of phylogenetic tree, respectively.

2.3. Tissue expression profiles

Quantitative real-time PCR (qRT-PCR) was used to compare the transcript abundance of *HaPGRP-A* in different tissues of *H. armigera*. Total RNA was isolated from the malpighian tube, epidermis, midgut, fat body and hemocytes of 6th instar larvae using Total RNA Purification System (Omega, Norcross, USA) combined with On-Column DNase (Qiagen, Hilden, Germany) digestion to remove any genomic DNA contamination. The first-strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, USA). The following primers were used for qRT-PCR analysis: *HaPGRP-A* forward 5′-ACAAGCATCACTAGACGCT-GTG-3′ and reverse 5′-ACAAGCATCACTAGACGCTGTG-3′; *rpS3* forward 5′-CGGCGTGGAGGTGCGCGTC-3′ and reverse 5′-CGATGGCGCACAGACCGCG-3′. The *rpS3* gene from *H. armigera* (*Ha-rpS3*, GenBank No.: KM064630) was used as the control. Each 15-μl reaction mixture contained 7.5 μl of 2× TransEco qPCR Mix (TransGen Biotech, Beijing, China), 2 μl of the cDNA template, and 0.3 μl of each primer (10 μM). The reactions were performed using CFX96™ Real-Time PCR System (Bio-Rad, Hercules, USA) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s. The relative expression level of *HaPGRP-A* was calculated using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008). Three replicates were performed for each template and three independent replicates were utilized for each tissue.

2.4. Expression profiles of HaPGRP-A post-bacterial or Sephadex bead stimulation

Escherichia coli and *Staphylococcus aureus* were harvested from cultures by centrifugation at 6000 rpm for 5 min, washed three times with phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 1.47 M KH_2PO_4, pH 7.4), and resuspended in PBS. After gentle mixing, the number of bacteria was counted at 400× magnification using a hemocytometer. Sephadex DEAE A-25 chromatography beads (Pharmacia, Uppsala, Sweden) were washed four times with PBS and resuspended in PBS. *H. armigera* larvae (6th instar, 1-day old) were anesthetized on ice and approximately 1×10^5 colony forming units (CFU) bacteria or 30 beads resuspended in 5 μl of PBS were injected into the hemocoel of each larva. Control larvae were injected with 5 μl of PBS. The fat body and hemocyte samples were collected 12 h post-injection and used to examine mRNA expression levels of *HaPGRP-A* by qRT-PCR as described in Section 2.3.

2.5. Recombinant expression and purification of HaPGRP-A

The sequence that codes for mature peptide of HaPGRP-A was amplified using a pair of gene-specific primers (forward 5′-*GGATCC*GACTGTGGCGTAGTCTCCAAA-3′ and reverse 5′-CGG*CTCGAG*TTAGTTCTTAATAGAGCTGAC-3′; the italicized sequences represent *Bam*HI and *Xho*I restriction sites, respectively). After cutting with *Bam*HI and *Xho*I, the DNA fragments were inserted into expression vector pET-32a (Novagen, Madison, WI) and then transformed into *E. coli* BL21 (DE3). The pET-32a vector lacking the insert DNA fragment was used as a negative control, which expressed 6× His-tagged thioredoxin (Trx) in the prokaryotic expression system. The transformants obtained were grown in LB medium at 37 °C under shaking conditions (200 rpm) until the optical density (OD) at 600 nm reached 0.6. IPTG was added at a final concentration of 0.8 mM, and the medium was shaken for another 8 h. Bacterial cells were harvested by centrifugation, resuspended in PBS, and sonicated on ice. The inclusion bodies were subjected to denaturation and renaturation according to Kuhelj et al. (1995). Protein refolding was accomplished by dialysis against 2 l of a solution containing 0.1 M Tris–HCl (pH 8.0), 5 mM EDTA, and 5 mM cysteine overnight at 4 °C. The solution was subsequently centrifuged and the supernatant was applied on High-Affinity Ni–NTA Resin (GenScript, Nanjing, China) to purify recombinant protein according to the manufacturer's instruction. The proteins were finally dissolved in PBS, and their concentrations were quantified by the Bradford method. The obtained proteins were stored at −80 °C before use.

2.6. Western blotting analysis

To further confirm the size of purified rHaPGRP-A, western blotting assay was performed according to the method described by Li et al. (2014). Briefly, purified rHaPGRP-A was first treated with loading buffer, electrophoresed on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane. Subsequently, the membrane was blocked with blocking buffer (3% BSA in PBS) for 1.5 h at room temperature and were incubated with mouse anti-His monoclonal antibody (diluted 1:2500 in blocking buffer) overnight at 4 °C and with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) for 2 h at room temperature. 4-Chloro-1-naphthol was used for color development.