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Identification and functional analysis of the peptidoglycan recognition protein LD gene in the mosquito, *Armigeres subalbatus*

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ABSTRACT

Peptidoglycan recognition proteins are important recognition proteins in many organisms ranging from echinoderms to humans. In an attempt to characterize all the PGRPs in the mosquito *Armigeres subalbatus*, two PGRP-LD isoforms, AsPGRP-LDa and AsPGRP-LDb, which are orthologs of the PGRP-LDs in several other insect species, were identified from this mosquito using homologous cloning. To date the functions of this PGRP gene have not yet been described in detail in other organisms with a known PGRP-LD gene. In the current study, we analyzed the sequences of these AsPGRP-LDs, their evolutionary relationships with their orthologs, their transcriptional expression in various developmental stages and different tissue samples, and their transcriptional responses to different bacterial stimuli. We then knocked down the expression of both AsPGRP-LDs by injection of double-stranded RNAs, and assessed the impact of AsPGRP-LD RNAi on mosquito survival after bacterial challenges and on the transcriptional expression of a number of antimicrobial peptides.

1. Introduction

The insect innate immune responses involve the activation of several humoral and cellular activities to battle pathogens. The activation of these immune responses depends on recognition of the pathogens as non-self by a number of recognition proteins. Peptidoglycan recognition proteins (PGRPs) are a group of proteins that specifically recognize peptidoglycan, a surface component of a variety of pathogens, such as bacteria.

A PGRP was first identified in the insect silkworm *Bombyx mori* (Linnaeus) and was shown to play an important role in this insect's immune response against bacteria (Yoshida et al., 1996). Other PGRP homologs were soon identified in a number of other insects including *Drosophila melanogaster* (Kang et al., 1998; Werner et al., 2000), and in a range of organisms from echinoderms to vertebrates.

D. melanogaster has been the most studied insect species, with 13 PGRP genes encoding 20 transcript variants and thus 20 PGRP proteins (Werner et al., 2000). Due to the recently available genome sequences for a number of organisms, PGRPs have been identified in more species, including four species of mosquitoes: *Anopheles gambiae* (Giles), *Aedes aegypti* (Linnaeus), *Culex quinquefasciatus* (Say), and *Armigeres subalbatus* (Coquillett). *A. gambiae* has seven PGRP genes that encode 19 transcripts, *A. aegypti* has seven PGRP genes encoding eight transcripts, and in *C. quinquefasciatus* there are four annotated PGRP genes that give rise to nine transcripts, and in *A. subalbatus*, four PGRP genes encoding 6 transcript variants have been identified and sequenced (Wang et al., 2012).

Insect PGRPs are divided into two classes, long form and short form, based on their sizes and protein structure (Werner et al., 2000). PGRPs have been proposed to be involved in insect immune systems through several different ways, including direct amidase enzyme activity (PGRP-SB1, PGRP-LB, and PGRP-SC1/2) (Mellroth et al., 2003; Mellroth and Steiner, 2006; Zaidman-Rémy et al., 2006), humoral responses, including the immune deficiency (IMD) pathway (PGRP-LC and PGRP-LE) (Choe et al., 2002; Gottar et al., 2002; Kaneko et al., 2006; Ramet et al., 2002; Takehana et al., 2004) and the Toll pathway (PGRP-SA, PGRP-SD) (Bischoff et al., 2004; Michel et al., 2001), and cellular responses including phagocytosis (PGRP-SC1) (Garver et al., 2006) and melanization (PGRP-LC, PGRP-LE) (Schmidt et al., 2008; Takehana et al., 2004; Tang et al., 2006). While functional studies have been performed for many of the PGRPs identified to date, some of the PGRPs have been relatively little studied, such as PGRP-LA, PGRP-LD and PGRP-LF.

In our previous studies (Wang et al., 2012; Wang and Beerntsen, 2013), we identified and cloned four PGRP genes (AsPGRP-S1, AsPGRP-LB, AsPGRP-LC, AsPGRP-LE) in the mosquito *A. subalbatus*

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encoding six transcripts, and assessed their functions in the immune responses against two types of bacteria, the gram negative bacteria *Escherichia coli*, and the gram positive bacteria *Micrococcus luteus*. In an attempt to identify additional PGRPs in this mosquito species, we performed homologous cloning using PGRP sequences from other mosquito species and identified a sequence that likely encoded another PGRP, PGRP-LD, based on sequence analysis. During full-length cloning of this sequence, we identified an additional sequence that was likely another isoform of the AsPGRP-LD gene. The functions of PGRP-LDs have not yet been studied in detail in any of the species to have known PGRP-LDs, except for a preliminary characterization in *D. melanogaster* (Werner et al., 2000) and a characterization of the *D. melanogaster* PMI protein, which is transcribed from the same gene as PGRP-LD but lacks a PGRP domain, has a completely different amino acid sequence, and regulates mitochondrial morphogenesis (Rival et al., 2011).

In the present study, we report the full-length sequence cloning, transcriptional profiling, and a functional analysis of the two isoforms of the PGRP-LD gene in the immune activities of *A. subalbatus*.

2. Materials and methods

2.1. Mosquito rearing

A. subalbatus were reared as previously described (Beerntsen et al., 1989). Briefly, larvae were raised in plastic pans with ddH_2O and standard TetraMin diet at 26.5 °C. Female pupae were mechanically separated from male pupae and ~70 female pupae were placed into 0.473-liter ice cream cartons covered with fine-mesh marquisette. Adult mosquitoes were maintained in an environmental chamber at 26.5 °C with a 16-h light and 8-h dark photoperiod, 75 ± 10% humidity, and fed 0.3 M sucrose.

2.2. Cloning of the AsPGRP-LD gene

Using the terms "peptidoglycan recognition protein LD", the National Center for Biotechnology Information (NCBI) database and Vectorbase (www.vectorbase.org) were searched for the presence of PGRP-LD sequences. The database search revealed the presence of PGRP-LD in three insect species: *D. melanogaster*, *A. aegypti*, and *A. gambiae*. *D. melanogaster* has four transcript isoforms: PGRP-LDa (GenBank accession number NM_001031942), -LDb (GenBank accession number NM_001031941), -LDc (GenBank accession number NM_001031940), and -LDd (GenBank accession number NM_001144421). However, -Lda, -LDb and -LDc give rise to the same protein product so these four transcripts only encode two different protein isoforms. *A. gambiae* has two cDNA/protein isoforms: PGRP-LDa (GenBank accession number XM_001688626) and -LDb (GenBank accession number XM_556195), and *A. aegypti* also has two likely cDNA/protein isoforms (GenBank accession numbers XM_001661739 and XM_001661740). The cDNA sequences of the PGRP-LDs from the three insect species were aligned and the alignment was used to design primers (Supp. Table S1) for homologous cloning. Cloned partial sequence was gel purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA), cloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced at the University of Missouri (MU) DNA Core facility. The partial sequence was subsequently used to design RACE primers for full-length cDNA cloning.

For the 5′ and 3′ RACE cloning, five 3-day old female *A. subalbatus* adults were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted according to the manufacturer's instructions. Total RNA was then treated with a Turbo™ RNase-free DNase kit (Ambion, Austin, TX) to remove genomic DNA contaminants. For the amplification of the 5′ end of the cDNAs, a gene specific primer (GSP, in Supp. Table S1) was used for the synthesis of first-strand cDNAs. Then the supplied abridged anchor primer (AAP, 5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′), the abridged universal amplification primer (AUAP, 5′-GGC CAC GCG TCG ACT AGT AC-3′) and the designed nested primers (5′ nested, in Supp. Table S1) were used for subsequent amplification. For the amplification of the 3′ end of the cDNAs, the first-strand cDNA was synthesized using the manufacturer-supplied adapter primer (AP, 5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3′). Then the supplied AUAP and the designed nested primers (3′ nested, in Supp. Table S1) were used in the subsequent amplification. The PCR products obtained from 5′ and 3′ RACEs were gel purified, cloned into the pGEM-T Easy vector, and sequenced as described above. Three clones were sequenced for each RACE product. The sequences acquired from both 5′ and 3′ RACEs were then combined together according to the overlapped sequences to obtain full-length cDNA sequences.

To verify these full-length AsPGRP cDNA sequences, forward and reverse primers (Supp. Table S1) were then designed according to the 5′ and 3′ ends of each AsPGRP-LD sequence, and PCR was performed using cDNA templates synthesized in the 3′ RACE experiment. PCR products were again gel purified, cloned into the pGEM-T Easy vector, and sequenced as described above. At least three clones were sequenced for each cDNA.

2.3. Protein sequence prediction and functional domain analysis

The putative open reading frames of the AsPGRP-LD transcripts were translated into protein sequences. The AsPGRP protein sequences were analyzed and functional domains predicted using the ExPASy (Expert Protein Analysis System) proteomics server (http://ca.expasy.org/) and the SMART (Simple Modular Architecture Research Tool) platform (http://smart.embl-heidelberg.de/). The AsPGRP protein sequences were analyzed for the presence of a signal peptide using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) and transmembrane domain using ProtComp Ver. 8.0 on SoftBerry (http://www.softberry.com).

2.4. Sequence alignments and evolutionary analysis

The predicted AsPGRP-LD protein sequences were aligned with their orthologs and other AsPGRPs using the Clustal Omega program (Sievers et al., 2011) from the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk). Multiple sequence alignments were viewed and edited using the Genedoc software (http://www.psc.edu/biomed/genedoc). For the phylogenetic analysis, AsPGRP-LD protein sequences were aligned with their orthologs in other insect species using Clustal Omega. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

2.5. Collection of mosquitoes and dissection of mosquito tissues

To examine the expression of AsPGRP-LDs in different developmental stages, *A. subalbatus* samples were collected at 1st, 2nd, 3rd, and 4th larval stages, early (day 1) and late (day 3) pupal stages, and day 0, day 1, day 3, day 5, day 7 and day 9 adult stages for analysis. Due to the difficulty in differentiating males and females in the early stages of *A. subalbatus*, larvae and pupae were collected as a random mixture of males and females, and only females were used for adults. Three independent cohorts of mosquitoes were collected as biological replicates.

To examine the expression profile of AsPGRP-LDs in different tissues/organs, day 4–5 old female adult *A. subalbatus* were cold