Peptidoglycan recognition protein-S5 functions as a negative regulator of the antimicrobial peptide pathway in the silkworm, *Bombyx mori*

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Abstract

Prophenoloxidase (proPO), immune deficiency (IMD), and Toll are the major signaling pathways leading to melanization and antimicrobial peptide production in insect hemolymph. Peptidoglycan recognition proteins (PGRPs) act as receptors and negative regulators in these pathways, and some PGRPs exhibit antimicrobial activity. Previously, we demonstrated that silkworm PGRP-S5 recognizes peptidoglycans (PGs) and triggers activation of the proPO pathway. It also acts as a bactericide, via its amidase activity (Chen et al., 2014). We generated a C177S site-mutated silkworm PGRP-S5 protein that lacked amidase activity but retained its PG-binding capacity. Functional studies showed that the mutation caused loss of its receptor function for activation of the proPO pathway, suggesting that processing of PG by PGRP-S5 is necessary for formation of the pathway initiation complex. Further, we found that PGRP-S5 negatively regulates antimicrobial peptide generation in an amidase-dependent manner, likely through the IMD pathway. Thus, silkworm PGRP-S5 acts as a sensor, a modulator, and an effector in the silkworm humoral immune system.

1. Introduction

Peptidoglycan (PG) recognition proteins (PGRPs) are characterized by a PGRP domain that is homologous to bacteriophage and bacterial type 2 amidase (Kang et al., 1998; Dziarski and Gupta, 2006), which hydrolyzes the bond between the *N*-acetylmuramyl group in the glycan strand and the L-alanine in the stem peptide of peptidoglycan. Some PGRPs possess amidase activity, but some PGRPs lost amidase activity during the course of their evolution (Mellroth et al., 2003; Dziarski and Gupta, 2006; Kurata, 2014). PGRPs act as sensors, regulators, and effectors in immune systems (Royet et al., 2011; Kurata, 2014). A recent study revealed that *Drosophila melanogaster* PGRP-LC, which primarily functions as a signal-transducing receptor in the immune deficiency (IMD) pathway (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002), is required for robust presynaptic homeostatic plasticity in the fly nervous system (Harris et al., 2015).

As signal-transducing receptors, PGRPs specifically recognize bacterial PGs and trigger host immune responses, such as antimicrobial peptide (AMP) generation and phenoloxidase (PO)-catalyzed melanization. Charroux et al. (2009) and Kurata (2014) summarized the scenario of detection of bacteria by *Drosophila* PGRPs. PGRP-SA,

SD, and Gram-negative binding protein-1 (GNBP-1) cooperate to detect extracellular Lys-type PG from bacterial cell walls and initiate activation of the Toll pathway; PGRP-LC or PGRP-LE detects extracellular meso-diaminopimelic acid (DAP)-type PG from the cell walls of most gram-negative bacteria and some gram-positive bacteria and activates the IMD pathway. Activation of the Toll and IMD pathways leads to transcription of AMP genes. PGRP-LE also mediates the proPO cascade upstream of the proPO-activating enzyme (Takehana et al., 2002; 2004). Intracellular PGRP-LE recognizes DAP-type PG and activates autophagy to eliminate intracellular bacteria such as *Listeria monocytogenes* (Charroux et al., 2009; Yano et al., 2008). PGRPs have also been found to act as receptors in immune pathways in organisms other than *Drosophila*. For instance, *Bombyx mori* PGRPs (Yoshida et al., 1996; Chen et al., 2014), *Tenebrio molitor* PGRP-SA (Park et al., 2006), *Holotrichia diomphalia* PGRP-1 (Lee et al., 2004), *Manduca sexta* PGRP-1 (Sumathipala and Jiang, 2010), and *Helicoverpa armigera* PGRP-A (Li et al., 2015) have been shown to participate in the proPO activation cascade. Mainly based on RNA interference (RNAi) results, it has been suggested that PGRPs from species other than *Drosophila* are responsible for transcriptional induction of AMP genes after bacterial challenge. RNAi against *PGRP-LC* reduced transcription levels of AMP *CEC1* and *DEF1* in *Anopheles gambiae* at the early stage of infection by *Staphylococcus aureus* (Meister et al., 2009). RNAi against *PGRP-LD* in the mosquito *Armigeres subalbatus* decreased transcription levels of certain AMPs (Wang and Beerntsen, 2014). In the mosquito

Aedes aegypti, PGRP-LC is a critical receptor that mediates antibacterial responses to both *Escherichia coli* and *Micrococcus luteus* (Wang and Beerntsen, 2015). In the red flour beetle *Tribolium castaneum*, PGRP-LA plays a major role as an IMD pathway sensor that responds to both gram-positive and gram-negative bacterial infection; PGRP-LC and PGRP-LE might also function as sensors in the IMD pathway to defend against gram-negative bacteria (Koyama et al., 2015). RNAi against *PGRP-SI* in the endoparasitoid wasp *Microplitis mediator* down-regulated defensin-1 at the transcriptional level (Wang et al., in press).

Some PGRPs function as immune regulators. In *Drosophila*, PGRP-LA is a positive regulator of the IMD pathway in barrier epithelia (Gendrin et al., 2013). PGRP-LF acts as a specific negative regulator of the IMD pathway on the basis of competition with PGRP-LCa for binding to PGRP-LCx (Maillet et al., 2008; Basbous et al., 2011). Several PGRPs with proven or predicted amidase activity play roles as negative regulators in immune systems. In *Drosophila*, PGRP-LB mainly functions as a scavenger to break down PG and dampen the IMD pathway and consequently controls the bacterial load in the gut; PGRP-SC2 degrades PG in the hemolymph and controls systemic immune responses (Zaidman-Rémy et al., 2006; Costechareyre et al., 2016). In tsetse flies, PGRP-LB scavenges PG and prevents IMD pathway activation, facilitating establishment of the symbiont *Wigglesworthia glossinidia* in the host (Wang and Aksoy, 2012). *Drosophila* PGRP-SC2 also functions as a longevity-promoting factor by preventing commensal dysbiosis, stem cell

hyperproliferation, and epithelial dysplasia through down-regulation of the IMD pathway (Guo et al., 2014). In the housefly *Musca domestica*, RNAi against *PGRP-SC* caused over-expression of AMP genes and delayed pupation (Gao et al., 2015).

Some PGRPs exhibit amidase-dependent bactericidal activity. *Drosophila* PGRP-SB1 shows enzymatic activity towards DAP-type polymeric PG (Zaidman-Rémy et al., 2011), and *in vitro* assays showed that it can kill *Bacillus megaterium* (Mellroth and Steiner, 2006). However, *in vivo* tests showed no protective effect after bacterial infection (Zaidman-Rémy et al., 2011). Our previous study of silkworm PGRP-S5 also demonstrated that it can break down a broad range of PGs and kill both gram-positive and gram-negative bacteria (Chen et al., 2014). Zebrafish PGRPs possess both amidase and bactericidal activities (Li et al., 2007).

We have already shown that silkworm PGRP-S5 plays roles both as effector that kill bacteria and as receptors for activation of the proPO pathway; we also hypothesize that it might play roles as an immune modulator (Chen et al., 2014). Here we present evidences supporting its function as a negative regulator to control AMP production through an amidase-dependent mechanism.

2. Material and methods

2.1 Structural modeling of PGRP-S5

The conserved domains and catalytic sites of PGRP-S5 (NP_001036858.1) were identified using SMART (http://smart.emblheidelberg.de/smart/set_mode.cgi) and BlastP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The signal peptide was predicted using SignalP4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). The domain illustration was plotted using IBS 1.0 (<http://ibs.biocuckoo.org/>). The tertiary structures of PGRP-S5 and PGRP-S5_C177S were predicted using Phyre 2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) according to the published protocol; “Intensive” was chosen as the “Modelling Mode” parameter (Kelley et al., 2015). Differences between 3D structural models of PGRP-S5 and PGRP-S5_C177S were determined using PyMOL (<http://www.pymol.org/>).

2.2 Production of recombinant PGRP-S5 mutant protein

The forward primer 5'-TATGACGACAGAAT**TCT**CCAGGA-3' and the reverse primer 5'-GCCTGATTGTGTCCTATTAG-3' were designed for generation of C177S mutant PGRP-S5. The “TGT” codon, which encodes a cysteine (C) residue in the wild-type PGRP-S5, was replaced by a “TCT” (shown in bold in the forward primer), which encodes a serine (S) residue. A previously constructed wild-type PGRP-S5 expression plasmid (Chen et al., 2014) was used as a template to produce the C177S mutant, according to the protocol provided with the MutanBEST Kit (TaKaRa, Dalian, China). The resulting C177S mutant plasmid was verified by sequencing. The C177S mutant vector and the pET-28a vector were digested with endonucleases NdeI and

XhoI, and then the C177S fragment was inserted into the pET-28a vector to construct the expression plasmid. The pET-28/PGRP-S5_C177S plasmid was transformed into *Escherichia coli* BL21 (DE3) cells. Expression and purification of PGRP-S5_C177S and wild-type PGRP-S5 were performed as previously described (Chen et al., 2014). The expression and purification of the recombinant His-tagged PGRP-S5_C177S were monitored by a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), visualized by staining with Coomassie Brilliant Blue R250 (Sigma-Aldrich, St. Louis, MO, USA) and immunoblotting with anti-His (1:10,000 dilution, CWbio, Beijing, China) as the primary antibody and goat-anti-rabbit IgG (1:20,000 dilution, Santa Cruz, Dallas, TX, USA) as the secondary antibody. The target band in SDS-PAGE was cut down and digested with trypsin, and then determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS, UltiMate3000, Thermo Scientific, Waltham, MA, USA; MicroTOF-QII, Bruker Daltonics, Bremen, Germany). The raw data after peaks analysis was used to search NCBI-*Bombyx mori* (28,777 sequences; 11,332,921 residues) with Mascot search engine (version 2.3.01). The search parameters were followed: Type of search: MS/MS Ion Search; Enzyme: Trypsin; Fixed modifications: Carbamidomethyl (C); Variable modifications: Gln->pyro-Glu (N-term Q), Oxidation (M); Mass values: Monoisotopic; Protein mass: Unrestricted; Peptide mass tolerance: ± 15 ppm; Fragment mass tolerance: ± 20 mmu; Max missed cleavages: 1; Instrument type: Default; Number of queries: 5,987.

2.3 Peptidoglycan binding and bacteria agglutination assays

Insoluble PGs from *Bacillus megaterium*, *Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus* were prepared as described previously (Sumathipala and Jiang, 2010). Ten microliters of PG (10 µg/µl) was incubated with 20 µl of recombinant PGRP-S5_C177S (200 µg/ml) for 2 h at room temperature. After centrifugation at 4°C, 10,000 × g for 15 min, the supernatant was transferred to a new Eppendorf tube for use as an unbound sample, and the pellet was washed sequentially with PBS containing 50 mM, 150 mM, and 500 mM of NaCl. After centrifugation at 4°C, 10,000 × g for 15 min, the supernatant was collected for use as the wash sample, and the pellet was resuspended with 30 µl of PBS for use as the bound sample (Chen et al., 2014). The samples were boiled in SDS-loading buffer for 3 min and then separated on a 12%SDS-PAGEgel followed by immunoblot analysis as described above. Next, we used green fluorescent protein (GFP)-expressing *Pseudomonas aeruginosa* (PAO1) and *E. coli* (TOP10) to examine whether PGRP-S5_C177S induces bacterial agglutination. PAO1 constitutively expressing GFP (a gift from Dr. Xihui Shen) was cultured in Luria-Bertani (LB) medium overnight at 37°C with shaking at 200 rpm. Competent cells of *E. coli* (Top10) were transformed with the pGLO plasmid containing an ampicillin resistance gene and a GFP reporter gene that can be induced by L-arabinose. The transformed *E. coli* was cultured to the logarithmic phase in LB broth containing 100 µg/ml ampicillin at 37°C with shaking at 200 rpm, and then induced with L-arabinose at a final concentration of 1 mg/ml for

12 h. The cultured bacterial cells were collected and washed three times and resuspended with PBS at a concentration of 2×10^8 cells/ml. Bacterial suspension (20 μ l) and 20 μ l of PGRP-S5_C177S (200 μ g/ml) or BSA (200 μ g/ml) were incubated in a 96-well plate at room temperature for 2 h. Images were captured under an inverted fluorescence microscope EVOS FL (Thermo Scientific, Waltham, MA, USA).

2.4 Amidase activity assay

Insoluble PGs from *M. luteus* and *E. coli* (InvivoGen, San Diego, CA, USA) (1 mg/ml) were used as substrates for amidase activity determination. PG (10 μ l) and 10 μ l of PGRP-S5 or PGRP-S5_C177S (200 μ g/ml) in PBS were incubated at room temperature on a Tube Tumbler. Lysozyme (10 μ l, 200 μ g/ml) was used as a positive control, and 1 μ l of Zn^{2+} (1 mM) was added to analyze the effect of Zn^{2+} on PGRP-S5 amidase activity. The total reaction volume was supplemented to 60 μ l with PBS. The decay of light scattering at 405 nm was measured after 0 h, 1 h, 2 h, 4 h, and 8 h on a Biophotometer (Eppendorf, Hamburg, Germany).

2.5 Antibacterial activity assay

M. luteus and *E. coli* were cultured to an $\text{OD}_{600\text{nm}}$ of approximately 0.6 in LB medium at 37°C with shaking at 220 rpm. Bacterial cultures (200 μ l) were spun down and resuspended in 20 ml of PBS. Diluted bacterial suspension (10 μ l) was incubated with 25 μ l of 40 μ M ZnCl_2 or 200 μ g/ml PGRP-S5_C177S in a 1.5-ml tube at room

temperature on a Tube Tumbler. After 5 h, the mixtures was supplemented with 1 ml of LB medium and cultured overnight. Finally, the absorbance of the cultures at 600 nm was measured.

2.6 Phenoloxidase activity assay

Eggs of nondiapausing silkworm strain *Nistari* were hatched, and then the larvae were reared to the 5th instar on fresh mulberry leaves at 27°C ± 1°C, with a photoperiod of 14L:10D, and 65% ± 10% relative humidity. The hemolymph was collected from day 3, 5th instar larvae after sterilization with 70% ethanol. Hemocytes were removed from the hemolymph by centrifugation at 16,000 × *g* for 30 sec (Clark and Strand, 2013). Four reactions were prepared: (1) 15 µl of PBS and 5 µl of plasma; (2) 2 µl of PGRP-S5 or PGRP-S5_C177S (200 µg/ml), 5 µl of plasma, and 13 µl of PBS; (3) 5 µl of plasma and 13 µl of PBS; (4) 2 µl of PGRP-S5 or PGRP-S5_C177S (200 µg/mL), 5 µl of plasma and 11 µl of PBS. After incubation on ice for 5 min, reaction mixtures (3) and (4) were mixed with 2 µl of PG (1 mg/ml) from *M. luteus*, *S. aureus*, *B. subtilis*, and *B. megaterium*. The mixtures were centrifuged at 1000 × *g* for 1 min after incubation on ice for 2 min. The supernatants (5 µl) were added to the wells of a microplate containing 100 µl of 2 mM dopamine for the PO activity assay, and their absorbance at 490 nm was read on a Tecan Infinite 200 Pro microplate reader (Tecan, Männedorf, Switzerland) (Jiang et al., 2003).

2.7 Detection of antimicrobial peptides by SDS-PAGE

Day 3, 5th instar silkworm larvae in the control group were injected with 4 µg of PGRP-S5 or PGRP-S5_C177S in 50 µl PBS; larvae in the treatment groups were injected with 1×10^4 cells of *P. aeruginosa* in 50 µl PBS, or 1×10^4 cells of *P. aeruginosa* and 4 µg of PGRP-S5 or PGRP-S5_C177S. Each group included at least 5 larvae. Six hours after the injections, the silkworms from each group were dissected to collect hemolymph and fat bodies. The fat bodies were stored in Tripure reagent (Roche, Basel, Switzerland) at -80°C. The hemolymph was centrifuged to remove hemocytes and the plasma was boiled at 95°C for 5 min, and after centrifugation at $15,000 \times g$ for 10 min at 4°C the supernatant was collected. The collected samples were then separated by electrophoresis on a 16% Tricine-SDS-PAGE, and visualized by staining with Coomassie Brilliant Blue R250. LC-MS/MS was used to identify protein bands showing apparent change. The search parameters and database were as the same in Section 2.2.

2.8 Expression analysis of antimicrobial peptide genes by quantitative real-time PCR

The fat body samples collected in Section 2.7 were homogenized with a pellet pestle (Kontes, Rockwood, TN, USA), and total RNA was extracted using Tripure reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. The total RNA was purified with an RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). One microgram of purified RNA was used to synthesize first-strand cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland)

following the manufacturer's instructions. The diluted cDNA (50 ng/μl) samples were used as templates for quantitative real-time PCR (qRT-PCR). qRT-PCR was used to analyze the changes of AMP mRNA levels in the fat bodies of silkworm larvae. The reactions were performed on a Rotor-Gene Q (Qiagen, Hilden, Germany) under the following conditions: 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 53°C for 15s, and extension at 72°C for 20s. A melting curve (60-95°C) was determined to confirm amplification of the desired PCR products. The housekeeping gene *IF4A* (DQ443290) was selected as a reference gene. The primers used in the qRT-PCR are given in Table 1.

3. Results

3.1 Prediction of activity sites and structural analysis of silkworm PGRP-S5

Tanaka et al. (2008) predicted that PGRP-S5 possesses amidase activity, and we confirmed this experimentally (Chen et al., 2014). To determine whether PGRP-S5 serves as a receptor or an effector (Chen et al., 2014), we attempted to produce a PGRP-S5 mutant without amidase activity. First, we analyzed the structure of PGRP-S5 and predicted the five amino acid residues, H60, Y95, H169, T175, and C177, required for PGRP-S5 amidase activity (Fig. 1A). We changed the C177 (purple circle in Fig. 1A) residue encoded by "TGT" to a serine residue, encoded by "TCT." To examine the effect of the C177S mutation on PGRP-S5 structure, the tertiary structures of PGRP-S5 and PGRP-S5_C177S were predicted using Phyre 2.

The five catalytic activity sites (H60, Y95, H169, T175, and C177) shown in Fig. 1A were located in the L-type groove, and PGRP-S5 and PGRP-S5_C177 showed no differences in any of the five sites, except C177 (Fig. 1B).

3.2 Expression, purification, and identification of recombinant PGRP-S5_C177S

To compare the function of C177S mutant and wild-type PGRP-S5, we expressed recombinant proteins in *E. coli*. Recombinant PGRP-S5_C177S, with a 6×His tag at the carboxyl terminus, was abundantly expressed in *E. coli* BL21 (DE3) cells after induction with 0.5 mM isopropyl β -D-1-thiogalactopyranoside; it was then purified with high-affinity Ni-NTA resin (Fig. 2A and 2B). The wild-type PGRP-S5 was expressed and purified as described previously (Chen et al., 2014). The C177S mutation was confirmed by mass spectrometry analysis. We compared the mass of peptides from wild-type and mutant PGRP-S5 containing residue 177 and found that the mass difference was equal to the mass difference between cysteine and serine (Fig. 2C). This confirmed successful change of cysteine to serine.

3.3 PGRP-S5_C177S retains the capacity to bind to PGs and may cause agglutination of bacteria

Insoluble DAP-type PGs from *B. megaterium* and *B. subtilis*, and Lys-type PGs from *M. luteus* and *S. aureus* were used for the PGRP-S5_C177S binding test. PGRP-S5_C177S showed strong binding to all of the above PGs (Fig. 3A), whereas

wild-type PGRP-S5 showed weak binding to PG from *S. aureus* (Chen et al., 2014). The difference between the mutant and wild-type PGRP-S5 in binding to PG from *S. aureus* is probably due to a smaller space occupation of serine than cysteine, which renders a closer contact with PG. We then tested whether PGRP-S5_C177S can agglutinate bacteria, and the results showed that the mutant protein caused agglutination of *E. coli* and *P. aeruginosa* cells (Fig. 3B).

3.4 C177 is required for PGRP-S5 amidase activity

C177 was one of the five predicted amidase activity sites, as shown in Fig. 1A. To verify our prediction, we used wild-type PGRP-S5 and lysozyme as positive controls to examine whether C177S has amidase activity (Fig. 4A and B). The wild-type PGRP-S5 showed equivalent activity to lysozyme when PGs from *E. coli* and *M. luteus* were used as substrates; PGRP-S5_C177S showed no amidase activity. These results suggest that C177 is a key site for PGRP-S5 amidase activity.

3.5 Amidase activity is essential for the antibacterial activity of PGRP-S5

The mechanism by which some PGRPs kill bacteria remains poorly understood. *Drosophila* PGRP-SC1b, which has amidase activity, has the equivalent activity of egg lysozyme towards PGs, but it does not exhibit antibacterial activity (Mellroth et al., 2003). *Drosophila* PGRP-SB1 with amidase activity can kill *Bacillus megaterium* (Mellroth and Steiner, 2006). Although mammalian PGRPs, such as PGLYRP-1,

PGLYRP-3, and PGLYRP-4, do not have amidase activity, they can kill bacteria by inducing suicide through the protein-sensing two-component system (Kashyap et al., 2011). Therefore, the correlation between the antibacterial activity and the amidase activity of PGRPs remains elusive. We tested the antibacterial activity of PGRP-S5_C177S and found that the mutation of cysteine to serine results in loss of antibacterial activity of PGRP-S5 against *M. luteus* (Fig. 5A) and *E. coli* (Fig. 5B). Our data indicate that amidase activity is a prerequisite for the antibacterial activity of PGRP-S5.

3.6 Amidase activity of PGRP-S5 is required for activation of the silkworm proPO pathway

Our previous study showed that binding of silkworm PGRP-S5 to bacterial PGs leads to activation of the proPO pathway (Chen et al., 2014). Unexpectedly, we found that PGRP-S5_C177S was unable to trigger proPO activation (Fig. 6), though it is able to bind to bacterial PGs (Fig. 3A). Therefore, it seems that the amidase activity of silkworm PGRP-S5 is also necessary for its involvement in activation of the proPO pathway.

3.7 PGRP-S5 down-regulates AMP production after bacterial infection in an amidase-dependent manner

Some PGRPs with amidase activity play roles as suppressors that down-regulate

expression of genes that encode antimicrobial peptides (Mellroth et al., 2003; Bischoff et al., 2006; Zaidman-Rémy et al., 2006; Paredes et al., 2011). Given that silkworm PGRP-S5 has amidase activity, we probed its effect on AMP production in silkworm larvae infected by bacteria. Two bands were detected in the plasma after *P. aeruginosa* infection (Fig. 7A). Mass spectrometry analysis identified these two bands as gloverin and cecropin B (data not shown). Under uninfected conditions, injection of PGRP-S5_C177S resulted in generation of gloverin and cecropin B, whereas injection of wild-type PGRP-S5 did not. Under infected conditions, injection of PGRP-S5_C177S caused greater production of gloverin and cecropin B than wild-type PGRP-S5 did. qRT-PCR analysis confirmed the gel-based results (Fig. 7B). We next examined transcription levels of other AMP genes in uninfected samples, and found that injection of the mutated PGRP-S5 induced a remarkable increase in the expression of these genes, whereas injection of the wild-type PGRP-S5 did not (Fig. 7C). Analysis of the transcription levels of *IKK γ* and *Dredd*, two key genes in the IMD signaling pathway, revealed a similar patterns of expression to the AMP genes (Fig. 7D), implying that PGRP-S5 down-regulates AMP expression through the IMD pathway.

4. Discussion

PGRPs have been identified in insects, mollusks, echinoderms, and vertebrates, but not in plants or nematodes. They interact with specific types of PGs to stimulate

or modulate immune responses. Some PGRPs have direct antibacterial activity (Dziarski and Gupta, 2006; Kurata, 2014). Previously, we found that silkworm PGRP-S5 functions as a receptor for activation of the proPO cascade and as a bactericidal protein (Chen et al., 2014). In this study, using a site-mutated protein we revealed that the activity of PGRP-S5 as a receptor and an effector rely on its amidase activity. More importantly, we demonstrated that it also plays a role as a negative regulator to limit AMP generation after systemic infection.

The PO pathway is critical for insects' defense against microbial infections (Cerenius et al., 2008; Binggeli et al., 2014). The activation of this pathway is initiated by specific interaction of pathogen-associated molecular patterns (PAMPs) and host pattern recognition proteins (PRPs) (Cerenius et al., 2008). PGRPs are a group of PRPs that specifically recognize bacterial PGs. Yoshida et al. (1996) first identified a PGRP without lysozyme activity, which is involved in the initiation of PO pathway activation, in the silkworm hemolymph. Binding assays also suggested that this PGRP recognizes repeating units of the glycan portion of PG. PGRP-SA binds to lysozyme-mediated partially digested lysine-type PG and recruits GGBP-1 and a modular serine protease to form the initiating complex, leading to activation of the PO pathway (Park et al., 2007). We herein demonstrate that loss of the amidase activity of silkworm PGRP-S5 resulted in failure of PO pathway activation. This result indicates that processing of PG is necessary for sensing of bacteria by silkworm PGRP-S5. To the best of our knowledge, this is the only PGRP with amidase activity that

participates in activation PO pathway identified to date. However, identification of the events that follow digestion of PG by PGRP-S5 and of the other molecules involved awaits further investigation.

In addition to PO-catalyzed melanization, production and release of AMPs into the hemolymph is another component of the humoral immune system. In *Drosophila*, Lys-type PGs from bacteria, β -1,3-glucan from yeast and, virulence-associated proteases released from other pathogens induce transcription of a certain type of AMP genes (e.g., Drosomycin) through the Toll pathway; DAP-type PGs from most gram-negative bacteria and some gram-positive bacteria induces transcription of other AMP genes (e.g., Diptericin) through the IMD pathway (Ganesan et al., 2011). In *B. mori*, AMPs seem to show a broad spectrum of antimicrobial activity, and most are induced by both gram-positive and gram-negative bacteria (Yang et al., 2011). Evidence has been reported implying that gram-positive and gram-negative bacterial infections activate the Toll and IMD pathways respectively in the silkworm, but evidence has also been reported that suggests cross-talk between these two pathways (Tanaka et al., 2008). Under uninfected condition, AMP genes are strongly induced upon injection of PGRP-S5_C177S into the silkworm (Fig. 7C). This observation implies that PGRP-S5 maintains immune homeostasis and loss of its function results in over-activated immune responses in the silkworm. Infection with the gram-negative bacterium *P. aeruginosa* strongly induces AMP gene expression, and PGRP-S5 negatively regulates induction in the silkworm (Fig. 7B). The suppression of *IKK γ* and

Dredd induction observed (Fig. 7D) confirms that these AMP genes are activated through the IMD pathway, although we cannot exclude the possibility that they may also be activated through other pathways. Costechareyre et al. (2016) recently demonstrated that *Drosophila* PGRP-SC2, along with PGRP-SC1, is required for full activation of the Toll pathway by gram-positive bacteria, in addition to its major role as a negative regulator of the IMD pathway in the fat bodies. It appears worthwhile to investigate the effect of silkworm PGRP-S5 on Toll pathway activation in future.

Thus, we have shown that silkworm PGRP-S5 plays multiple roles, i.e., as a receptor for activation of the proPO pathway, as a negative regulator for the IMD pathway, and as a bactericide. Investigating the benefits to the silkworm when PGRP-S5 plays different roles in the immune system may yield valuable insights. PO-mediated melanization occurs within minutes to hours of detection of invaders, whereas production of AMPs in the fat body and their release to the hemolymph take hours to days (Imler, 2014). Rapid initiation of the proPO pathway by PGRP-S5 ensures killing of the invading bacteria at the early stages of infection. Meanwhile, down-regulation of the IMD pathway by PGRP-S5 in the late stages controls over-activation of immune responses that could have detrimental effects on the silkworm. Analysis of the induction, intensity, and duration of different immune responses in a given host after infection with a specific pathogen, along with study of host survival and pathogen viability in the host, will improve our understanding of the interaction and regulation of immune responses and pathways.

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References

Basbous, N., Coste, F., Leone, P., Vincentelli, R., Royet, J., Kellenberger, C., Roussel, A., 2011. The *Drosophila* peptidoglycan-recognition protein LF interacts with peptidoglycan-recognition protein LC to downregulate the Imd pathway. *EMBO Rep* 12, 327-333.

Binggeli, O., Neyen, C., Poidevin, M., Lemaitre, B., 2014. Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*. *PLoS Pathog* 10, e1004067.

Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., Hoffmann, J.A., Royet, J., 2006. Downregulation of the *Drosophila* immune response by peptidoglycan-recognition proteins SC1 and SC2. *PLoS Pathog* 2, e14.

Cerenius, L., Lee, B.L., Soderhall, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in immunology* 29, 263-271.

Chen, K., Liu, C., He, Y., Jiang, H., Lu, Z., 2014. A short-type peptidoglycan recognition protein from the silkworm: expression, characterization and involvement in the prophenoloxidase activation pathway. *Developmental and comparative immunology* 45, 1-9.

Charroux, B., Rival, T., Narbonne-Reveau, K., Royet, J., 2009. Bacterial detection by *Drosophila* peptidoglycan recognition proteins. *Microbes Infect.*, 11, 631-636.

Choe, K.M., Werner, T., Stöven, S., Hultmark, D., Anderson, K.V., 2002. Requirement for a Peptidoglycan Recognition Protein (PGRP) in Relish Activation and Antibacterial Immune Responses in *Drosophila*. *Science* 296, 359-362.

Clark, K.D., Strand, M.R., 2013. Hemolymph melanization in the silkworm *Bombyx mori* involves formation of a high molecular mass complex that metabolizes tyrosine. *The Journal of biological chemistry* 288, 14476-14487.

Costechareyre, D., Capo, F., Fabre, A., Chaduli, D., Kellenberger, C., Roussel, A., Charroux, B., Royet, J., 2016. Tissue-Specific Regulation of *Drosophila* NF- κ B Pathway Activation by Peptidoglycan Recognition Protein SC. *Journal of innate immunity* 8, 67-80.

Dziarski, R., Gupta, D., 2006. The peptidoglycan recognition proteins (PGRPs). *Genome Biol* 7, 232.

Ganesan, S., Aggarwal, K., Paquette, N., Silverman, N., 2011. NF- κ B/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. *Current topics in microbiology and immunology* 349, 25-60.

Gao, Y., Tang, T., Gu, J., Sun, L., Gao, X., Ma, X., Wang, X., Liu, F., Wang, J., 2015. Downregulation of the *Musca domestica* peptidoglycan recognition protein SC (PGRP-SC) leads to overexpression of antimicrobial peptides and tardy pupation. *Mol Immunol* 67, 465-474.

Gendrin, M., Zaidman-Remy, A., Broderick, N.A., Paredes, J., Poidevin, M., Roussel, A., Lemaitre, B., 2013. Functional analysis of PGRP-LA in *Drosophila* immunity. *PLoS One* 8, e69742.

Guo, L., Karpac, J., Tran, S.L., Jasper, H., 2014. PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. *Cell* 156, 109-122.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., Royet, J., 2002. The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature* 416, 640-644.

Harris, N., Braiser, D.J., Dickman, D.K., Fetter, R.D., Tong, A., Davis, G.W., 2015. The Innate Immune Receptor PGRP-LC Controls Presynaptic Homeostatic Plasticity. *Neuron* 88, 1157-1164.

Imler, J.L., 2014. Overview of *Drosophila* immunity: a historical perspective. *Developmental and comparative immunology* 42, 3-15.

Jiang, H., Wang, Y., Yu, X.Q., Kanost, M.R., 2003. Prophenoloxidase-activating proteinase-2 from hemolymph of *Manduca sexta*. A bacteria-inducible serine proteinase containing two clip domains. *The Journal of biological chemistry* 278, 3552-3561.

Kang, D., Liu, G., Lundstrom, A., Gelius, E., Steiner, H., 1998. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc Natl*

Acad Sci U S A 95, 10078-10082.

Kashyap, D.R., Wang, M., Liu, L.H., Boons, G.J., Gupta, D., Dziarski, R., 2011. Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems. *Nature medicine* 17, 676-683.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10, 845-858.

Koyama, H., Kato, D., Minakuchi, C., Tanaka, T., Yokoi, K., Miura, K., 2015. Peptidoglycan recognition protein genes and their roles in the innate immune pathways of the red flour beetle, *Tribolium castaneum*. *J Invertebr Pathol* 132, 86-100.

Kurata, S., 2014. Peptidoglycan recognition proteins in *Drosophila* immunity. *Developmental and comparative immunology* 42, 36-41.

Lee, M.H., Osaki, T., Lee, J.Y., Baek, M.J., Zhang, R., Park, J.W., Kawabata, S., Soderhall, K., Lee, B.L., 2004. Peptidoglycan recognition proteins involved in 1,3-beta-D-glucan-dependent prophenoloxidase activation system of insect. *The Journal of biological chemistry* 279, 3218-3227.

Li, X., Wang, S., Qi, J., Echtenkamp, S.F., Chatterjee, R., Wang, M., Boons, G.J., Dziarski, R., Gupta, D., 2007. Zebrafish peptidoglycan recognition proteins are bactericidal amidases essential for defense against bacterial infections. *Immunity* 27, 518-529.

Li, Y.P., Xiao, M., Li, L., Song, C.X., Wang, J.L., Liu, X.S., 2015. Molecular characterization of a peptidoglycan recognition protein from the cotton bollworm, *Helicoverpa armigera* and its role in the prophenoloxidase activation pathway. *Mol Immunol* 65, 123-132.

Maillet, F., Bischoff, V., Vignal, C., Hoffmann, J., Royet, J., 2008. The *Drosophila* peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. *Cell host & microbe* 3, 293-303.

Meister, S., Agianian, B., Turlure, F., Relogio, A., Morlais, I., Kafatos, F.C., Christophides, G.K., 2009. *Anopheles gambiae* PGRP-LC-mediated defense against bacteria modulates infections with malaria parasites. *PLoS Pathog* 5, e1000542.

Mellroth, P., Karlsson, J., Steiner, H., 2003. A scavenger function for a *Drosophila* peptidoglycan recognition protein. *The Journal of biological chemistry* 278, 7059-7064.

Mellroth, P., Steiner, H., 2006. PGRP-SB1: an N-acetylmuramoyl L-alanine amidase with antibacterial activity. *Biochemical and biophysical research communications* 350, 994-999.

Paredes, J.C., Welchman, D.P., Poidevin, M., Lemaitre, B., 2011. Negative regulation by amidase PGRPs shapes the *Drosophila* antibacterial response and protects the fly from innocuous infection. *Immunity* 35, 770-779.

Park, J.W., Je, B.R., Piao, S., Inamura, S., Fujimoto, Y., Fukase, K., Kusumoto, S., Soderhall, K., Ha, N.C., Lee, B.L., 2006. A synthetic peptidoglycan fragment as a competitive inhibitor of the melanization cascade. *The Journal of biological chemistry* 281, 7747-7755.

Park, J.W., Kim, C.H., Kim, J.H., Je, B.R., Roh, K.B., Kim, S.J., Lee, H.H., Ryu, J.H., Lim, J.H., Oh, B.H., Lee, W.J., Ha, N.C., Lee, B.L., 2007. Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. *Proc Natl Acad Sci U S A* 104, 6602-6607.

Ramet, M., Manfrulli, P., Pearson, A., Mathey-Prevot, B., Ezekowitz, R.A.B., 2002. Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. *Nature* 416, 644-648.

Royet, J., Gupta, D., Dziarski, R., 2011. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* 11, 837-851.

Sumathipala, N., Jiang, H., 2010. Involvement of *Manduca sexta* peptidoglycan recognition protein-1 in the recognition of bacteria and activation of prophenoloxidase system. *Insect Biochem Mol Biol* 40, 487-495.

Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki, T., Kurata, S., 2002. Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. *Proc Natl Acad Sci USA* 99, 13705-13710.

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., Kurata, S., 2004. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. *EMBO J* 23, 4690-4700.

Tanaka, H., Ishibashi, J., Fujita, K., Nakajima, Y., Sagisaka, A., Tomimoto, K., Suzuki, N., Yoshiyama, M., Kaneko, Y., Iwasaki, T., Sunagawa, T., Yamaji, K., Asaoka, A., Mita, K., Yamakawa, M., 2008. A genome-wide analysis of genes and gene families

involved in innate immunity of *Bombyx mori*. *Insect Biochem Mol Biol* 38, 1087-1110.

Wang, J., Aksoy, S., 2012. PGRP-LB is a maternally transmitted immune milk protein that influences symbiosis and parasitism in tsetse's offspring. *Proc Natl Acad Sci US A* 109, 10552-10557.

Wang, R.J., Lin, Z., Jiang, H., Li, J., Saha, T.T., Lu, Z., Lu, Z., Zou, Z. Comparative analysis of peptidoglycan recognition proteins in endoparasitoid wasp *Microplitis mediator*. *Insect Science*, In press.

Wang, S., Beerntsen, B.T., 2015. Functional implications of the peptidoglycan recognition proteins in the immunity of the yellow fever mosquito, *Aedes aegypti*. *Insect molecular biology* 24, 293-310.

Yang, W., Cheng, T., Ye, M., Deng, X., Yi, H., Huang, Y., Tan, X., Han, D., Wang, B., Xiang, Z., Cao, Y., Xia, Q., 2011. Functional divergence among silkworm antimicrobial peptide paralogs by the activities of recombinant proteins and the induced expression profiles. *PLoS One* 6, e18109.

Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., Takada, H., Goldman, W., Fukase, K., Silverman, N., Yoshimori, T., Kurata, S., 2008. Autophagic control of listeria through intracellular innate immune recognition in *Drosophila*. *Nat Immunol* 9, 908-916.

Yoshida, H., Kinoshita, K., Ashida, M., 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *The Journal of biological chemistry* 271, 13854-13860.

Zaidman-Remy, A., Herve, M., Poidevin, M., Pili-Floury, S., Kim, M.S., Blanot, D., Oh, B.H., Ueda, R., Mengin-Lecreulx, D., Lemaitre, B., 2006. The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. *Immunity* 24, 463-473.

Zaidman-Remy, A., Poidevin, M., Herve, M., Welchman, D.P., Paredes, J.C., Fahlander, C., Steiner, H., Mengin-Lecreulx, D., Lemaitre, B., 2011. *Drosophila* immunity: analysis of PGRP-SB1 expression, enzymatic activity and function. *PLoS One* 6, e17231.

Figure legends

Fig. 1. (A) *Bombyx mori* PGRP-S5 domain architecture. The signal peptide (SP, residues 1-17) is shown in green; the peptidoglycan recognition protein (PGRP) domain (53-180) is shown in red; the amino acid residues required for amidase activity (H60, Y95, H169, T175, and C177) are circled. **(B)** Comparison of 3D structural models of *B. mori* wild-type PGRP-S5 and its C177S mutant. The wild type is shown in yellow and the C177S mutant is shown in blue. The residues H60, Y95, H169, T175, and C177 of wild-type PGRP-S5 are shown in green while those of the mutant are in magenta. An enlarged image of the C177 site is shown in the upper left corner. The chemical structures of cysteine and serine are shown in the lower right corner. Template: *Drosophila melanogaster* PGRP-LB (PDB entry: 1OHT), Identity: 48% for silkworm PGRP-S5.

Fig. 2. Purification and identification of recombinant C177S site-mutated silkworm peptidoglycan recognition protein (PGRP)-S5. **(A)** Gel stained with Coomassie Brilliant Blue. **(B)** Western blot probed with anti-His antibody. M, protein size markers with their molecular masses (kDa) shown on the left. Samples in (A) and (B): Lane 1, *Escherichia coli* cell lysate supernatant without induction; lane 2, cell lysate supernatant with isopropyl β -D-1-thiogalactopyranoside induction; lane 3, purified PGRP-S5_C177S. **(C)** LC-MS/MS results of the fragments containing C177

and S177 from the wild-type and C177S mutant PGRPs, respectively. Mr (expt), experimental molecular weight; Mr (calc), calculated molecular weight.

Fig. 3. (A) Binding of silkworm peptidoglycan recognition protein (PGRP)-S5_C177S to peptidoglycans (PGs). PGs used in the assays were from *Bacillus megaterium* (Bm-PG), *Bacillus subtilis* (Bs-PG), *Micrococcus luteus* (Ml-PG) and *Staphylococcus aureus* (Sa-PG). Control, PGRP-S5_C177S only; Unbound, supernatant after PGs were incubated with PGRP-S5_C177S; Wash: wash residue of PGs after incubation with PGRP-S5_C177S; Bound: SDS sample buffer elutes from PGs incubated with PGRP-S5_C177S. PGRP-S5_C177S in the above samples was immunoblotted using anti-His as the primary antibody. **(B)** Bacterial agglutination induced by PGRP-S5_C177S. *Pseudomonas aeruginosa* is able to constitutively express GFP, and *Escherichia coli* transformed with the pGLO plasmid containing a GFP reporter gene can be induced by L-arabinose to express GFP. *Pseudomonas aeruginosa* and *Escherichia coli* suspensions (10 µl) and PGRP-S5_C177S (10 µl, 200 µg/ml) were incubated for 2 h and then photographed under a EVOS FL microscope (Thermo Scientific, Waltham, MA, USA). BSA was used as a control. The magnification is 40×, and the scalebar shown in the lower right corner is 100 µm.

Fig. 4. Substitution of the cysteine at position 177 results in loss of amidase activity of silkworm Peptidoglycan recognition protein (PGRP). Insoluble PGs

from *Escherichia coli* (Ec-PG) (A) and *Micrococcus luteus* (Ml-PG)(B) were incubated with phosphate buffered saline (Cont), PGRP-S5_C177S (C177S, with or without Zn^{2+}). Wild-type PGRP-S5 (WT) and lysozyme were used as positive controls. Light scattering at 405nm was monitored for 8 h. Asterisks indicate significant differences: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for pairwise comparisons by Student's *t*-test. ns, not significant.

Fig. 5. Antibacterial activity of silkworm peptidoglycan recognition protein (PGRP)-S5 depends on its amidase activity. *Micrococcus luteus*(A) and *Escherichia coli*(B) were incubated with purified PGRP-S5_C177S (C177S), Zn^{2+} , or both for 5 h at room temperature, then cultured in LB overnight. Turbidity of the cultures was measured at 600nm. PGRP-S5 (WT) was used as a positive control. Asterisks indicate significant differences: ** $p < 0.01$; *** $p < 0.001$ for pairwise comparisons by Student's *t*-test. ns, not significant.

Fig. 6. Amidase activity of peptidoglycan recognition protein (PGRP)-S5 is required for the silkworm proPO activation. Silkworm plasma was incubated with purified PGRP-S5_C177S (C177S), PGs, or both. PGs used in the assays were from *Bacillus megaterium* (Bm-PG), *Bacillus subtilis* (Bs-PG), *Micrococcus luteus* (Ml-PG) and *Staphylococcus aureus* (Sa-PG). PGs were added after the plasma had been incubated with PGRP-S5 for 5 min at room temperature. Two minutes after PG addition, the PO activity of the reactions was measured using dopamine as a substrate.

PGRP-S5 (WT) was used as controls. Asterisks indicate significant differences: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for pairwise comparisons by Student's t -test. ns, not significant.

Fig. 7. Down-regulation of induction of antimicrobial peptides and the immune deficiency (IMD) pathway in the silkworm by peptidoglycan recognition protein (PGRP)-S5 after bacterial challenge. Silkworm larvae were separately injected with buffer (PBS), *Pseudomonas aeruginosa*, PGRP-S5 (WT, with or without *P. aeruginosa*), or PGRP-S5_C177S (C177S, with or without *P. aeruginosa*). (A). After 6 h of treatment, the hemolymph samples were prepared as described in the methods section and separated on a 16% Tricine-SDS-PAGE gel. (B), (C). qRT-PCR analysis of transcription levels of genes encoding antimicrobial peptides. Att2, attacin 2; CecB6, cecropin B6; CecD, cecropin D; Def2, defensin 2; Glov2,3,4, gloverin 2,3,4; Leb, lebecin; Mor, moricin. (D). qRT-PCR analysis of transcription levels of *IKK γ* and *Dredd*. M, protein molecular marker; PBS, the group injected with PBS; Pa, the group injected with *P. aeruginosa*; WT, the group injected with PGRP-S5; C177S, the group injected with PGRP-S5_C177S; Pa+WT, the group injected with PGRP-S5 and *P. aeruginosa*; Pa+C177S, the group injected with PGRP-S5_C177S and *P. aeruginosa*. The housekeeping gene *IF4A* was selected as a reference gene to calculate the relative expression in the qRT-PCR analysis. Asterisks indicate significant differences: * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$ for pairwise comparisons by Student's t -test.

Fig1.

A

B

C: Cysteine

S: Serine

Fig2.

Fig 3.

Fig 4.

A

B

Fig5.

Fig6.

Fig7.

Table 1

Name	Acc. number	Primers (5'-3')	Amplicon size (bp)
Attacin 2	XM_004934124	F: TTCAAACAGAAGGTGGGC R: GACGGAGATTGGAACAGG	104
Cecropin B6	AK386784	F: TCCTTCGTCTTCGCTCTG R: GATGCCGTCACGGATGTT	114
Cecropin D	NM_001043368	F: CTCCCGGCAACTTCTTCA R: CGAACCCTCTGACCCATT	49
Defensin 2	NM_001135205	GAGCCGTGTACGAGTGAACA TAAGGCCCTCCTGGATCGAA	154
Dredd	NM_001114865	F: GCAAGATGAGCCGTATGACA R: AATAAGAAAGTGCCTCCAGGAAC	199
Gloverin 2	AK383878	F: ACGGACCTTCTGATTACGC R: CATTCTTGTTGCCCCAGT	279
Gloverin 3	AK383909	F: GACACGAGAATGGGAGGAG R: AAGACCCTGGTGCCGTAA	143
Gloverin 4	AK383596	F: CTTGACAAGAACACCCGCCT R: GTCTTGAAGGGATCTTCTGGAT	126
IF4	DQ443290	F: AATCAACGTGAGCGTGAAGTG R: TCCCTTACGACCAAAACGTC	192
IKK γ	XM_012688738	F: GACGACGACACCATGAA R: AACTATATGCTCCAGGG	120
Lebocin	NM_001126260	GAAGGTATGTTTCGCAGTGTC CTTGTGTTACGGTGGCTCTC	115
Moricin	NM_001043364	F: CAAGGCCATTAAGACTGT R: TTTCTTTTCTTCGGTTTC	119