The prophenoloxidase (proPO) system is activated upon recognition of pathogens by pattern recognition proteins (PRPs), including a lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP). However, shrimp LGBPs that are involved in the proPO system have yet to be clarified. Here, we focus on characterizing the role of a *Penaeus monodon* LGBP (PmLGBP) in the proPO system. We found that PmLGBP transcripts are expressed primarily in the hemocytes and are increased at 24 h after pathogenic bacterium *Vibrio harveyi* challenge. The binding studies carried out using ELISA indicated that recombinant (r)PmLGBP binds to β-1,3-glucan and LPS with a dissociation constant of 6.86 \times 10^{-7} \text{ M} and 3.55 \times 10^{-7} \text{ M}, respectively. Furthermore, we found that rPmLGBP could enhance the phenoloxidase (PO) activity of hemocyte suspensions in the presence of LPS or β-1,3-glucan. Using dsRNA interference-mediated gene silencing assay, we further demonstrated that knockdown of PmLGBP in shrimp *in vivo* significantly decreased the PmLGBP transcript level but had no effect on the expression of the other immune genes tested, including shrimp antimicrobial peptides (AMPs). However, suppression of proPO expression down-regulated PmLGBP, proPO-activating enzyme (PmPPAE2), and AMPs (penaeadin and crustin). Such PmLGBP down-regulated shrimp showed significantly decreased total PO activity. We conclude that PmLGBP functions as a pattern recognition protein for LPS and β-1,3-glucan in the shrimp proPO activating system.

The innate immune system is important to invertebrates (1), where cellular immune responses, such as phagocytosis, nodule formation, and encapsulation, are important in arthropods, whereas the prophenoloxidase (proPO) activation system, the clotting system, and synthesis of antimicrobial peptides are important humoral defense mechanisms (1–5). These immune responses are triggered by the specific recognition of microorganisms by host proteins referred to as pattern recognition proteins (PRPs), which are capable of binding to a variety of microbial cell wall components, referred to as pathogen-associated molecular patterns (PAMPs). PAMPs include lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycan (PGN) from Gram-negative and Gram-positive bacteria and β-1,3-glucan from fungi (1, 6).

The melanization cascade is initiated by the activation of the proPO system and plays key roles in the host defense against microbial infections in invertebrates (5, 7–9). ProPO activation can be triggered by PAMPs after their recognition by specific PRPs, leading to activation of a serine proteinase cascade that results in the activation of proPO-activating enzymes (PPAEs) (5). Then, the activated PPAE(s) convert the zymogen proPO to the functionally active phenoloxidase (PO) by specific proteolytic cleavage. Subsequently, PO catalyzes the formation of quinone-reactive intermediates for melanin synthesis at the injury site or around invading microorganisms (10–12).
Recognition of invasive pathogens by PRPs is an essential step for the activation of the proPO system. To date, various types of PRPs in the proPO system have been reported such as peptidoglycan recognition proteins (2, 13–20), C-type lectins (21, 22), β-glucan-binding proteins (bGBPs) (23, 24) and LPS- and β-1,3-glucan binding proteins (LGBPs) (25–27). In crustaceans, the binding of LGBP to LPS or β-1,3-glucan has been documented to activate the proPO system of the freshwater crayfish, *Pacifastacus leniusculus* (27). In shrimp, several LGBPs have been cloned and characterized, such as in the blue shrimp *Penaeus stylirostris* (28), white shrimp *Litopenaeus vannamei* (29), Chinese shrimp *Fenneropenaeus chinensis* (30, 31) and kuruma shrimp *Marsupenaeus japonicus* (32). In the shrimp *Penaeus monodon*, a PRP was initially identified as a bGBP, based on its binding activity to β-glucan but not to LPS (33).

However, shrimp LGBPs that are involved in the proPO system have yet to be clarified. Previously, two proPOs (*PmproPO1* and *PmproPO2*) and two PPAEs (*PmPPAE1* and *PmPPAE2*) genes from *P. monodon* were functionally characterized and shown to both function in the proPO system and be important components in the *P. monodon* shrimp immune system (34–36). Therefore, in the present study, the molecular characterization of a PRP in the shrimp *P. monodon* proPO system is described. The transcript expression profiles in various tissues and in response to the pathogenic bacterium, *Vibrio harveyi*, were examined, as were the binding activity and the proPO activation of the recombinant (r) protein. The protein was named *PmLGBP* and its involvement in the proPO system was elucidated.

**EXPERIMENTAL PROCEDURES**

**Shrimp and Sample Preparation**—Healthy black tiger shrimp (*P. monodon*) with an average wet weight of 15 g were maintained in aerated seawater (20 ppt) for a week prior to the experiment. To determine the tissue expression pattern of *PmLGBP* transcripts, six different tissues (hemocytes, hepatopancreas, gill, lymphoid organ, intestine, and heart tissue) from three shrimp were separately collected as described previously (34). All samples were then stored at −8 °C until used for RNA isolation. In the immune challenge experiments, shrimp were injected with a suspension of *V. harveyi* suspension of 10^8 colony-forming units (cfu) in 50 μl of sterile 0.85% (w/v) sodium saline solution (SSS) into the last abdominal segment of each shrimp. SSS-injected control shrimp using the mathematical model of Paffl (39) to determine the relative expression ratio.

**Construction and Expression of Recombinant (r)*PmLGBP* Protein**—A gene fragment encoding the mature peptide of *PmLGBP* was amplified using *Pfu* DNA polymerase with the specific primers *PmLGBP*/F/-/R that contain 5′-flanking EcoRI and Xhol restriction enzyme sites, respectively (Table 1). The purified PCR product was digested with EcoRI and Xhol, ligated into the EcoRI/Xhol sites of the pET28b(+) expression vector (Novagen), and transformed into competent *Escherichia coli* JM109. The positive clones were confirmed by nucleotide sequencing. The selected recombinant plasmid (pET28b-*PmLGBP*) was transformed into *E. coli* Rosetta (DE3)-pLysS cells (Novagen) for recombinant protein expression and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. At 6 h after induction, cells were harvested by centrifugation at 8,000 rpm for 15 min. The pellets were resuspended in 20 mM Tris-HCl (pH 8.0) and disrupted by an ultrasonic oscillator. The *rPmLGBP* protein was purified and refolded as described previously (40). The *rPmLGBP* protein preparation was evaluated for purity through SDS-PAGE. The concentration of the *rPmLGBP* protein was quantified by the Bradford assay. For Western blot analysis, the *rPmLGBP* protein sample was resolved on a SDS–polyacrylamide gel as above and then electroblotted onto a PVDF membrane (Amersham Biosciences). The membrane was blocked by incubation in Tris buffer solution (TBS: 137 mM...
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Table 1
Nucleotide sequences of the primers used

| Primer | Sequence (5’–3’) | GenBank accession number |
|--------|------------------|-------------------------|
| LGBP   |                  |                         |
| SpmLGBP-F | 5’-TTCCTATCCACAGCAGATG-3’ | JN415536 |
| SpmLGBP-R | 5’-ATGTGACATATTCCGTCAG-3’ | JN415536 |
| LGBPexp-F | 5’-GCCCTCTGGTACTATGATAGT-3’ | JN415536 |
| LGBPexp-R | 5’-CTCCATGCGGCGGCTGATG-3’ | JN415536 |
| Prophenoloxidase |                 |                         |
| PO1RT-F | 5’-GTCCTGCTCCGCTCTCTG-3’ | AF099741 |
| PO1RT-R | 5’-GCCCGCTGATCTGAGC-3’ | AF099741 |
| PO2RT-F | 5’-GCCAGGAGGACGAGTATG-3’ | FJ025814 |
| PO2RT-R | 5’-TTCCTGATCCGCGGCTGATG-3’ | FJ025814 |
| Prophenoloxidase-activating enzyme |                 |                         |
| PmPPE1-F | 5’-ATGAAAGGCGTGACGTTCTCTAG-3’ | FJ952155 |
| PmPPE1-R | 5’-CTCTCTGATCCGCGACCTCTCTCT-3’ | FJ952155 |
| PPAE2-F | 5’-AGCAGATGCGTGTCCTTCTG-3’ | FJ620868 |
| PPAE2-R | 5’-CTAGGGTTAGATCTGACG-3’ | FJ620868 |
| Penaein |                  |                         |
| PEN3-F | 5’-GTCCTGGTCCGCTCTCCG-3’ | FJ680616 |
| PEN3-R | 5’-TGTCAATGAGGATGACGCTCA-3’ | FJ680616 |
| Crustin-like peptide |                 |                         |
| Crus72-F | 5’-CCAGAGTTCCGCAACACGTC-3’ | EF546568 |
| Crus72-R | 5’-AATTGATGACGCGACGCTCTAT-3’ | EF546568 |
| Single WAP domain-containing protein |                 |                         |
| SWD/PW2-F | 5’-GCCGCTTAAAGATCAAGGAGG-3’ | EU623980 |
| SWD/PW2-R | 5’-TCGTAACCTTTCCGAGGAC-3’ | EU623980 |
| Lysozyme |                  |                         |
| PmLyce-F | 5’-GCCGAGGCGGATATGACGAG-3’ | GQ478702 |
| PmLyce-R | 5’-TGGGAACGACGGACCAGCT-3’ | GQ478702 |
| Toll receptor |                |                         |
| PmToll-F | 5’-GATGTCCTTCGGCTGTCCTGC-3’ | EF117252 |
| PmToll-R | 5’-GCTCCAACGACGAGGATCTC-3’ | EF117252 |
| EFla |                  |                         |
| EFla-F | 5’-GTCCTGGACACAACTGAAAGC-3’ | EU623980 |
| EFla-R | 5’-GCGTCCGATGTCATGCTGTCG-3’ | EU623980 |
| Gene silencing |               |                         |
| LGBP   |                  |                         |
| LGBPexp-F | 5’-AGCGTCTTCGTTACGCTGTCG-3’ | JN415536 |
| LGBPexp-R | 5’-CGAAGGACCTGTTCTCTTCCATC-3’ | JN415536 |
| T7PmLGBP-F | 5’-GACGCTTCTTAAGACTCTCTTGAGA-3’ | JN415536 |
| T7PmLGBP-R | 5’-GACGCTTTATGACGACCGTGCTC-3’ | JN415536 |
| GFP    |                  |                         |
| GFP-F | 5’-AATTGATGACGCGACGCTCTAT-3’ | U55761 |
| GFP-R | 5’-AATTGATGACGCGACGCTCTAT-3’ | U55761 |
| GPT7-F | 5’-AGCAGGAGGCGGCTGATG-3’ | U55761 |
| GPT7-R | 5’-AATTGATGACGCGACGCTCTAT-3’ | U55761 |

NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.6)) containing 0.05% (v/v) Tween 20 (TBST) and 5% BSA and then probed with a 1:10,000 dilution of the mouse anti-His tag monoclonal antibody (GenScript) in TBS, washed twice in TBST, and probed in a 1:10,000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma) secondary antibody in TBS. The alkaline phosphatase antibody-protein band complex was detected by incubation in bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as the chromogenic substrate.

Binding Assay of rPmLGBP—The PAMPS binding assay was performed according to Yu et al. (22) with some modification. Briefly, 20 μg of LPS from *E. coli* O127:B8 (Sigma, L4130), laminarin (β-1,3-glucan, L9634) from *Laminaria digitata* (Sigma) and soluble Lys-type PGN from *Staphylococcus aureus* (InvivoGen) in 100 μl of TBS were used to coat each well of a 96-well microtiter plate (Costar) and air-dried overnight at 37 °C. The plate was incubated at 60 °C for 1 h to fix the ligands, and the wells were then blocked with 200 μl of 1 mg/ml BSA in TBS at 37 °C for 2 h. After washing (see below), 100 μl of rPmLGBP (0–10 μM in TBS) was added to each well and incubated for 3 h at room temperature. After washing (see below), the bound protein was detected immunochromatically. First, 100 μl of a 1:10,000 dilution of the mouse anti-His tag monoclonal antibody in TBS was added and incubated at 37 °C for 3 h, washed, and then incubated for 3 h with 100 μl of alkaline phosphatase-conjugated rabbit anti-mouse IgG (diluted 10,000-fold in TBS) as the second antibody. After each stage above the wells were washed four times with TBST (TBS with 0.1% (v/v) Tween 20) for 15 min and once with 0.5 mM MgCl₂/10 mM diethanolamine. Finally, after the last wash, 50 μl of p-nitrophenyl phosphate (1.0 mg/ml in the diethanolamine buffer) was added to each well and incubated at room temperature for 30 min. The
reaction was stopped by the addition of 0.4 m NaOH (100 μl), and the absorbance was measured with an ELISA reader at 405 nm. Wells with 0 μM rPmLGBP protein (100 μl of TBS) were used as the negative control (blank). The apparent dissociation constant (K_d) values were calculated using Prism 5.00 software (GraphPad software) with a one-site binding model and nonlinear regression analysis, as A = A_{max}[L]/(K_d + [L]), where A is the absorbance at 405 nm and [L] is the concentration of the rPmLGBP protein.

ProPO Activation Assay of the rPmLGBP Protein—To investigate the potential involvement of rPmLGBP in the shrimp proPO system, the PO activity was determined by measuring the oxidation of L-DOPA to dopachromes as described (34). HLS was prepared as described (35). For the PO activation assay, HLS (250 μg) was added to a LPS (0.1 μg/ml), β-1,3-glucan (laminarin) (0.1 μg/ml), or PGN (0.1 μg/ml) solution with or without rPmLGBP protein (4 μg/ml), and then incubated at room temperature for 30 min. Subsequently L-DOPA (3 mg/ml) was added to each reaction and incubated at room temperature for 30 min. The absorbance at 490 nm was then measured using a spectrophotometer.

In Vivo Gene Silencing and Semiquantitative RT-PCR Analysis—Double-stranded RNAs (dsRNAs) were generated and purified as described previously (34) using the T7 RibomAX™ Express Large Scale RNA Production Systems (Promega) with the PmLGBP gene-specific primers T7PmLGBPpIF/-R and PmLGBPpIF/-R (Table 1). For dsRNA-mediated gene silencing, the dsRNAs solution of PmLGBP (5 μg/g shrimp, wet body weight) in 25 μl of SSS was intramuscularly injected into P. monodon shrimp (∼3 g, fresh weight). For a sequence-independent dsRNA control, shrimp were injected with GFP dsRNA at the same concentration, whereas shrimp in the handling control group were injected with 25 μl of SSS only. At 24 hpi, a second repeat injection of dsRNA (5 μg/g) or SSS into the shrimp was repeated but together with 20 μg of LPS (E. coli 0111:B4 (Sigma) and laminarin (β-1,3-glucan (Sigma)). Shrimp were reared for a further 48 h after the second dsRNA injection prior to RT-PCR analysis.

To determine the efficiency of the PmLGBP gene knockdown, the total RNA extraction from the hemocytes of the knockdown and control group shrimp was extracted and reverse transcribed into cDNA. The efficiency of PmLGBP gene silencing was then analyzed by semiquantitative RT-PCR using the gene-specific PmLGBPpIF/-R primer pair (Table 1). All PCRs and amplification steps were performed (see above), including the use of the EF1α fragment as an internal control for cDNA template normalization. The PmLGBP protein level of knockdown shrimp was estimated by performing immunoblotting analysis with a crayfish LGBP antibody (27) (kindly provided by Prof. K. Söderhäll and Dr. K. Sritunyalucksana).

Hemolymph PO Activity of PmLGBP Knockdown Shrimp—To investigate the involvement of PmLGBP in the shrimp proPO system, hemolymph collected from the PmLGBP knockdown and the two control group shrimp (GFP dsRNA and SSS-injected) at 48 h after the second dsRNA or SSS injection was analyzed for its PO activity levels using L-DOPA as the substrate, as reported previously (34). The hemolymph PO activity was defined in terms of ΔA_{490}/mg total protein/min. All experiments were performed in triplicate. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Duncan’s test.

Effect of PmLGBP Gene Silencing on Expression of Other Immune Genes—The effect of the dsRNA-mediated PmLGBP gene silencing on the transcript expression level of other immune genes was checked by RT-PCR amplification with gene-specific primers for the P. monodon shrimp antimicrobial peptides (penaeidin, PEN3 [FJ686016]; crustin-like peptide, Crus-likePm [EF654658]; single WAP domain-containing protein, SWDPn2 [EU623980]; and lysozyme, PmLyzc [GQ478702]); the shrimp proPO-associated genes (PmproPO1 [AF099741], PmproPO2 [FJ025814], PmPPAE1 [FJ592515], and PmPPAE2 [FJ620685]); and the Toll receptor (EF117252) (Table 1). Amplification of the EF1α fragment served as the internal control for cDNA template normalization.

RESULTS

Sequence Characterization of PmLGBP cDNA—The ORF of PmLGBP, comprising a putative signal peptide of 17 amino acid residues and a mature peptide of 349 amino acid residues (accession number JN415536), the mature peptide having a calculated molecular mass and isoelectric point (pI) of 39.8 kDa and 4.28, respectively. Protein sequence characterization of PmLGBP by the SMART program revealed a conserved domain that could be classified as glycoside hydrolase family 16 at amino acid positions 79—290. This comprised a polysaccharide binding, glucanase, and β-glucan recognition motifs. In addition, two integral recognition motifs (RGD) at positions 106 and 157 and two putative N-glycosylation sites (NRS; NLS) at positions 66 and 318, respectively, were also found. The BLASTX results showed that PmLGBP significantly matched with the bGBP cDNA from P. monodon (AF368168; 99% amino acid sequence identity), suggesting that PmLGBP is the same gene as that reported previously (33). However, PmLGBP displays the highest amino acid sequence similarity to the crustacean LGBPs (81—95% identities). Moreover, amino acid sequence comparison of PmLGBP with kuruma shrimp M. japonicus LGBP (32) indicated that PmLGBP contained the conserved motifs for a potential polysaccharide binding, glucanase, and β-glucan recognition motifs, and so PmLGBP likely belongs to the family of crustacean LGBPs.

PmLGBP Transcript Is Expressed in Hemocytes and Up-regulated in Response to Gram-negative Bacterium V. harveyi—Based on the semiquantitative RT-PCR results, using the EF1α transcript as an internal control for normalizing the total RNA samples, the PmLGBP mRNA transcript is specifically expressed in shrimp hemocytes, with no detectable transcript expression in the other five shrimp tissues tested (Fig. 1A). SYBR Green-based real-time PCR was carried out to analyze the temporal expression after V. harveyi infection in hemocytes. After systemic V. harveyi infection, the mRNA expression of PmLGBP decreased slightly (0.33- and 0.34-fold) below the control level at 6 and 12 hpi, respectively, but at 24 hpi it dramatically increased to 4.77-fold higher than that at 0 hpi, before returning back to normal expression levels at 48 and 72 hpi (Fig. 1B). Thus, PmLGBP is expressed in the hemocytes (Fig.
**LGBP as Pattern Recognition Protein in Shrimp ProPO System**

![Graph](image)

**FIGURE 1.** *PmLGBP* transcript is expressed in the hemocyte and increased post *V. harveyi* infection. A, tissue distribution analyses of *PmLGBP* transcripts by semiquantitative RT-PCR in the hemocytes (HC), hepatopancreas (HP), gills (G), lymphoid organs (L), intestines (I), and heart (HT). Gel images shown are representative of those seen from two independent replication samples and PCRs. B, relative expression of *PmLGBP* transcripts in the hemocytes of *V. harveyi*-injected shrimp as evaluated by SYBR Green real-time PCR at the indicated times. Relative expression levels of mRNA were calculated according to Pfaffl [39], using EF1α as the internal reference gene. The average relative expressions are representative of three independent repeats ± 1 S.D. (error bars).

1A), and its expression level in that tissue is transiently significantly up-regulated after *V. harveyi* infection (Fig. 1B).

**Expression and Enrichment of rPmLGBP**—The mature *rPmLGBP* protein was successfully expressed in *E. coli* cells. The *rPmLGBP* protein, after enrichment and refolding, appeared as a single band with an estimated molecular mass of ~40 kDa after SDS-PAGE resolution (data not shown). Western blotting with the anti-His antibody revealed a single band of the same apparent size (data not shown). Thus, the *rPmLGBP* was likely to have been enriched to apparent homogeneity.

**PmLGBP Binds to Both LPS and β-1,3-Glucan (Laminarin), but Not Peptidoglycan**—To determine whether *PmLGBP* possesses the properties of a PRP, the binding activity of the purifying *rPmLGBP* protein with three different types of PAMPs, namely LPS, β-1,3-glucan, and Lys-type PGN, was performed by ELISA. As shown in Fig. 2, the *rPmLGBP* bound to LPS and β-1,3-glucan (laminarin) directly in a concentration-dependent manner and with a saturable process. However, the *rPmLGBP* did not exhibit any detectable binding activity to the Lys-type PGN (data not shown). The apparent dissociation constant (*Kd*) of the *rPmLGBP* to LPS and β-1,3-glucan, calculated from the saturation curve fitting according to the one-site binding model, was 3.55 ± 1.03 × 10⁻⁷ M and 6.86 ± 1.86 × 10⁻⁷ M, respectively (Fig. 2). These data support that *rPmLGBP* is a PRP that can bind specifically to both LPS and β-1,3-glucan.

**PmLGBP Enhanced Phenoloxidase Activity after β-1,3-Glucan or LPS Binding in Vitro**—To understand the mechanisms of PAMP binding and proPO activation, incubation of HLS, which contains zymogen proteins involved in the proPO system, with *rPmLGBP* together with PAMP was investigated. In the presence of either laminarin (a β-1,3-glucan) or LPS, but not in the present of PGN (Fig. 3D) or in their absence, *rPmLGBP* significantly enhanced the PO activity of HLS by 72% (Fig. 3B) and 88% (Fig. 3C), respectively, compared with the control groups (nonactivated HLS; Fig. 3A). On the other hand, incubation of HLS with either laminarin or LPS in the absence of *rPmLGBP* showed only a numerically slightly higher PO activity than the control group, which was not statistically significant (p > 0.05).

**Effect of in Vivo Gene Silencing of PmLGBP and PmproPO on Expression of Shrimp Immune Genes**—To investigate the involvement of *PmLGBP* in the shrimp proPO system, dsRNA-mediated gene knockdown of *PmLGBP* transcripts was performed. The semiquantitative RT-PCR analysis showed that the *PmLGBP* transcript level was specifically decreased in *PmLGBP*-silenced shrimp, whereas injection of the GFP dsRNA had no significant effect on the transcript levels of *PmLGBP* compared with the SSS-injected control shrimp (Fig. 4A). Immunoblotting results suggested *PmLGBP* dsRNA also could suppress *PmLGBP* at the protein level (Fig. 4B).

To examine the effect, if any, of *PmLGBP* transcript expression disruption on the expression of other immune genes, cDNA samples that exhibited a *PmLGBP* gene silencing were analyzed further by RT-PCR for the transcript expression levels of the other genes. Silencing of the *PmLGBP* gene had no significant effect on the transcript expression levels of any of the...
other tested *P. monodon* shrimp genes from the proPO system (*PmproPO1, PmproPO2, PmPPAE1*, and *PmPPAE2*), antimicrobial peptides (PEN3, Crus-like*Pm*, SWD*Pm*2, and lysozyme) or the Toll receptor (Fig. 5).

Furthermore, we performed the gene silencing of proPO genes (*PmproPO1 and PmproPO2*) (34) and also examined the effect of gene silencing on the gene expression levels of immune genes. We found that the knockdown of proPO genes significantly decreased the transcription of two genes in proPO system (*PmLGBP* and *PmPPAE2*) and two antimicrobial peptides (PEN3 and Crus-like*Pm*) (Fig. 5). This result suggests that the proPOs not only contributes to control the expression of genes in proPO cascade but also AMPs in shrimp.

**Figure 3. Enhancement of PO activity by laminarin or LPS in the presence of rPmLGBP.** The proPO activation of shrimp HLS by rPmLGBP alone (A) or after preincubation in vitro with β-1,3-glucan (laminarin) (B), LPS (C), or PGN (D). PO activity was defined as ΔA490/mg of total protein per min. The data are shown as the mean ± 1 S.D. (error bars) and are derived from three independently replicated experiments. Means with a different lowercase letter (above each bar) are significantly different at the *p* < 0.05 level.

**Figure 4. RNAi-mediated suppression of PmLGBP resulted in a reduction of PmLGBP transcript and protein levels of *P. monodon*.** The efficiency of dsRNA-mediated gene silencing of PmLGBP transcripts (A) and proteins (B) after the dsRNA injection was examined using semiquantitative RT-PCR and Western blot analysis. Shrimp injected with GFP dsRNA in SSS or with SSS alone served as controls. EF1α was used as the internal reference gene for RT-PCR whereas β-actin was used as a loading control for Western blot analysis. In the shown gel, the lanes for each condition represent the results from individual shrimp.

**Double Strand RNAi-mediated Knockdown of PmLGBP Transcript Resulted in Reduction of Hemolymph Phenoloxidase Activity**—To determine the importance of any role of PmLGBP in the shrimp proPO system, the hemolymph from PmLGBP knockdown shrimp was subjected to a PO activity assay. A significant reduction in the PO activity to 53% of that of the control (PAMP-stimulated shrimp) was observed in the PmLGBP knockdown shrimp, whereas no significant change in the PO activity (101%) was observed in the GFP dsRNA injected shrimp (Fig. 6). The results of basal PO activity in normal (PAMP-untreated) shrimp clearly showed no significant difference of PO activities of...
both normal shrimp and PmLGBP knockdown shrimp (Fig. 6).

Thus, PmLGBP is a PRP member that functions in the shrimp proPO system.

DISCUSSION

The proPO system is an important component of the immune reaction in the host defense against microbial or parasitic infections in many arthropods (5, 8, 9). The first process of the proPO system is to detect the PAMPs, such as LPS, lipoteichoic acid, /H9252-1,3-glucan and PGN molecules of the pathogens. LGBP is an important PRP in crustaceans. In crayfish, LGBP has been demonstrated to be a required PRP for the activation of the proPO system (27). However, the role of shrimp LGBPs in the proPO activating system is still somewhat elusive and poorly elucidated.

In the black tiger shrimp, P. monodon, the first LGBP gene (previously named bGBP) was reported to be constitutively expressed in the hemocyte and to exhibit binding to /H9252-1,3-glu- can but not to LPS (33). In this study, we report on the further functional characterization of the LGBP from P. monodon (PmLGBP). After analysis, we found that the high nucleotide and deduced amino acid sequence identity (99%) of PmLGBP and the previously reported bGBP from P. monodon (33) sug-

FIGURE 6. RNAi-mediated silencing of PmLGBP gene significantly decreased the hemolymph PO activity in shrimp. Hemolymph was collected at 48 h after the second dsRNA (or SSS) injection. The PO activity of normal shrimp hemolymph was used as the basal activity in PAMP-unstimulated shrimp. The total hemolymph PO activity was defined as ΔA490/mg of total protein per min. The data are shown as the mean ± 1 S.D. (error bars) and are derived from three independently replicated experiments. Significant difference compared with control is indicated by an asterisk (p < 0.05).

FIGURE 5. Effect of RNAi-mediated suppression of PmLGBP and PmproPOs on gene expression of the shrimp antimicrobial peptides, proPO-associated gene, and Toll receptor after. The effect of gene knockdown on the expression level of P. monodon shrimp antimicrobial peptide transcripts (PEN3, Crus-likePm, SWDPm2, and lysozyme), genes involved in the proPO system (PmproPO1, PmproPO2, PmPPAE1, and PmPPAE2), and the Toll receptor were evaluated by semiquantitative RT-PCR using the gene-specific primers for each gene (Table 1). EF1α served as the internal reference gene to normalize the amount of cDNA template. The average relative expressions are representative of three independent repeats ±1 S.D. (error bars). Significant difference compared with control is indicated by an asterisk (p < 0.05).

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cytes, including the proPO genes (34) and PPAEs (35, 36). In the present study, semiquantitative RT-PCR analysis revealed that PmLGBP transcripts were only detected in hemocytes, consistent with that reported previously for bGBP in P. monodon (33). In other crustacean species, where the tissue distribution of LGBP has been investigated, LGBP transcripts are expressed mainly in the hemocytes of crayfish (27) and shrimp (29–32). Moreover, in shrimp *F. chinensis* both LGBP mRNA and protein were reported to be synthesized mainly in the granular hemocytes and located on the hemocyte surface, respectively (30).

In the present study, the response of PmLGBP expression to systemic infection with the Gram-negative bacteria *V. harveyi* was investigated. The expression of PmLGBP mRNA did not significantly change at 6–12 hpi, but was increased significantly (4.77-fold) at 24 hpi and then dropped to near the initial level at 48–72 hpi. Similarly, the *P. monodon* LGBP (bGBP) showed no change in transcriptional levels within 12 h after heat-killed *V. harveyi* and curdlan (a β-1,3-glucan) injection (33). Moreover, in *M. japonicus*, LGBP mRNA was up-regulated in hemocytes at 12–48 h after LPS challenge (32). In contrast, in *L. vannamenei*, LGBP transcript levels were significantly up-regulated in hemocytes at 3 and 6 hpi and then returned to the original level at 12–24 hpi with live *Vibrio alginolyticus* (29). In *F. chinensis*, FcLGBP was significantly up-regulated at 6 hpi but returned to the original level at 12–24 hpi with a mixture of heat-killed *V. anguillarum* and *S. aureus* (31). The differences in LGBP transcript expression could be due to variations between the response to live and dead pathogen cells or to different microorganisms and so cell wall components, in addition to differences between host species or strains.

PRP receptors are essential molecules that have an ability to recognize and initiate the host defense mechanism, especially in the activation of the proPO cascade in many invertebrate species. However, in shrimp, no PRPs that activate the proPO system have been described. LPS and PGN are the major cell wall components of Gram-negative and Gram-positive bacteria, respectively, whereas β-1,3-glucan is a major component of fungal cell walls. In crustaceans, including shrimp and crayfish, the activation of the proPO system can be triggered upon recognition of LPS, PGN, and β-1,3-glucans (27, 41). To investigate the binding activity of *PmLGBP* to these microbial elicitors, we examined the binding activity of *rPmLGBP* to LPS, PGN, and β-1,3-glucan and found a high affinity binding to both LPS and β-1,3-glucan, but not to the l-lysine-type PGN. Thus, *PmLGBP* may only serve as a PRP receptor for LPS and β-1,3-glucan. According to the assumption of a single-site binding model, the apparent *Kd* value of *rPmLGBP* for LPS (*Kd* = 3.55 ± 1.03 × 10⁻⁷ m) is approximately 2-fold lower than that of β-1,3-glucan (*Kd* = 6.86 ± 1.86 × 10⁻⁷ m), and so the binding of *rPmLGBP* to LPS is ~2-fold tighter than to β-1,3-glucan. These binding constants are in broad agreement with the *Kd* values obtained from other known PRPs that have previously been reported to be involved in the activation of the proPO system, including the *Kd* values obtained from the *Manduca sexta* β-1,3-glucan-recognition protein (*Kd* = 1.5 × 10⁻⁶ m for *Saccharomyces cerevisiae* and 1.2 × 10⁻⁶ m for *E. coli*) (42) and *M. sexta* microbe-binding protein (*Kd* = 3.77 ± 0.90 × 10⁻⁸ m for LPS) (43).

In contrast, *P. monodon* LGBP (as bGBP) was reported to bind to β-1,3-glucan only and not LPS when using a pulldown assay (33). However, it is unclear whether this apparent different LPS binding activity is due to the different experimental assay systems used. In comparison, the results presented here correlate well with the binding data of the LGBPs from other crustacean species. In the crayfish *P. leniusculus*, a purified LGBP protein exhibited binding activity to both LPS and β-1,3-glucans (laminarin and curdlan) but could not bind to PGN (27). In *F. chinensis*, rLGBP exhibited a stronger binding activity to Gram-negative bacteria (*Klebsiella pneumoniae*) than that of *E. coli*, or Gram-positive bacteria (*Micrococcus luteus* and *Bacillus megaterium*) and yeast (*Pichia pastoris*) (30). In the scallop *Chlamys farreri*, rLGBP exhibited a strong binding to LPS and β-1,3-glucan and a moderate binding to PGN and also showed an agglutination activity toward the Gram-negative bacteria *E. coli*, the Gram-positive bacteria *B. subtilis*, and the fungus *P. pastoris* (44). Significantly, all of these data indicate that LGBP functions as a PRP in invertebrates and that the binding of these proteins is involved in the recognition of invading microorganisms to activate the host immune defense and the proPO-activating system.

To confirm that *rPmLGBP* could activate PO activity, the *rPmLGBP* protein was incubated with either LPS or β-1,3-glucan, where it was found to trigger the PO activity in HLS (cell-free hemolymph) in the presence of LPS or β-1,3-glucan. In accord, the LGBP protein from the crayfish *P. leniusculus* was reported to bind to both LPS and β-1,3-glucan and activates the proPO system (27). Sequence alignment of the shrimp *PmLGBP* and the crayfish *PLGBP* indicated that the amino acid sequence of the domains that are believed to be responsible for LPS and β-1,3-glucan binding are highly conserved. These regions are composed of the polysaccharide binding motif, glucanase motif, and β-glucan recognition motif (32). Recently, two serine proteinase homologues (*PISP1* and *PISP2*) and *PLGBP* from *P. leniusculus* have been reported to be involved in the PGN-induced proPO activation, and that *PISP1* may act as a PGN-binding protein and *PISP2* and *PLGBP* probably function as cofactors in a PGN-binding complex (41). In the insect *M. sexta*, immulectin-2 specifically binds with LPS and is involved in stimulating the proPO activation system (45). Moreover, a β-1,3-glucan recognition protein and a β-1,3-glucan recognition protein-2 of *M. sexta* were found to bind and to aggregate the bacterial and fungal cell wall components and to stimulate the proPO pathway (42, 46). In addition, microbe-binding protein, a β-1,3-glucanase-related protein from *M. sexta*, specifically binds to lipoteichoic acid, LPS, and a dianimopimelic acid-type PGN from *E. coli* and *B. subtilis*, and this binding is involved in triggering the insect proPO system (43). Consistent with this is that in this study reported here in *P. monodon*, the knockdown of *PmLGBP* transcript levels significantly decreased the enzymatic PO activity. Overall, our data support that *PmLGBP* functions as a PRP that can recognize LPS and β-1,3-glucan and activate the proPO system.

In *Drosophila*, the Gram-negative-binding protein1 (GNBP1), a PRP, is required for Toll activation in response to Gram-posit
tive bacterial infection. Silencing of GNBPI reduces the induction of *Drosomycin*, an antifungal peptide, after Gram-positive bacteria infection but not after fungal infection (47). In the shrimp *L. vannamei*, knockdown of the Toll-like receptor did not significantly alter the transcript expression levels of a crustin antimicrobial peptide (48). In this study, RNAi treatment significantly repressed the mRNA expression of *PmLGBP*, but this had no apparent affect on the expression level of any of the immune defense genes (antimicrobial peptides, proPO system, and Toll receptor) tested in this study. These results suggest that the expression of these antimicrobial peptides is independent of *PmLGBP* signaling pathway or that these are redundant and that the expression of these genes is compensated through alternative signaling pathways of PRPs. Surprisingly, mRNA expressions of some genes in proPO cascade, and antimicrobial peptides were significantly decreased in proPO gene-silenced shrimp, which is consistent with the previous report (49), which demonstrated that silencing of kuruma shrimp proPO results in down-regulation of AMP transcripts (penaeidin, crustin, and lysozyme). The down-regulated expression of these genes in proPO-silenced shrimp illustrated that proPO are not only involved in expression of genes in proPO cascade but also in antimicrobial peptide.

In conclusion, *in vitro* and *in vivo* results clearly demonstrate for the first time that *PmLGBP* is a PRP involved in the shrimp proPO system, exhibits LPS and β-1,3-glucan binding activity, and can activate the proPO system.

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