Molecular and functional characterization of a short-type peptidoglycan recognition protein, PGRP-S in the amphibian *Xenopus laevis*

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**A R T I C L E  I N F O**

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**A B S T R A C T**

Peptidoglycan recognition proteins (PGRPs) are a family of pattern recognition receptors (PRRs) involved in host antibacterial responses, and their functions have been characterized in most invertebrate and vertebrate animals. However, little information is available regarding the function of frog PGRPs. In this study, a short-type PGRP (termed Xl-PGRP-S) gene was identified in the African clawed frog, *Xenopus laevis*. The predicted protein of Xl-PGRP-S contains several structural features known in PGRPs, including a typical PGRP domain and two closely spaced conserved cysteines. Xl-PGRP-S gene was constitutively expressed in all tissues examined, with the highest expression level observed in muscle. As a typical PRR, Xl-PGRP-S is inducible after peptidoglycan (PGN) stimulation, and has an ability to bind PGN. In addition, Xl-PGRP-S has been proven to have Zn\(^{2+}\)-dependent amidase activity and antibacterial activity against *Edwardella tarda*. The present study represents the first discovery on the function of frog PGRPs, thus contributing to a better understanding of the functional evolution of PGRPs in early tetrapods.

**1. Introduction**

As the front line of host defense, the innate immune system relies on a series of pattern recognition receptors (PRRs) for detecting pathogen-associated molecular patterns (PAMPs) derived from microbial pathogens (Brubaker et al., 2015). Among various PAMPs, peptidoglycan (PGN) is an essential and unique component of the cell wall of virtually all bacteria, which provides an excellent target for host to sense bacterial invasion (Royet and Dziarski, 2007). It has been shown that multiple classes of PRRs can mediate PGN recognition, such as peptidoglycan recognition proteins (PGRPs), and some members of Toll-like receptor (TLRs) and nucleotide oligomerization domain-like receptor (NLRs) families (Brubaker et al., 2015). PGRPs are a family of PRRs homologous to bacteriophage type 2 amidase, and play vital roles in material invasion (Royet and Dziarski, 2007). It has been shown that amidase activity and antibacterial activity of PGRPs in early tetrapods.

From an evolutionary point of view, PGRPs are ubiquitously present in most invertebrates such as insects, mollusks and echinoderms, as well as in all vertebrate animals (Royet and Dziarski, 2007; Royet et al., 2011; Wang et al., 2018, 2019). According to the size of the mRNA transcripts, PGRPs can be classified into two main types, i.e. short and long types, which are termed PGRP-S and PGRP-L, respectively (Royet and Dziarski, 2007; Royet et al., 2011). Insects appear to harbour the highly complex PGRP repertoire (Kurata, 2014; Wang et al., 2019). For example, at least 13 PGRP genes are identified in *Drosophila melanogaster*, including PGRP-SA, -SB1, -SB2, -SC1A, -SC1B, -SC2, and -SD, which express short transcripts, and PGRP-LA, -LB, -LC, -LD, -LE, and -LF, which express long transcripts (Kurata, 2014; Wang et al., 2019). By contrast, vertebrates may contain fewer PGRP members when compared with insects. For instance, to date only four PGRP genes have been identified in human, which are named PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4 (Dziarski and Gupta, 2006b). Similarly, fish appear to have three main types of PGRPs, i.e. PGRP2, PGRP5, and PGRP6, among which PGRP2 is homologous to mammalian PGLYRP-2, while PGRPS and PGRP6 are only found in fish (Li et al., 2007, 2013, 2014).

Structurally, all PGRPs contain at least one PGRP domain, and two closely spaced and conserved cysteines involved in the formation of...
disulfide bond (Dziarski and Gupta, 2006b; Michel et al., 2001; Wang et al., 2003). In addition, all PGRPs with amidase activity have a conserved Zn\(^{2+}\)-binding site in the peptidoglycan-binding groove, which is composed of two histidines, one tyrosine, and one cysteine (Chang et al., 2004; Guan et al., 2005; Reiser et al., 2004). Interestingly, insect and mammalian PGRPs may provide immune protections in host cells against infection through different mechanisms. Insect PGRPs activate Toll or IMD signal transduction pathways or trigger proteolytic cascades, thus inducing the production of antimicrobial products and the phagocytosis (Kurata, 2014; Royet et al., 2011; Zhang et al., 2019), whereas mammalian PGRPs function as bactericidas by direct contact (Dziarski and Gupta, 2006a).

Amphibians have an unique evolutionary position as the first terrestrial tetrapods, and provide a valuable subject for the research on comparative immunology (Roelants et al., 2007). Previous studies revealed that two short-type PGRPs from the Chinese giant salamander (Andrias davidianus) play essential roles in the recognition of PGN and the immune responses against the bacterial infection (Qi et al., 2017; Yang et al., 2018). Nevertheless, little information is available to date regarding the function of frog PGRPs, especially the ones from the clawed frog, *Xenopus*, the most important model species among amphibians. In the present study, a short-type PGRP gene named as Xi-PGRP-S, was identified in the African clawed frog, *Xenopus laevis*, and its PGN-binding ability, amidase activity and antibacterial activity against the *Edwardsiella tarda* was characterized. The present research thus contributes to a better understanding of the function of the amphibian PGRPs.

2. Materials and methods

2.1. Experimental animals and cells

*X. laevis* were acclimated in freshwater tank at 22 °C under natural photoperiod for one month prior to experiments with the approval of the institute in terms of the use of laboratory animals. Human Embryonic Kidney 293T (HEK293T) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO\(_2\). The A6 cells, a kind of kidney fibroblast cell line from *X. laevis*, were cultured in 75% NCTC-109 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 15% distilled water, 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies) at 26 °C with 5% CO\(_2\).

2.2. Cloning of PGRP-S cDNA sequence in X. laevis

The putative open reading frame (ORF) of XI-PGRP-S gene was predicted by in silico analysis. Using Trizol Reagent (Invitrogen), total RNA from various tissues including liver and intestine of healthy *X. laevis* was extracted and mixed. The first-strand cDNA was synthesized from above mixed total RNA using RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and served as template to amplify ORF sequence of XI-PGRP-S by PCR using specific primers designed on the basis of predicted data. All PCR products were ligated into the pMD18-T vector (TaKaRa, Japan) and transformed into competent *Escherichia coli* cells. Then the positive clones were sequenced by Sangon Biotech (Shanghai, China). The sequences of all PCR primers used in this study are summarized in Supplementary Table 1.

2.3. Sequence analysis of PGRP-S gene in X. laevis

The protein sequences were deduced from the nucleic acid sequences using the program on the Expasy Web site (http://ca.expasy.org/tools). Signal peptides were identified with the SignalP 4.1 server (www.cbs.dtu.dk/services/SignalP). Homology between sequences was calculated using the Megalign program within the DNASTAR package. Multiple sequence alignments for amino acids were generated using the Clustal X program (Larkin et al., 2007), and phylogenetic tree for PGRP-S in *X. laevis* was constructed using MEGA5.1 package (Tamura et al., 2011) with neighbor-joining (NJ) algorithm, with 1000 time repeat of bootstrap analysis. All sequences used in the analysis were listed in Supplementary Table 2.

2.4. Quantitative analysis of PGRP-S mRNA expression in X. laevis and A6 cells

For analyzing the expression of target genes in healthy *X. laevis*, organs/tissues were collected from three animals to extract total RNA with Trizol (Invitrogen). For analyzing the expression after stimulation, 1 × 10\(^6\) A6 cells were seeded into individual wells of 24-well plates and were stimulated with lyszyme-treated PGN from *Staphylococcus aureus* (50 μg/ml; Sigma), which were divided into four groups, each with three samples. A6 cells as control were stimulated with amphibian phosphate buffered saline (APBS; pH = 7.2) and were also divided into four groups. At 3, 6, 12 and 24 h post-stimulation, the cells in each group were collected for extracting RNA with Trizol (Invitrogen), and total RNA from different simples was used separately for the synthesis of the first strand cdNA sequence using RevertAid\(^{TM}\) first strand cdNA synthesis kit (Thermo Fisher Scientific) by following the manufacturer’s instruction.

Specific primers (Supplementary Table 1) were used to amplify PGRP-S and β-actin fragments by PCR. All PCR products were sequenced as described above. Sequenced plasmid DNA of positive clones was extracted with Plasmid Mini Kit (Omega Bio-tek) and measured using spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific). The copy number was calculated following a method described previously (Gan et al., 2017, 2018). To establish a standard curve, plasmids were diluted in serial 10-fold dilution, ranging from 10\(^{-6}\) to 10\(^{-2}\), before being quantified with PCR using CFX96 Real-Time PCR Detection System (BioRad). A final volume of 20 μl PCR reaction system contained 10 μl IQ\(^{®}\) SYBR\(^{®}\) Green Supermix (BioRad), 1 μl of each primer, 7 μl sterile water and 1 μl cdNA template, with the PCR protocol as the followings: one cycle of 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 40 s. Each sample was run in triplicate, and the gene expression for each sample was normalized against β-actin. Data analysis was performed using the 2\(^{-\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001), and fold changes were calculated relative to control group.

2.5. Production of recombinant PGRP-S from X. laevis

For the production of recombinant PGRP-S from *X. laevis*, XI-PGRP-S-expression plasmid was transfected into HEK293T cells as previously reported with minor modification (Gan et al., 2018; Qi et al., 2017). Briefly, the entire ORF of XI-PGRP-S was amplified and inserted with the coding sequence of HA-tag between the last protein-coding codon and stop one for XI-PGRP-S gene, before being subcloned into pcDNA3.1/myc-His (−) A vector (Invitrogen; called pcDNA3.1 in this research), which was named as pcDNA3.1-XI-PGRP-S. Then, pcDNA3.1-XI-PGRP-S and empty pcDNA3.1 vector (as control) were transfected into HEK293T cells, respectively, following the protocol of Lipofectamine (2000) Transfection Reagent (Invitrogen). Thirty-six hours after the transfection, the supernatants and lysates of transfected HEK293T cells were collected for analyzing the expression of target genes in healthy *X. laevis*, organs/tissues were collected from three animals to extract total RNA with Trizol (Invitrogen), and total RNA from different simples was used separately for the synthesis of the first strand cdNA sequence using RevertAid\(^{TM}\) first strand cdNA synthesis kit (Thermo Fisher Scientific) by following the manufacturer’s instruction.

Specific primers (Supplementary Table 1) were used to amplify PGRP-S and β-actin fragments by PCR. All PCR products were sequenced as described above. Sequenced plasmid DNA of positive clones was extracted with Plasmid Mini Kit (Omega Bio-tek) and measured using spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific). The copy number was calculated following a method described previously (Gan et al., 2017, 2018). To establish a standard curve, plasmids were diluted in serial 10-fold dilution, ranging from 10\(^{-6}\) to 10\(^{-2}\), before being quantified with PCR using CFX96 Real-Time PCR Detection System (BioRad). A final volume of 20 μl PCR reaction system contained 10 μl IQ\(^{®}\) SYBR\(^{®}\) Green Supermix (BioRad), 1 μl of each primer, 7 μl sterile water and 1 μl cdNA template, with the PCR protocol as the followings: one cycle of 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 40 s. Each sample was run in triplicate, and the gene expression for each sample was normalized against β-actin. Data analysis was performed using the 2\(^{-\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001), and fold changes were calculated relative to control group.
2.6. Assays for PGN-binding activity of PGRP-S from X. laevis

PGN-binding activity of Xl-PGRP-S was measured as previously reported with minor modification (Li et al., 2013; Qi et al., 2017). Briefly, 40 mg lysozyme-treated PGN from S. aureus was incubated with 50 μg protein extract of recombinant Xl-PGRP-S or the pcDNA3.1-vector control from transfected HEK293T cells (Supplementary Fig. 1) for 4 h at 4 °C in a rocking incubator. Then, the bound proteins were separated by centrifugation at 16,000 relative centrifugal force (rcf) for 15 min and washed 4 times with TBS buffer (50 mM TrisHCl, 50 mM NaCl, 10 μM ZnCl2, pH 7.5). The bound proteins were recovered from PGN by boiling in 2 × SDS-PAGE loading buffer. Then, the proteins were loaded onto the SDS-PAGE gels for gel electrophoresis and detected with anti-HA antibody by Western blotting. The empty pTurboGFP-N vector (as a negative control) was also transfected into HEK293T cells, and the extracted protein from pTurboGFP-N vector-transfected cells was detected with anti-GFP antibody by Western blotting as described above.

2.7. Assays for amidase activity of PGRP-S from X. laevis

Amidase activity of Xl-PGRP-S was measured as previously reported with minor modification (Choi et al., 2018; Gan et al., 2016; Li et al., 2013). Briefly, 40 mg lysozyme-treated PGN from S. aureus was incubated with 50 μg protein extract of recombinant Xl-PGRP-S or the vector control in Tris-ZnCl2 buffer (20 mM TrisHCl, 150 mM NaCl, 10 μM ZnCl2, pH 7.2) or Tris buffer (20 mM TrisHCl, 150 mM NaCl, pH 7.2). The optical density (OD) at 540 nm was recorded every 10 min during a 120 min period. All assays were performed in triplicate.

2.8. Assays for antibacterial activity of PGRP-S from X. laevis against E. tarda

Antibacterial activity of Xl-PGRP-S against E. tarda was measured as previously reported with minor modification (Li et al., 2013; Qi et al., 2017). To assess the antibacterial activity of Xl-PGRP-S against intracellular E. tarda, 3 × 10^5 HEK293T cells were transfected with 0.5 μg pcDNA3.1-Xl-PGRP-S or empty pcDNA3.1 vector and cultured at 37 °C for 36 h. The cells were infected with E. tarda at the multiplicity of infection (MOI) of 10 (2 × 10^7 cfu/ml) for 1 h at 37 °C. After the cells were washed with DMEM for three times, the 10% FBS DMEM containing 16 μg/ml gentamicin was added to each well to kill extracellular bacteria. The cells were collected at 3 h post incubation with gentamicin, and then washed with DMEM for four times and then lysed in 500 μl PBS containing 1% Triton X-100 for 20 min. The number of bacteria was calculated by plate colony-counting methods. To assess the antibacterial activity of Xl-PGRP-S against extracellular E. tarda, HEK293T cells were transiently transfected with pcDNA3.1-Xl-PGRP-S or empty pcDNA3.1 vector, and the cells were then infected with E. tarda as described above. At 3 h post infection, the medium was collected and the number of bacteria were calculated by plate colony-counting methods. All assays were performed in triplicate.
PGRP-S, including a typical PGRP domain and two closely spaced and conserved cysteines (C55 and C61) participated in the formation of disulfide bonds (Dziarski and Gupta, 2006b; Michel et al., 2001; Wang et al., 2003) (Fig. 1). Importantly, four conserved Zn2+-binding residues (H48, Y83, H157 and C165) that are essential for amidase activity of PGRPs, are also present in predicted protein of Xl-PGRP-S (Chang et al., 2004; Guan et al., 2005; Reiser et al., 2004) (Fig. 1).

To understand the phylogenetic relationship between Xl-PGRP-S and other PGRPs in vertebrates, an NJ tree was constructed using protein sequences (Fig. 2). In general, all the long-type PGRPs in vertebrates were clustered into a major clade at the base of the phylogenetic tree, and all the short-type PGRPs in vertebrates were grouped together to form another major clade, in which Xl-PGRP-S and other short-type PGRPs in amphibians were grouped together to form a separate clade with the support of high bootstrap value (Fig. 2).

3.2. Expression pattern of PGRP-S gene in X. laevis and A6 cells

In healthy X. laevis, the mRNA of Xl-PGRP-S was detected in all organs/tissues examined, with the highest level of Xl-PGRP-S observed in muscle (Fig. 3a). Following PGN stimulation, the transcript level of Xl-PGRP-S was significantly up-regulated in A6 cells, with the highest level observed at 3 h post-stimulation (Fig. 3b).

3.3. PGN-binding activity of PGRP-S from X. laevis

For the production of recombinant PGRP-S from X. laevis, XI-PGRP-S-expression plasmid and the empty vector (as control) was transfected separately into HEK293T cells. Western blotting showed that recombinant XI-PGRP-S protein was mainly expressed in the lysates of transfected cells (Supplementary Fig. 1), which was used in assays for testing the PGN-binding activity and amidase activity of XI-PGRP-S. To test the PGN-binding activity of XI-PGRP-S, recombinant protein was extracted from HEK293T cells transfected with pcDNA3.1-Xl-PGRP-S, empty pcDNA3.1 vector, or empty pTurboGFP-N vector was incubated with PGN, and the bound proteins were detected by Western blotting. As shown in Fig. 4, XI-PGRP-S was able to bind PGN, whereas such binding was not observed for the empty pcDNA3.1 vector control and the empty pTurboGFP-N vector control.

3.4. Amidase activity of PGRP-S in X. laevis

Based on the conservation of all four residues required for amidase activity, XI-PGRP-S was hypothesized as a kind of PGRP with amidase activity. To confirm this notion, recombinant XI-PGRP-S or the empty vector control were incubated with PGN in Tris-ZnCl2 buffer or Tris buffer, and the OD value at 540 nm of the reactive system was recorded. As PGN was degraded by recombinant XI-PGRP-S in Tris-ZnCl2 buffer, the absorbance at 540 nm of reactive system decreased significantly, indicating that recombinant XI-PGRP-S possesses PGN-lytic activity in the presence of Zn2+ (Fig. 5a). However, the PGN-lytic activity of recombinant XI-PGRP-S was significantly reduced in absence of Zn2+ (Fig. 5a). By contrast, no matter whether Zn2+ was added into the reactive system, the empty vector control showed no PGN-degrading activity (Fig. 5b). These results demonstrate that XI-PGRP-S possesses Zn2+-dependent amidase activity.
3.5. Antibacterial activity of PGRP-S from *X. laevis* against *E. tarda*

To test the antibacterial activity of Xl-PGRP-S, HEK293T cells were infected with *E. tarda* after transfection with pcDNA3.1-Xl-PGRP-S and empty pcDNA3.1 vector, respectively, and the number of *E. tarda* was counted using the plate count method. In cells transfected with Xl-PGRP-S, it was observed that the number of both extracellular and intracellular bacteria was significantly lower than that of control cells transfected with empty pcDNA3.1 vector (Fig. 6).

4. Discussion

PGRPs are a family of PRRs that play crucial roles in host innate immune responses (Dziarski and Gupta, 2006a, b; Royet and Dziarski, 2007), and their functions have been characterized in most invertebrates and vertebrates, including insects, mollusks, echinoderms, fish, and mammals (Royet and Dziarski, 2007; Royet et al., 2011; Wang et al., 2018, 2019; Zhao et al., 2018a, 2018b). However, little information is available to date regarding the functions of frog PGRPs. In intracellular bacteria was significantly lower than that of control cells transfected with empty pcDNA3.1 vector (Fig. 6).
the present study, a PGRP gene named XI-PGRP-S, was identified in an amphibian model species, X. laevis. The predicted protein of XI-PGRP-S possesses two structural features known in PGRPs, including a typical PGRP domain and two closely spaced conserved cysteines (Dziarski and Gupta, 2006b; Michel et al., 2001; Wang et al., 2003), and XI-PGRP-S and other short type PGRPs in amphibians were grouped together to form a separate clade in phylogenetic tree, indicating that XI-PGRP-S is indeed a kind of short-type PGRP in amphibians.

Although different PGRPs have very similar structural characteristics, they appear to exhibit distinct tissue-specific expression profiles. For instance, catfish PGRP-S was highly expressed in gill, whereas the high expression level of PGRP-6 was observed in spleen, gill, and liver (Sun et al., 2014). In contrast, both grass carp PGRP-S and PGRP-6 exhibited abundant expression in intestine (Li et al., 2013, 2014). Notably, the highest expression level of XI-PGRP-S was observed in muscle, a nonimmune organ, where tilapia, rock bream, and Chinese giant salamander PGRP-SC2 exhibited most abundant expression (Choi et al., 2018; Gan et al., 2016; Yang et al., 2018), implying that XI-PGRP-S and PGRP-SC2 in lower vertebrates may have similar functions regarding some fundamental physiological process in hosts. In addition, XI-PGRP-S is inducible in A6 cells in response to PGN stimulation, and recombinant XI-PGRP-S has an ability to bind PGN, reflecting the importance of XI-PGRP-S in the recognition of PGN.

One common function between invertebrate and vertebrate PGRPs is amidase activity, which hydrolyzes the amide bond between MurNAc and l-alanine in bacterial PGN (Royet and Dziarski, 2007). However, not all PGRPs possess amidase activity, and the experimental evidence from previous studies suggest that amidase-active PGRPs must contain four conserved Zn$^{2+}$-binding residues, including two histidines, one tyrosine, and one cysteine (Chang et al., 2004; Guan et al., 2005; Reiser et al., 2004). In the case of human, these four residues are conserved in PGLYRP-2, which has amidase activity (Wang et al., 2003). By contrast, PGLYRP-1, -3, and -4 are not amidase-active PGRPs, as they do not have all these four residues with serine instead of cysteine in the position (Wang et al., 2003). In the present study, the identified XI-PGRP-S harbours these four Zn$^{2+}$-binding residues, and recombinant XI-PGRP-S has been proven to have Zn$^{2+}$-dependent amidase activity, indicating that XI-PGRP-S is a kind of amidase-active PGRP.

Interestingly, amidase-active PGRPs from different species may play distinct roles in immune response during bacterial infection. Drosophila PGRPs with amidase activity are the key negative regulators of antibacterial response by targeting the IMD pathway (Bischoff et al., 2006; Paredes et al., 2011), whereas all known fish amidase-active PGRPs promote host defense against bacterial infection (Choi et al., 2018; Gan et al., 2016; Kim et al., 2010; Li et al., 2007, 2012, 2013). Consistent with this finding in fish, XI-PGRP-S not only possesses amidase activity, and also can inhibit the proliferation of E. tarda in vitro, providing important evidence to suggest that amidase-active PGRPs in lower vertebrates may function as the key effectors to promote antibacterial response in host.

In conclusion, a short-type PGRP, named XI-PGRP-S, was identified in X. laevis. XI-PGRP-S gene was constitutively expressed in all tissues examined, with the highest expression level observed in muscle, and was inducible after PGN stimulation. In addition, XI-PGRP-S has been proven to have PGN-binding activity, Zn$^{2+}$-dependent amidase activity and antibacterial activity against E. tarda. To our knowledge, the present study represents the first discovery on the function of PGRPs in frogs, thus contributing to a better understanding of the functional evolution of PGRPs in early tetrapods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2019.04.003.

Disclosures

The authors have no financial conflicts of interest.

References


