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A short-type peptidoglycan recognition protein from tongue sole (*Cynoglossus semilaevis*) promotes phagocytosis and defense against bacterial infection

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ABSTRACT

Peptidoglycan recognition proteins (PGRPs) are members of the innate immune system that interact with bacteria by binding to bacterial peptidoglycan. In this study, we examined the expression and function of a short type of PGRP, CsPGRP-SC2, from tongue sole (*Cynoglossus semilaevis*). CsPGRP-SC2 contains 164 amino acid residues and shares 54.5%–65.3% overall sequence identities with other teleost PGRPs. CsPGRP-SC2 possesses an amidase domain with a conserved zinc binding site. *CsPGRP-SC2* expression occurred in multiple tissues and was upregulated by bacterial and viral infection. Purified recombinant CsPGRP-SC2 (rCsPGRP-SC2) was able to bind and agglutinate Gram-positive bacteria in a Zn^{2+}-dependent manner. rCsPGRP-SC2 enhanced the uptake of the bound bacteria by host phagocytes and reduced bacterial dissemination and colonization in host tissues. These results indicate that CsPGRP-SC2 is an innate immune factor that participates in host defense against bacterial infection.

1. Introduction

Peptidoglycan recognition proteins (PGRPs) are pattern recognition receptors that recognize and interact with peptidoglycan, a bacterial cell wall component [1]. PGRPs are widely distributed in invertebrates and vertebrates [2–4]. In insect, PGRPs are classified into three types based on molecular weight, i.e. short-, intermediate-, and long-PGRPs (PGRP-S, PGRP-I, and PGRP-L, respectively), which exhibit molecular weights of approximately 20 kDa, 40–45 kDa, and 90 kDa, respectively [5]. In fruit fly, PGRPs are grouped into two classes: PGRP-SA, -SB1, -SB2, -SC1A, -SC1B, -SC2, and -SD, which have short transcripts, and PGRP-LA, -LB, -LC, -LD, and -LE, which have long transcripts [6]. In humans, four types of PGRPs have been identified, which were initially named PGRP-S, PGRP-L, PGRP-Iα, and PGRP-Iβ [2,5]. Subsequently, these proteins were re-named PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4, respectively [5].

All PGRPs contain a conserved peptidoglycan binding type 2 amidase domain, which is also called the PGRP domain. The PGRP domain is composed of approximately 165 amino acids and structurally homologous to bacteriophage and bacterial type 2 amidases, which hydrolyze the amide bond in peptidoglycan [2,7]. In general, short PGRPs contain mainly a single PGRP domain, while long PGRPs have one or two PGRP domains at the C-terminal region and a varied-sized N-terminal region that is not conserved. Crystal structure analysis revealed that the PGRP domain forms a peptidoglycan-binding groove, and that in amidase-active PGRPs, the peptidoglycan-binding groove contains a conserved Zn^{2+}-binding site consisting of two histidines, one tyrosine, and one cysteine [5]. Functionally, both invertebrate and vertebrate PGRPs are known to act as antimicrobial factors, and some mammalian PGRPs, such as bovine PGLYRP-1, exhibit killing activity against a wide range of Gram-positive and Gram-negative bacteria [8–10].

To date, only three types of PGRPs, i.e. PGLYRP-2, PGLYRP-5, and PGLYRP-6, have been identified in teleost including channel catfish, grass carp, large yellow croaker, red drum, rainbow trout, and zebrafish [11–19]. PGLYRP-2 is homologous to mammal PGLYRP-2, while PGLYRP-5 and PGLYRP-6 are only found in teleost [11]. PGLYRP-5 is also known as PGRP-SC, which has been identified in zebrafish and grass carp and is involved in defense against bacterial infection [14,19]. In addition, zebrafish PGRP-SC is able to regulate

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immune responses and other processes such as development and apoptosis [20].

Tongue sole (*Cynoglossus semilaevis*) is one of the most popular and economically important fish species farmed in China. However, the working mechanism of the immune system in tongue sole is unclear. In the current study, with an aim to identify factors involved in innate immune defense against bacterial infection, we examined the expression and function of a PGRP homologue of the SC2 type (named CsPGRP-SC2) identified in tongue sole. We found that CsPGRP-SC2 expression was regulated by pathogen infection, and that CsPGRP-SC2 agglutinated Gram-positive bacteria, facilitated phagocytosis, and promoted host resistance against bacterial infection.

2. Materials and methods

2.1. Fish

Clinically healthy tongue sole (average 12.5 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. The pH and salinity of the seawater were 7.8 and 32.5‰. Before experiment, fish were randomly sampled and verified to be absent of bacterial pathogens in liver, kidney, and spleen as reported previously [21]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA).

2.2. Sequence analysis

The cDNA and amino acid sequences of CsPGRP-SC2 (GenBank accession no. XP_008306717.1) were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 [22] and the conserved domain search program of NCBI. Multiple sequence alignment was created with ClustalX.

2.3. Quantitative real time reverse transcription-PCR (RTqPCR)

Kidney, blood, intestine, gill, spleen, brain, muscle, heart, and liver were taken aseptically from five tongue sole (as described above) and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, USA). RTqPCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript RTqPCR Kit (Takara, Dalian, China) as described previously [23]. Beta-actin (ACTB) was used as an internal reference [23]. The mRNA level of ACTB was calculated with the $2^{-\Delta\Delta CT}$ method [24]. For bacterial infection, *Streptococcus iniae* SF1 [25] was cultured in Luria-Bertani broth (LB) medium at 28 °C to OD_{600} 0.8; the cells were washed with PBS and resuspended in PBS to 1×10^6 CFU/ml. For viral infection, the fish viral pathogen megalocytivirus RBIV-C1 [26] was resuspended in PBS to 5×10^5 copies/ml. The copy number of the virus was determined as reported previously [27]. Tongue sole (as described above) were divided randomly into three groups and injected intraperitoneally with 50 μl *S. iniae*, megalocytivirus, or PBS. Kidney and spleen were taken from the fish (five at each time point) at 6 h, 12 h, 24 h, and 48 h post-bacterial infection and at 1 d, 3 d, 5 d, and 7 d post-viral infection. RTqPCR analysis of CsPGRP-SC2 expression in the tissues was performed as above. For bacterial infection, the internal reference genes for kidney and spleen were ACTB and ribosomal protein L18 (RPL18), respectively [23]; for viral infection, the internal reference genes for kidney and spleen were ACTB [23]. The experiment was performed three times, each time with five fish.

2.4. Construction of pEtCsPGRP-SC2

To construct pEtCsPGRP-SC2, which expresses His-tagged recombinant CsPGRP-SC2 (rCsPGRP-SC2), the coding sequence of CsPGRP-SC2 was amplified by polymerase chain reaction (PCR) with primers PGRPF (5′-GATATCATGGAGTCGCAGGTGACCAT-3′, underlined sequence, EcoRV site) and PGRPR (5′-GATATCGCTCAGCTGTGGAAGTGCAG-3′, underlined sequence, EcoRV site); the PCR products were ligated with the T-A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the CsPGRP-SC2-containing fragment, which was inserted into pET259 [28] at the SwaI site, resulting in pEtCsPGRP-SC2.

2.5. Purification of recombinant CsPGRP-SC2 (rCsPGRP-SC2) and Trx (rTrx)

Escherichia coli BL21(DE3) (purchased from TransGen Biotech Beijing, China) was transformed with pEtCsPGRP-SC2 and pET32a (Novagen, San Diego, USA), the latter expressing Trx. rTrx, which was purified under the same condition as rCsPGRP-SC2, was used in this study as a control protein for rCsPGRP-SC2. The transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 16 °C for overnight, the cells were harvested by centrifugation, and His-tagged proteins were purified using Ni-NTA Agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer. The proteins were treated with Triton X-114 to remove endotoxin as reported previously [29]. The proteins were then concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, USA). The concentrated proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250.

2.6. Agglutination

Micrococcus luteus was purchased from China General Microbiological Culture Collection Center (CGMCC). Agglutination assay was performed as reported previously [28]. Briefly, *M. luteus* and *S. iniae* were cultured in LB medium at 37 °C and 28 °C, respectively, to an OD_{600} of 0.8. The cells were washed with PBS for three times and resuspended in PBS or PBS containing 10 μM $ZnCl_2$ (PBS—Zn) to 10^9 CFU/ml. rCsPGRP-SC2 and rTrx were added to the bacterial cells to the final concentration of 10 μM. After incubation at 25 °C for 2 h, bacterial cells were stained with 4,6-diamino-2-phenyl indole (DAPI) (Invitrogen, USA), and agglutination was observed with a fluorescence microscope (Nikon E800, Japan).

2.7. Binding of rCsPGRP-SC2 to bacterial cells

Binding of rCsPGRP-SC2 to bacterial cells was performed as reported previously [28]. Bacterial cells were cultured in LB medium as above. The cells were washed and diluted in PBS to 10^8 CFU/ml. Four hundred microliters of bacterial suspension was dropped onto a glass slide, and the slide was incubated at room temperature for 4 h rCsPGRP-SC2 and rTrx were diluted to 10 μM in PBS containing 10 μM $ZnCl_2$. Three hundred microliters of each of the diluted proteins were added to the slide. The slide was incubated at 25 °C for 1 h and washed three times with PBS. The cells were fixed, treated with mouse anti-His antibody and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Bioss, Beijing, China) (1/1000 dilution), followed by observation with a fluorescence microscope (Nikon E800, Japan) as reported previously [28]. The experiment was repeated three times.