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Rainbow trout peptidoglycan recognition protein has an anti-inflammatory function in liver cells

Ju Hye Jang, Hyun Kim, Ju Hyun Cho*

Department of Biology, Research Institute of Life Science, Gyeongsang National University, 501 Jinju Dae-ro, Jinju 660-701, South Korea

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ABSTRACT

Peptidoglycan recognition proteins (PGRPs) are innate immune molecules that are structurally conserved through evolution in both invertebrate and vertebrate animals. PGRPs exert diverse host-defense functions both through direct antibacterial activity and through indirect effects, including the induction of antimicrobial peptides and the modulation of inflammation and immune responses. In this study, we identified the gene encoding a long form of PGRP (OmPGRP-L1) from the rainbow trout, *Oncorhynchus mykiss*, and investigated whether it has immunomodulating activity in a rainbow trout hepatoma cell line RTH-149 challenged with fish pathogenic bacteria. OmPGRP-L1 contains the conserved PGRP domain and the four Zn^{2+}-binding amino acid residues required for amidase activity. In RTH-149 cells, OmPGRP-L1 expression was increased by bacterial stimulation. Loss-of-function and gain-of-function experiments indicated that OmPGRP-L1 is involved in the expression of pro-inflammatory cytokines. Silencing of OmPGRP-L1 in RTH-149 cells challenged with *Edwardsiella tarda* dramatically increased the expression of IL-1β and TNF-α. In contrast, overexpression of OmPGRP-L1 or its amidase-inactive mutant OmPGRP-L1(C472S) resulted in down-regulation of IL-1β and TNF-α expression. When overexpressed in RTH-149 cells, OmPGRP-L1 inhibited NF-κB activity with or without bacterial stimulation. Collectively, these findings suggest that OmPGRP-L1 has an anti-inflammatory function, independent of its amidase activity, possibly via NF-κB inhibition in liver cells.

1. Introduction

The immune system provides protection from a wide range of pathogens. One component of immunity, the phylogenetically ancient innate immune response, fights infections from the moment of first contact and is the fundamental defensive weapon of multicellular organisms [1]. Innate immunity can detect the pathogen and start a rapid defensive response through pattern recognition receptors (PRRs), which detect pathogens via the recognition of pathogen-associated molecular patterns (PAMPs) [2,3]. PAMPs include polysaccharides, lipopolysaccharide (LPS), peptidoglycan (PGN), bacterial DNA and double stranded viral RNA and other molecules not normally found on the surface of multicellular organisms [4]. Among them, PGN, a polymer of β (1-4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid, is an essential and unique component of the cell walls of virtually all bacteria. Since eukaryotic organisms do not contain PGN in their cellular structures, PGN is an ideal target molecule for detecting bacterial invasion in eukaryotic organisms [5]. For this purpose, both invertebrate and vertebrate animals possess proteins specifically recognize PGN, known as peptidoglycan recognition proteins (PGRPs) [6–8].

The first PGRP was discovered in the hemolymph of a silkworm (*Bombyx mori*) by Ashida and coworkers in 1996 [6]. Subsequently, PGRP homologues have been identified in many species especially in insects and mammals [7,9–12]. These studies revealed similarities and differences between invertebrates and vertebrates. The genome sequencing of *Drosophila melanogaster* revealed 13 PGRP genes that are transcribed into 19 proteins [5,10]. These PGRPs play a role in activating the immune system in various ways. For example, PGRP-SA induces drosomycin and defensin through Toll pathway [13]. PGRP-LC also induces antimicrobial peptide synthesis, such as diptericin and cecropin, and apoptosis through Imd pathway [14,15], while PGRP-SC1 activates phagocytosis [16]. Additionally, PGRP-SC1, -LB, and -SB1 are a PGN-lytic enzyme, *N*-acetylmuramoyl-L-alanine amidase that functions to prevent excessive activation of the immune system by bacteria [17–19]. Therefore, PGRPs are vitally important to the immune system of invertebrates that do not have adaptive immunity. Mammals have four PGRPs, PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4 [7,11].

* Corresponding author. Tel.: +82 55 772 1347; fax: +82 55 772 1349.
E-mail address: juhyun.cho@gnu.kr (J.H. Cho).

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Compared to the insect PGRPs, the mammalian PGRPs are originally believed to function as effector molecules rather than inducers of signaling cascades in antimicrobial defenses, because mammals have other PRRs that recognize PGN, such as Toll-like receptor (TLR) 2 and nucleotide-binding oligomerization domain-containing proteins (NODs) [20]. PGLYRP-1, PGLYRP-3, and PGLYRP-4 are directly bactericidal [21–23], whereas PGLYRP-2 is an *N*-acetyl-muramoyl-$_L$-alanine amidase that hydrolyzes PGN [24,25]. Recently, however, other functions of mammalian PGRPs, which are independent of their bactericidal and PGN-hydrolytic activities, have been reported. For example, porcine PGLYRP-2 has been implicated in regulating β-defensin expression [26], and mouse PGLYRP-2 has pro-inflammatory properties in PGN-induced arthritis [27].

Teleost fish occupy a key evolutionary position in the development of the immune responses in that they are the earliest class of vertebrates possessing the elements of both innate and adaptive immunity [28]. Therefore, teleost fish are a good model to study the basic functions of various components of the innate immune response such as PGRPs. Recently, several PGRPs have been identified in teleost fish, including zebrafish (*Danio rerio*) [29], pufferfish (*Tetraodon nigroviridis*) [30], rockfish (*Sebastes schlegeli*) [31], and large yellow croaker (*Pseudosciaena crocea*) [32]. However, little is known about the function of fish PGRPs except that fish PGRPs, unlike mammalian PGRPs, have both amidase and bactericidal activities in one molecule [29,31]. Ectothermic fish depend more heavily on innate immunity against bacterial infection compared to the endothermic vertebrates such as mammals due to the temperature constraint on fish metabolism. Indeed, it has been recently reported that the innate immunity is essential in defense against infections in zebrafish, and that the responsible innate immune component is the PGRP family [29]. Zebrafish Pglyrp2 is highly expressed in eggs and in developing oocytes, and Pglyrp5 is induced during early embryogenesis and is strongly expressed at 72 h post fertilization. These proteins protect the developing embryo from infection by bacteria that are present in the surrounding water [29]. Fish PGRPs might also affect multiple intracellular pathways. Chang et al. reported that inhibition of zebrafish Pglyrp5 expression in the developing embryo using small interfering RNA modified the expression of genes involved in several pathways, including immune and inflammatory responses, signaling pathways, transcription and metabolism [33,34]. Inhibition of Pglyrp5 increases the expression of TLR2, TLR3, MAPK-interacting serine/threonine kinase 2, the interleukin-17 receptor and NF-κB [34], thereby indicating that fish PGRPs may directly or indirectly down-regulate the immune response to bacteria, thus preventing a constant state of inflammation. However, additional studies confirming these roles of fish PGRPs are needed. In this study, we identified a long form of PGRP (OmPGRP-L1) from the rainbow trout (*Oncorhynchus mykiss*), and showed, by silencing and overexpressing this protein, that OmPGRP-L1 down-regulates the expression of pro-inflammatory cytokines IL-1β and TNF-α, independently of its amidase activity in a rainbow trout hepatoma cell line RTH-149 challenged with fish pathogenic bacteria.

2. Materials and methods

2.1. Fish cell culture

The rainbow trout hepatoma cell line RTH-149 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Eagle's minimal essential medium (EMEM), supplemented with non-essential amino acids, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM HEPES, 10,000 units/mL penicillin and 10 mg/mL streptomycin. Cells were grown at 19 °C in the absence of CO_2. Trypsin-EDTA (0.05%) was used to detach cells in subculturing. All the cell culture media and reagents were purchased from Lonza (Basel, Switzerland).

2.2. Cloning cDNA and genomic sequences of OmPGRP-L1

Total RNA was extracted from rainbow trout fingerlings (10 g each, provided from a fish farm near Geochang, Korea), using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). A first-strand cDNA was synthesized from 1 μg of total RNA using a CapFishing Kit (Seegene, Seoul, Korea) according to the manufacturer's procedure. The expressed sequence tag (EST) sequences in the Computational Biology and Functional Genomic Laboratory (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi) were searched using the rockfish SsPGRP-L2 protein sequence (GenBank Accession number GU126382) as a bait sequence for TBLASTX analysis. Several candidate ESTs were found with high sequence homology to known PGRPs, among which a EST sequence BX868341 (*O. mykiss*) aligned well with the C terminal region of SsPGRP-L2. Based on the sequence of a partial rainbow trout PGRP (BX868341), a forward primer (FP) was designed and used as a gene specific sense primer for 3′-RACE. The 3′-RACE product was cloned into pGEM-T easy vector (Promega, Madisom, WI, USA) and the selected clone was sequenced in both directions by using T7 and SP6 primers. Based on the sequence of the partial cDNA clone obtained from the 3′-RACE, an intragenic primer (IGP) was designed, and used as a gene-specific primer for 5′-RACE to get the 5′-end of the putative PGRP fragment. The 5′-RACE product was cloned and sequenced as described above. Then, a pair of primers was designed based on tentative full-length cDNA (FL-1F/FL-1R), and the sequence of resulting PCR product (named OmPGRP-L1) was verified again.

Genomic DNA was extracted from rainbow trout fingerlings using an i-genomic CTB DNA extraction mini kit (Intron, Seongnam, Korea) according to the manufacturer's procedure. The genomic sequence of OmPGRP-L1 was amplified from the prepared genomic DNA using the primer set (FL-1F/FL-1R). An mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/sidey/) was employed to form the organization of OmPGRP-L1 gene. All primers used in the study were listed in Table 1.

2.3. Sequence analysis

The deduced amino acid sequence of OmPGRP-L1 was searched for similarity using BLAST program at the ExPASy Molecular Biology Server (http://www.expasy.org/tools/blast/), and the presence of signal peptide was assumed by the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Alignment of multiple sequences was performed using the MEGALIGN program within Lasergene (DNASTAR, Madison, WI, USA) and a phylogenetic tree was constructed using the MEGA4 Neighbor-joining [35]. All sequences used in the analysis were listed in Table 2.

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR

To determine tissue expression pattern of OmPGRP-L1, gill, kidney, spleen, head kidney, liver, intestine, skin and eye were collected separately from two healthy fish for RNA extraction. To examine the variation in expression of OmPGRP-L1 in RTH-149 cells following the stimulation with heat-killed bacteria (*Edwardsiella tarda* ATCC 15947 and *Streptococcus iniae* ATCC 29178), 1×10^6 RTH-149 cells were seeded per well into 6-well plates, and stimulated for 0, 4, 8, 12, 24 and 48 h with 2×10^8 cells/ml of heat-killed bacteria (70 °C, 30 min). Heat-killed bacteria were used to stimulate cells, because heat inactivation of bacteria has been established