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A novel lipopolysaccharide-binding protein (LBP) gene from sweetfish *Plecoglossus altivelis*: Molecular characterization and its role in the immune response of monocytes/macrophages

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ABSTRACT

Lipopolysaccharide-binding protein (LBP) belongs to the lipid transfer/LBP (LT–LBP) family, and plays a crucial role in the recognition of bacterial components that modulate cellular signals in phagocytic cells. Although several LBPs have been identified in teleosts, the effects of LBP homologs on teleost phagocytic cells are still obscure. Here, we report the cloning a novel full-length cDNA sequence of LBP-like protein (paLBP) gene from sweetfish, *Plecoglossus altivelis*. The paLBP cDNA encoded a 464 aa polypeptide, which was closest to that of rainbow smelt (*Osmerus mordax*). paLBP mRNA was detected mainly in the spleen, liver, and head kidney and levels dramatically increased in various tissues after *Listonella anguillarum* infection. In contrast to mammalian studies, paLBP mRNA could also be detected in sweetfish monocytes/macrophages. Recombinant paLBP showed LPS-binding activity and Western blot results revealed a significant increase of paLBP in the supernatant of sweetfish monocytes/macrophages challenged with *L. anguillarum*. Moreover, paLBP neutralization led to up-regulation of IL-1β and TNF-α mRNA as well as respiratory burst activity in sweetfish monocytes/macrophages in response to *L. anguillarum* or LPS challenge. Therefore, these results suggest that paLBP is an inducible acute-phase protein mediating the immune response of sweetfish monocytes/macrophages upon bacterial challenge.

1. Introduction

An important function of innate immunity in animals is to recognize invariant pathogens in a coordinated fashion and to eradicate them. The effectiveness of a particular pathogen defense relies on the subcellular compartment occupied by the pathogen [1]. Lipopolysaccharide (LPS) is the main component of the asymmetric outer membrane in gram-negative bacteria. As the infection progresses, bacteria spread through the blood and lymph, and release LPS from their membranes into the circulation of the host [2]. LPS commonly induces a wide series of biological responses in various organisms, including mammals and fish [3]. The main target cells of LPS in the innate immune system are professional phagocytes like macrophages [2].

Two mammalian proteins, lipopolysaccharide-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI) have been shown to recognize LPS. They belong to the lipid transfer/LBP (LT–LBP) family of proteins [4]. LBP opsonizes LPS-bearing particles for subsequent binding to CD14 on the surface of phagocytes [5]. In addition, LBP also assists the binding of LPS to the soluble form of CD14 [6]. Although the CD14-dependent pathway is important in LBP signaling, LBP can also play a role in alveolar macrophages in the absence of CD14, suggesting a CD14 independent pathway for LBP action [7]. Bactericidal/permeability increasing protein (BPI), which is similar to LBP in sequence, can also bind to LPS [8]. BPI acts to damage bacterial membranes and opsonizes particles for phagocytosis [9,10]. Both LBP and BPI have two common domains: an N-terminal BPI/LBP/Cholesteryl ester transfer protein (CETP)-specific sequence, and a C-terminal BPI/LBP/CETP domain [11].

In the innate immune response, monocytes and macrophages play an essential role in recognizing pathogens. These cells can recognize bacterial components such as LPS, and activate the innate immune system by releasing an array of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 [12]. LBP binds and transfers LPS to toll-like receptor 4 (TLR4) via CD14, and other proteins on the surface of mammalian

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monocytes or macrophages [13]. TLR4 triggers signaling pathways that produce inflammatory cytokines in response to LPS [14]. Interestingly, LBP has different effects on the LPS response depending on their relative concentrations and the cellular environment [5]. In mammals, LBP expression is limited to a few organs and tissues, such as the liver and intestinal epithelial cells [15]. Besides mammals, many members of LT–LBP family have been identified in other vertebrates including fish, birds, and also various invertebrates [16]. Many non-mammalian LBP members have the basic "boomerang" two-domain fold present in human LBP [17]. In fish, several LBPs have been cloned in rock bream (*Oplegnathus fasciatus*), tiger puffer (*Takifugu rubripes*), Japanese ricefish (*Oryzias latipes*), Nile tilapia (*Oreochromis niloticus*), southern platyfish (*Xiphophorus maculatus*), rainbow smelt (*Osmerus mordax*), zebrafish (*Danio rerio*), and other species. Recently, a teleost LBP of rock bream (*O. fasciatus*) was detected in all tested tissues including blood, kidney, spleen, liver, intestine, gill, and muscle [18]. However, the molecular characterization and cellular function of other LBPs in teleosts are still obscure.

Sweetfish is an economically important fish in East Asian countries. Bacterial diseases caused by *Listonella anguillarum* have been a major cause of losses in the sweetfish culture industry [19]. Since the important role of monocytes and macrophages in the fish immune response, the sweetfish monocyte/macrophage transcriptome has been recently characterized [20]. Interestingly, the CD14 transcript, which is highly expressed in mammalian monocytes/macrophages, could not be detected in the sweetfish monocyte/macrophage transcriptome. This raises the interesting question of how LBP regulates LPS signaling pathway in sweetfish. Here, we cloned a novel LBP (paLBP) from sweetfish and studied its possible role involved in the immune response. Moreover, we investigated the effects of paLBP on monocytes/macrophages in response to *L. anguillarum* or LPS challenge.

2. Materials and methods

2.1. Fish

About 100 healthy sweetfish, weighing 40–50 g each, were obtained from a commercial farm in Huangtan Reservoir, Ningbo city, China. Fish were kept in 100 L tanks at 20–22 °C. The fish were regularly fed with pelleted dry food once a day, and acclimatized to laboratory conditions for two week before experiments. The fish used for investigation were healthy without any history of pathological signs and the experiment was approved by the Committee on Animal Care and Use and the Committee on the Ethics of Animal Experiments of Ningbo University.

2.2. Primary culture of sweetfish head kidney-derived monocytes/macrophages

Sweetfish head kidney-derived monocytes/macrophages were isolated and cultured as previously described [21] with minor modifications. Briefly, sweetfish were anaesthetized and sacrificed to obtain head kidney. Then, head kidney tissues were cut into small pieces and pushed through a 100 μm nylon mesh. The cell suspension was layered onto Ficoll (Invitrogen, Shanghai, China), following centrifugation at 2000 rpm for 25 min at room temperature to collect the band of cells layering above the interface. The cells were seeded on 35-mm culture plates. Non-adherent cells were washed off and the attached cells were incubated with RPMI 1640 medium containing 6% fetal calf serum, 4% sweetfish serum, and 1% P/S throughout the experiment after overnight incubation at 24 °C. According to Giemsa staining results, over 96% of adherent cells were monocytes/macrophages.

2.3. Molecular cloning of paLBP cDNA

We used the LBP cDNA sequence of rainbow smelt (*O. mordax*) BT074556 to screen a transcriptomic database of sweetfish head kidney-derived monocytes/macrophages [20] and BLAST analysis revealed only one related unigene. Total RNA was extracted from sweetfish monocytes/macrophages using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. The first-strand cDNA was synthesized from total RNA of monocytes/macrophages using M-MLV reverse transcriptase (TaKaRa). The full-length cDNA sequence of paLBP was subsequently confirmed by using the rapid amplification of cDNA ends method (TaKaRa) and the sequence specific primers (paLBP-C) (Table 1). The PCR amplification product was sequenced using an ABI 3730 automated sequencer (Invitrogen). Multiple alignments were analyzed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [22].

2.4. Bacterial challenge

L. anguillarum was grown in nutrient broth on a rotary shaker at 200 rpm and 28 °C, and harvested in the logarithmic phase of growth, which was monitored by optical density assay and plate counting. *L. anguillarum* were washed, resuspended, and diluted to the appropriate concentration in sterile PBS. The final concentration of bacteria was verified by serial dilutions and plating on solid media. Sweetfish were challenged by intraperitoneal injection of *L. anguillarum* (3.8×10^5 CFU/ml in 100 μl PBS) and PBS was used as control. After injection 4, 8, 12, and 24 h, we collected sweetfish tissues including brain, gill, heart, head kidney, liver, and spleen and preserved them in at −80 °C until further examination.

2.5. Real-time quantitative PCR (RT-qPCR)

RT-qPCR of target genes was performed in a RT-Cycler™ Real-time Fluorescence Quantitative PCR thermocycler (CapitalBio, Beijing, China). Total RNA was extracted from sweetfish tissues and monocytes/macrophages using RNAiso reagents (TaKaRa), followed by deoxyribonuclease I digestion. The primers of target genes including LBP, TNF-α, IL-1β, and reference gene β-actin from sweetfish are listed in Table 1. Amplifications were carried out in a 25 μl reaction volume, containing sample cDNA, primers, SYBR premix Ex Taq (Perfect Real Time) (TaKaRa). The reaction mixture was incubated for 300 s at 95 °C, followed by 40 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C. Ct values of LBP, TNF-α,

Table 1
Oligonucleotide primers used in this work.

Primers	Nucleotide sequence (5′–3′)[a]	Sequence information
paLBP-C	TCCCGTTCAGCGCATCCGCA	Sequence confirmation
paLBP-T (+)	GGTCTCCATCTCAGGGAACA	RT-qPCR
paLBP-T (−)	GGCTGCAGTCTTGAAAGGTC	RT-qPCR
TNF-α (+)	ACATGGGAGCTGTGTTCCTC	RT-qPCR
TNF-α (−)	GCAAACACACCGAAAAAGGT	RT-qPCR
IL-1β (+)	TACCGGTTGGTACATCAGCA	RT-qPCR
IL-1β (−)	TGACGGTAAAGTTGGTGCAA	RT-qPCR
β-actin-T (+)	TCGTGCGTGACATCAAGGAG	RT-qPCR
β-actin-T (−)	CGCACTTCATGATGCTGTTG	RT-qPCR
paLBP-P (+)	CCATATGTTGTCCCACCTTCTGTT	Prokaryotic expression
paLBP-P (−)	CCTCGAGTTAGACATCAAAGTTGCTGGAA	Prokaryotic expression

[a] The underlined sequences represent the restriction sites for *NdeI* and *XhoI*, respectively.