Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Immunology 43 (2006) 2061–2069
Molecular Immunology
www.elsevier.com/locate/molimm

Cloning and characterization of a LPS-regulatory gene having an LPS binding domain in kuruma prawn *Marsupenaeus japonicus*

Hiroki Nagoshi [a], Hiroyuki Inagawa [a,b,c], Kayoko Morii [a], Hideaki Harada [a], Chie Kohchi [b,c], Takashi Nishizawa [b], Yoshie Taniguchi [b], Maya Uenobe [b], Teruko Honda [d], Masakazu Kondoh [a], Yukinori Takahashi [a], Gen-Ichiro Soma [b,c,*]

[a] *National Fisheries University, Applied Aquabiology, 2-7-1 Nagata-Honmachi Shimonoseki, Yamaguchi 759-6595, Japan*
[b] *Institute for Health and Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima 770-8514, Japan*
[c] *Institute for Drug Delivery Systems, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*
[d] *Department of Histology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Honjo, Kumamoto 860-8556, Japan*

Received 13 November 2005; received in revised form 13 December 2005; accepted 20 December 2005
Available online 25 January 2006

Abstract

LPS is known as an effective stimulator of the immune system in various animals, including mammals and horseshoe crabs (HSC). Both of these animal groups have suppressive regulatory proteins for the LPS response, e.g. the bactericidal/permeability increasing protein in mammals and anti-LPS factor (ALF) in HSC. Prawns are a valuable aquaculture species, but the regulatory molecules and/or mechanisms that respond to LPS are largely unknown. To investigate the molecular mechanism of the LPS response in kuruma prawns, we cloned a cDNA having a LPS binding domain. A full-length cDNA gene, denoted as M-ALF (*Marsupenaeus japonicus* ALF-like peptide) was cloned that consisted of 746 bp and encoded 123 amino-acid residues. The 3′ non-translated region of this gene had the pentamer of ATTTA repeated four times; this is known as sequences for messenger RNA stabilization. Deduced amino-acid sequences showed a 42% homology with Japanese HSC-ALF. In particular, both have clusters of basic and hydrophobic amino acids, indicating that the region is probably binding to lipid A. The mRNA expression was determined for hemocytes, lymphoid organs, hearts, intestines and gills by RT-PCR. The mRNA expression was augmented 1.5–3 h after LPS administration in lymphoid organs, but then decreased to normal level at 6 h. Synthetic peptides containing Cys30 to Cys51 had LPS neutralizing activity to the *Limulus* reaction and NO production in RAW264.7 cells. These data suggest that in kuruma prawns, M-ALF acts as a LPS regulator during the acute phase response after invasion of pathogens.

Keywords: LPS binding protein; Innate immunity; Comparative immunology; LPS inducible protein

1. Introduction

Innate immunity is the first line of defense against infection by pathologic microbes. The essential initiating step of the innate immune response is recognition of invasive pathogens followed by activation of cellular responses that leads to elimination of the microbes. Following recognition of a specific bacterial product such as lipopolysaccharide (LPS), there are two different functional methods for the immune response (either stimulation or inhibition). In a variety of animals, LPS appears to be the most effective bacterial product for activating the innate immune responses. Vertebrates, and mammals in particular, have an extremely high susceptibility to LPS. Concentrations as low as 80–100 pg/ml of LPS initiated production of TNF and LFA-1 by macrophages in vitro (Dery et al., 2000; Strassmann et al., 1985). LPS binding protein (LBP) is well characterized in its role of constructing LPS–LBP complexes, which bind to LPS receptors, CD14-Toll like receptor-4 (TLR-4) and MD-2 complexes on phagocytes. These complexes are more efficient for inducing phagocyte activation than LPS alone (Jia et al., 2004).

Mammals also have a bactericidal/permeability increasing protein (BPI) which strongly binds LPS and suppresses LPS

Abbreviations: BPI, bactericidal/permeability-increasing protein; LBP, LPS binding protein; LPS, lipopolysaccharide; M-ALF, kuruma prawn (*Marsupenaeus japonicus*) anti-LPS factor

* Corresponding author. Tel.: +81 88 622 9611; fax: +81 88 622 3217.
E-mail address: sma5628@tokushima.bunri-u.ac.jp (G.-I. Soma).

0161-5890/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2005.12.009

activity as part of the stimuli of innate immune responses (Weiss, 2003). It has been reported that invertebrates (represented by the horseshoe crab) have high sensitivity to LPS and have proteins that exhibit the same function as BPI. In horseshoe crabs, small amount of LPS (0.1 pg/ml) interacts with a factor of the coagulation system, and this is followed by sequential activation of proteases to generate proactivator and proclotting enzymes, which ultimately results in clotting of the blood (Nakamura et al., 1977; Niwa et al., 1990; Roth and Levin, 1992). In addition to the hemocytes, horseshoe crabs have LPS binding proteins that act as suppressive regulators of coagulation. Research papers have reported the purification and functional analyses of a protein from the hemocytes of Japanese horseshoe crab (*Tachypleus tridentatus*; T-ALF) and Atlantic horseshoe crab (*Limulus polyphemus*; L-ALF). It was named the anti-LPS factor (ALF), and it could neutralize LPS activity by forming a clot (Morita et al., 1985; Ohashi et al., 1984; Wang et al., 2002; Warren et al., 1992; Weiss et al., 2000). An analysis of the crystal structure indicated that the LPS binding domain of L-ALF is presented in a loop conformation (Hoess et al., 1993). Both L-ALF and BPI exhibit neutralizing activity to LPS and share substantial sequence homology between the LPS binding region of each molecule (Weiss et al., 2000).

The mechanisms by which LPS either stimulates or suppresses innate immune responses have been intensively investigated in the horseshoe crab and in mammals. Molecular information is quite limited for crustaceans, even though they are very important economically in world fisheries. Previously, we reported that oral administration of low molecular weight LPS (*Pantoea agglomerans* LPS: LPSp) to kuruma prawns, *Marsupenaeus japonicus*, could induce significant protective effect from infection by white-spot virus (WSV) (Takahashi et al., 2000), a virus which has caused mass prawn mortalities in culture operations (Tsai et al., 2004). As little as 20 μg/kg body weight/day oral administration of LPSp was sufficient to develop a significant protective effect against WSV and to enhance phagocytosis by granulocytes (a hemocyte) in kuruma prawns (Takahashi et al., 2000). Moreover, as little as 5 pg/ml of LPSp induced significant amounts of nitric oxide (NO) by kuruma prawn granulocytes in vitro (Itami et al., 2001). These facts indicate that kuruma prawn has molecule(s) that have a high affinity to LPS and that stimulate innate immune responses (as was also observed in mammals and horseshoe crabs). There is no information on suppressive regulatory molecules related to LPS and its receptors in crustaceans, though recently there was a report on the antibacterial activity of a recombinant ALF protein in tiger shrimp (Somboonwiwat et al., 2005). However, the LPS neutralization activity of this protein was not clarified.

As part of the research for this paper, we cloned a cDNA of kuruma prawn that codes a homologue of the anti-LPS factor of horseshoe crab, denoted as M-ALF. The kuruma prawn anti-LPS factor-like peptide encoded 123 amino acids including 25 signal peptides. The M-ALF gene could be induced after LPS administration and by *Vibrio penaeicida* infection. Moreover, the synthetic 24mer peptide (based on amino-acid sequence of LPS binding site estimated from the homology of amino-acid sequence of L-ALF) had LPS neutralizing activity.

2. Materials and methods

2.1. Prawn

Healthy kuruma prawns, *M. japonicus*, weighing 18–22 g (average 20 g), were used. They were maintained in a tank with fresh running water at 23.5–24.5 °C and fed commercial pelleted prawn food at a feeding rate of 1% of the total body weight per day.

2.2. RNA preparation and construction of a cDNA library from hemocytes

Whole hemolymphs were individually collected from the base of the walking legs of kuruma prawns using a 2.5 ml syringe with a 21-gauge needle preloaded with 1 ml of marine anticoagulant KHE buffer (510 mM NaCl, 10 mM HEPES, 10 mM EDTA, pH 7.6). The hemocytes were isolated by centrifugation (1000 × *g*, 10 min). Supernatant was decanted, and the cells were collected. Total RNA of the hemocytes from the kuruma prawns was isolated using a RNA extraction kit (Trisol, Invitrogen, Tokyo). Total RNA (5 μg) was subjected to synthesis of double-stranded cDNA using a SMART polymerase chain reaction (PCR) cDNA synthesis kit (Clonetech Laboratories Inc., CA), followed by ligation with an *Eco*RI adapter and with λZAPII vector (Stratagene, CA). After in vitro packaging with Gigapack Gold III (Stratagene), the library (5.8×10^9 pfu/ml) was amplified once before use.

2.3. Analysis of cDNA sequences and peptide structures

To check the inserted cDNA in λZAPII vectors, PCR was performed to amplify the inserted DNA with λZAPII primers (Takara Shuzo, Tokyo). Briefly, PCR was performed using a PC-700 (Astech, Japan) for 35 cycles of 1 min each at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. For sequence analysis, partially purified PCR products were analyzed by the dideoxy-chain termination method using a fluorescence DNA sequencer (Beckman CEP2000). The deduced peptides sequences were compared with those in the database by using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The cleavage sites of signal peptides were analyzed by the SignalP program (Version 1.1; Center for Biological Sequence Analysis http://www.cbs.dtu.dk/services/SignalP/). Protein alignment and percentage identities were calculated by Clustal W.

2.4. Synthetic peptides

From an intensive analysis of the biologically active site of L-ALF (the β-hairpin structure between Cys31 and Cys52), a lipid A binding portion was demonstrated using the various synthetic peptides (Andra et al., 2004; Dankesreiter et al., 2000). In this study, we also synthesized a putative LPS binding region of M-ALF (synM-ALF: GCNFYVEPKFRNWQLRFKGRMWCG, Invitrogen Corporation) with disulfide bonds between the 2nd and 23th cysteine residues, L-ALF