Fish & Shellfish Immunology 42 (2015) 335–344

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Activation of *Pm*Relish from *Penaeus monodon* by yellow head virus

Suwattana Visetnan [a], Premruethai Supungul [a, b], Ikuo Hirono [c], Anchalee Tassanakajon [a], Vichien Rimphanitchayakit [a, *]

[a] Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand
[b] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 10120, Thailand
[c] Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo 108-8477, Japan

ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form
12 November 2014
Accepted 13 November 2014
Available online 21 November 2014

Keywords:
Relish
Imd pathway
Yellow head virus
Penaeus monodon
Antimicrobial proteins

ABSTRACT

Humoral innate immune response against pathogenic infection is partly responsible by the Imd pathway in which a transcription factor Relish relays the infection signals to the nuclei for the expression of antimicrobial proteins. A *PmRelish* gene which encoded a protein of 1195 amino acids was cloned. The *PmRelish* was constitutively expressed in all tissues tested and mostly up-regulated upon YHV infection. In hemocytes, the *PmRelish* expression was up-regulated upon *Vibrio harveyi*, yellow head virus (YHV) and white spot syndrome virus (WSSV) challenges. Using dsRNA silencing of *PmRelish* gene, it was shown that the expression of *penaeidin5* but not anti-lipopolysaccharide factor *ALFPm3*, *crustinPm1* and *penaeidin3* was under the regulation of Imd pathway. Under *PmRelish* silencing, the shrimp were more susceptible to infection by YHV with the 50% survival rate reduced from about 72 h to 42 h. The *Pm*Relish was detected in the cytoplasm of all the hemocytes from both uninfected and YHV-infected shrimp. The accumulation of activated *Pm*Relish in the nuclei was not clearly observed but the activated *Pm*Relish was detected in the YHV-infected hemocytes by Western blot analysis. Thus, the *Pm*Relish and, hence, the Imd pathway respond to the YHV infection.

1. Introduction

Immediate responses to pathogenic infection of animals involve the innate immunity which consists of humoral and cellular responses, for example, blood coagulation system, agglutination, nodule formation, phagocytosis, prophenoloxidase activating system and various immune active proteins. Antimicrobial proteins are a group of immune active proteins in humoral response capable of inhibiting and eradicating the pathogens [1,2]. The synthesis of antimicrobial proteins in innate immunity in response to infection is signaled by two signaling pathways, the Toll and immune deficiency (Imd) pathways. The two signaling pathways have been studied extensively in insects, particularly the *Drosophila* [3,4].

The pathways begin with the sensing of infection in the circulating system by some pattern recognition proteins that recognize the common constituents of the pathogens such as peptidoglycans of both Gram-positive and Gram-negative bacteria, lipopolysaccharide of Gram-negative bacteria and β-glucans of fungi, bind and are activated. The signals of infection as the activated recognition proteins are carried to the antimicrobial protein-synthesizing cells by binding to the cell surface receptors which, in turn, activate in series the intracellular proteins involved in the pathways. The signals are relayed as the proteins in the pathways are activated. In the end, the NF-κB transcription factors, Dorsal/DIF of Toll pathway and Relish of Imd pathway are activated and are able to move into the nucleus to commence the synthesis of antimicrobial proteins [4]. The Toll pathway is responsible for sensing the invasion of Gram-positive bacteria with the Lys-type peptidoglycan and fungi. The Imd pathway is for the Gram-negative bacteria with the diaminopimelic acid (DAP)-type peptidoglycan as well as some Gram-positive bacteria that contain the DAP-type peptidoglycan [4].

In crustaceans, the two signaling pathways have only recently been recognized. Protein components of the pathways have been identified. For the Toll pathway, the Spätzle proteins from *Fenneropenaeus chinensis* Fc-Spz and *Litopenaeus vannamei* LvSpz1–3 are identified and they are up-regulated against Vibrio species and

* Corresponding author. Tel.: +66 2 2185436; fax: +66 2 2185418.
E-mail address: kpvr2@yahoo.com (V. Rimphanitchayakit).

http://dx.doi.org/10.1016/j.fsi.2014.11.015
1050-4648/

WSSV infection. They also affect the expression of certain antimicrobial proteins [5,6]. The Toll receptors from *Penaeus monodon* PmToll, *L. vannamei* LvToll1-3 and *Marsupenaeus japonicus* MjToll are found to express in all tissues [6–9]. The myeloid differentiation factor 88 (MyD88) from *Scylla paramamosain* SpMyD88 is able to bind SpToll while that from *F. chinensis* FcMyD88 is up-regulated transcriptionally against bacterial infection [10,11]. The LvPelle was cloned for functional study. It is up-regulated in gills against WSSV infection and able to activate *P. monodon* penaeidin promoter [12]. The Cactus from *F. chinensis* can regulate the expression of some antimicrobial proteins and antiviral factor [13]. The Dorsal from *L. vannamei* LvDorsal can regulate the transcription of shrimp penaeidin-4 gene [14].

For the Imd pathway, the IMDs from *L. vannamei* is able to induce the expression of penaeidin-4 [15]. Two IMDs from *F. chinensis* FcIMD and *Procambarus clarkia* PcIMD are involved in regulating the expression of crustins, anti-lipopolysaccharide factors and lysozymes in shrimp and crayfish, respectively [16]. The Relish from horseshoe crab *Carcinoscorpius rotundicauda* CrRelish is up-regulated by *Pseudomonas aeruginosa* infection [17]. Two isoforms of LvRelish, short and full length, can be found in *L. vannamei*. The LvRelish can regulate the transcription of penaeidin-4 [18]. A Relish from *F. chinensis* FcRelish is essential for the expression of penaeidin-5 [19]. The expression of Relish from *Eriocheir sinensis* EsRelish is induced by fungi and bacteria [20].

Similar to Dorsal, the NF-κB/Rel transcription factor Relish is a key signal mediator and a regulatory factor for the synthesis of cognate antimicrobial proteins. The Relish is able to move into the nucleus and activate the synthesis of antimicrobial proteins providing that it is activated. Normally, the full length Relish consists of a Rel homology domain (RHD), a nucleus localization signal, an IκB-like domain containing six ankyrin repeats and a death domain. The Relish is activated by cleaving away its C-terminal half, ankyrin repeats and a death domain, with a caspase enzyme [21,22].

In *Drosophila*, it has been shown that the Toll and Imd pathways are involved in antiviral responses. In particular, the Imd pathway responds to the RNA virus [23,24]. Studies in shrimp show that the Toll pathway and Imd pathway are also responses to viral infections. The expression of LvDorsal and LvRelish is significantly up-regulated by WSSV [25]. In this study, a Relish from *P. monodon* (*Pm*Relish) was characterized. The expression of *PmRelish* gene in tissues and in response to bacterial and viral infection were investigated. In particular, we tested whether the Imd pathway help protect the shrimp from YHV, an RNA viral pathogen of shrimp. The cellular localization and the activation of *Pm*Relish in YHV-infected hemocytes was also investigated.

2. Materials and methods

2.1. Shrimp, bacterium and viruses

Healthy black tiger shrimp with body weight of 5–7 g for gene silencing experiment and 10–15 g for other experiments were purchased from a local farm and acclimatized in aquaria at an ambient temperature of about 29 ± 1 °C and a salinity of 15 ppt for a few days before the experiments.

The *Vibrio harveyi* 639 suspension was prepared by culture the bacterium in tryptic soy broth until the OD_{600} reached 0.6. The number of colonies was determined by agar plating. The colony forming unit (CFU) was adjusted before the bacterium suspension was used in the experiment. The yellow head virus (YHV) suspension was the hemolymph drawn with anticoagulant from the moribund YHV-infected *P. monodon* according to Pongsomboon et al. (2008) [26]. In this study, the YHV suspension used induced a cumulative mortality of 50% within 3 days post-injection. The WSSV was prepared from gills of WSSV-infected *L. vannamei* according to Xie et al. (2005) [27] and the number of virions was determined by real time PCR.

2.2. Cloning and nucleotide sequencing of PmRelish

The *PmRelish* was PCR amplified from the hemocyte cDNA of 48-h YHV-infected shrimp using specifically designed primers. The cDNA was prepared from the shrimp hemocytes. The hemolymph was drawn from shrimp ventral sinus and centrifuged 250× g for 10 min at 4 °C to collect the hemocytes for total RNA extraction. The total RNA was extracted using Trizol® Reagent (Molecular Research Center) and treated with RNase-free DNase I (Promega). First-strand cDNA was synthesized from 1 μg of total RNA by a cDNA First-strand Synthesis Kit (Fermentas) according to the manufacturer's instruction.

For the ease of cloning the gene fragments and DNA sequencing, the *PmRelish* was amplified as two overlapping fragments. Two primer pairs of *PmRelish*, PmRelish_F1/PmRelish_R1 and PmRelish_F2/PmRelish_R2, were designed based on the Relish sequences from two shrimp species, *LvRelish* and *FcRelish* [18,19]. The PmRelish_F1 and PmRelish_R1 (Table 1) annealed to sequences a few bases upstream of start codon and at around the middle of Relish gene, respectively. The PmRelish_F2 and PmRelish_R2 (Table 1) annealed to sequences at around the middle of Relish gene and a few bases downstream of stop codon, respectively.

The PCR amplification was performed using 1 μl cDNA template in 50 μl reaction volume containing 1 × PCR buffer, 0.2 mM dNTP, 0.2 μM each primer and 1 × Advantage 2 Polymerase Mix) (Clontech). The mixtures were subjected to denaturation at 94 °C for 2 min, 5 cycles of 94 °C for 25 s, 62 °C for 45 s and 72 °C for 3 min, 30 cycles of 94 °C for 25 s, 60 °C for 45 s and 67 °C for 3 min, and the final extension step at 67 °C for 10 min. The uses of Relish_F1/Relish_R1 and Relish_F2/Relish_R2 in PCR gave rise to two gene fragments with overlapping sequences. The two gene fragments were cloned into pGEM-T easy vector (Promega) and sequenced using T7 promoter and M13 reverse primer by Macrogen Inc., South Korea. Then, the two sequences were connected using a restriction site *Kpn*I in the overlapping sequence. The connection of the *PmRelish* fragments also removed the primer sequences in the overlapping sequence.

Nucleotide and protein sequence similarities were analyzed with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The protein motifs features were predicted using Simple Molecular Architecture Research Tool SMART (http://smart.embl-heidelberg.de/).

2.3. Tissue distribution of PmRelish in normal and YHV-infected shrimp

The expression of *PmRelish* in six different shrimp tissues of normal and YHV-infected shrimp was determined by RT-PCR using the primers PmRelish_RT_F and PmRelish_RT_R (Table 1). Experiments were performed in triplicate for statistical analysis. Shrimp were challenged with YHV by injecting 100 μl of YHV suspension in 1 × phosphate buffered saline (PBS) into each shrimp through the abdominal muscle. The control shrimp were injected with 100 μl 1 × PBS.

At 48 h post infection (hpi) when the shrimp were in fully YHV-infected stage, the various tissues: hearts, hepatopancreata, hemocytes, gills, lymphoid organs and stomachs, were dissected out, homogenized in TRI® Reagent (Molecular Research Center) and total RNA isolated. The total RNAs obtained were subjected to cDNA synthesis as described above.