Fish & Shellfish Immunology 70 (2017) 720–730

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

The TLR13-MyD88-NF-κB signalling pathway of *Cyclina sinensis* plays vital roles in innate immune responses

Yipeng Ren [a, b], Dan Ding [a], Baoping Pan [a, *], Wenjun Bu [b]

[a] Tianjin Key Laboratory of Animal and Plant Resistance, School of Life Sciences, Tianjin Normal University, Tianjin, 300387, PR China
[b] Institute of Entomology, College of Life Sciences, Nankai University, Tianjin, 300071, PR China

ARTICLE INFO

Article history:
Received 13 May 2017
Received in revised form
24 August 2017
Accepted 23 September 2017
Available online 25 September 2017

Keywords:
Toll-like receptors
Pattern recognition receptors
Cyclina sinensis
Innate immune response
TLR13-MyD88-NF-κB signalling pathway

ABSTRACT

Toll-like receptors, the best known pattern recognition receptors, play important roles in recognizing non-self molecules and binding pathogen-associated molecular patterns in the innate immune system. In the present research, the cDNA and protein characterization of the TLR signalling pathway genes including *IRAK4*, *TRAK6* and *IKKα* (named *CsIRAK4*, *CsTRAF6* and *CsIKKα*, respectively) with the typical motifs from *Cyclina sinensis* showed significant similarity with their homologues from other shellfish. Furthermore, the mRNA transcripts of these three genes are ubiquitously expressed in all tissues tested and are dominantly expressed in *C. sinensis* haemocytes ($P < 0.05$). Moreover, *IRAK4*, *TRAK6* and *IKKα* cDNA expression levels were all up-regulated after injection with *Vibrio anguillarum*, *Micrococcus luteus* and poly I:C ($P < 0.01$) as shown by quantitative real-time PCR, indicating that they were involved in responding to pathogenic stimulation. We explored the function of the TLR13-MyD88-NF-κB signalling pathway in the innate immune responses of *C. sinensis* by RNA interference and immune challenges. The results suggested the mRNA expression patterns of *CsMyD88*, *CsIRAK4*, *CsTRAF6*, *CsIKKα*, *CsIκB*, *CsNF-κB*, *CsC-LYZ* and *CsAMP* were all down-regulated ($P < 0.01$) in normal and stimulated *C. sinensis* haemocytes, revealing the involvement of the TLR13-MyD88-NF-κB signalling pathway in innate immunity by positively adjusting internal signalling factors and immune-related genes. In summary, a TLR13-MyD88-NF-κB signalling pathway exists and plays vital roles in innate immune responses in *C. sinensis*. These findings collectively lay the foundation for studying the functional characterization of internal signalling factors and establishing a regulatory network for the TLR signalling pathway in molluscs.

1. Introduction

Organisms depend on rapidly mounted defence mechanisms in response to potentially harmful microbes, mechanisms that are evolutionarily conserved in all multicellular organisms and are collectively referred to as innate immunity. In the innate immune system, these mechanisms are based on a small number of receptors called pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) such as viral nucleic acids, components of bacterial and fungal cell walls, flagellar proteins and more [1]. Until now, four types of PRRs have been identified and characterized in different species, and the first family of PRRs studied in detail was the Toll-like receptors (TLRs) family [2]. The first intracellular PRRs to be discovered were the NOD-like receptors (NLRs), and (RIG-I)-like receptors (RLRs) are involved in recognizing viral double-stranded RNA (dsRNA) [3,4]. In addition, the C-type lectin receptors (CLRs) can recognize many diverse PAMPs and activate downstream signalling pathways [5,6].

TLRs are type I integral membrane glycoproteins including an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) domain and are well-studied PRRs in both vertebrate and invertebrate species [7]. The LRR domains of TLRs recognize foreign pathogenic organisms composed of an α-helix or a β-sheet [8]. The TLR signal transduction domains known as Toll/IL-1 receptor (TIR) domains are directed towards the cytoplasm, transduce their signals through interaction with cytoplasmic adaptor proteins containing TIR domains, and then recruit and activate downstream signalling proteins, eventually triggering host-defence responses such as the production of antimicrobial peptides, proinflammatory chemokines, adhesion molecules and enzymes [9]. In previous reports, TLRs have been divided into two groups: *TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6* and *TLR11* are

* Corresponding author.
E-mail address: baopingpan@126.com (B. Pan).

https://doi.org/10.1016/j.fsi.2017.09.060
1050-4648/

transmembrane proteins for detection of membrane components of microbes, while *TLR3*, *TLR7*, *TLR8* and *TLR9* are intracellular molecules that recognize nucleic acids of microbes [10,11].

In 1996, Jules Hoffman et al. discovered the first innate immune signalling receptor, Toll, which can activate the NF-κB signalling pathway to produce the antimicrobial peptide *drosomycin* in *Drosophila melanogaster* [12]. Furthermore, Janeway's group identified a human homologue of the Toll protein, and Bruce Beutler et al. showed the first innate immune signalling Toll-like receptor 4 (TLR4), which can recognize lipopolysaccharide (LPS) and trigger a downstream signalling pathway in mammals [13,14]. To date, a growing number of studies have reported the structural and functional characterization of TLR family genes from vertebrate and invertebrate species. Recently published genome and transcriptome information offers new opportunities to understand and explore their functions in invertebrates. For example, there are 253, 83 and 72 TLR genes in the genomes of sea urchin, oyster and amphioxus, respectively [15–17]. In the mollusc *Chlamys farreri*, a primitive TLR1 signalling pathway was preliminarily characterized and found to be involved in immune responses via activating diverse downstream pathways and genes [18]. In addition, the TLR-TRAF6-mediated signalling pathway might play a role in the shrimp *Penaeus monodon* response to WSSV [19].

The aquaculture industry of *Cyclina sinensis*, a commercially important marine shellfish, encounters tremendous challenges from a variety of chemical contaminants, environmental factors and stressors that threaten the healthy development of Chinese mariculture [20,21]. In the Venus clam *C. sinensis*, we identified two TLRs (*TLR4* and *TLR13*) and a myeloid differentiation factor 88 (MyD88) molecule gene and proved that a MyD88 signalling pathway is involved in inducing the expression of immune-related genes. Additionally, heat shock protein 70 and kazal-type serine proteinase inhibitor genes participated in immune responses and signal pathway regulation [21–23]. However, reports of the function and regulatory mechanisms of TLR13 signalling pathways in molluscs remain very limited. In the present study, we obtained and characterized three signalling proteins, namely IL-1R-associated kinase 4 (IRAK4; termed as *CsIRAK4*), tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6, termed as *CsTRAF6*), and inhibitor of kappa-B kinase alpha (IKKα, termed as *CsIKKα*), from a transcriptome library [24] and investigated their expression profiles in several healthy tissues and in haemocytes injected with various pathogens. Finally, we applied RNA interference to illuminate the regulatory patterns of the TLR13 signalling pathway in *C. sinensis*. These data might provide important information for exploring the regulatory mechanisms of the TLR signalling pathway in phylum Mollusca.

2. Materials and methods

2.1. Microorganisms and animals

Healthy *C. sinensis*, with an average length of 28.22 ± 1.49 mm, average shell height of 28.96 ± 1.57 mm, average shell width of 18.46 ± 0.47 mm, and average mass of 25.50 ± 0.5 g, were collected from the Binhai District (Tianjin, China) and cultured in aerated seawater and 5% Chlorella sp. at 15-20 °C for one week before the experiments. *Vibrio anguillarum* and *Micrococcus luteus* bacteria were cultured in 2216E media at 28 °C and 37 °C, respectively, for 24 h and then washed and re-suspended with sterilized sea water (SSW) to 1×10^8 cells/mL ($OD_{600} = 0.4$) after centrifugation. In addition, poly I:C (Sigma-Aldrich, China; Catalogue No. P1530) was added to SSW to the optimized final concentration (1 mg/mL).

2.2. Gene identification and bioinformatics analysis

In this study, the nucleotide sequences of *CsIRAK4*, *CsTRAF6* and *CsIKKα* were obtained from our transcriptome library [24] and analysed using the BLAST algorithm of NCBI (http://www.ncbi.nlm.nih.gov/blast/). Furthermore, we deduced amino acid sequences from three nucleotide sequences using the Expert Protein Analysis System (http://www.expasy.org/) and predicted the protein domains with the simple modular architecture research tool, SMART (http://smart.embl_heidelberg.de). The protein molecular weight and theoretical isoelectric point were estimated with an online tool (http://web.expasy.org/compute_pi/). All amino acid sequences were aligned using MUSCLE software because of its high accuracy and speed [25]. Finally, the neighbour-joining (NJ) phylogenetic trees were constructed based on the deduced amino acid sequences of other published genes using MEGA 6 software tested by 1000 bootstrap resampling [26].

2.3. Tissue distribution and immune stimulation of CsIRAK4, CsTRAF6 and CsIKKα

Five healthy clams were randomly sampled, and haemocytes (haemolymph was centrifuged at 5000 *g*, 4 °C for 10 min to collect the haemocytes), hepatopancreas, adductor muscle, gills, mantle and gonad were dissected to analyse the tissue expression of *CsIRAK4*, *CsTRAF6* and *CsIKKα*. Each tissue type from individual clams was pooled and immediately stabilized in RNAlater solution (Ambion) for subsequent RNA purification and cDNA synthesis. The relative gene expression levels of *CsIRAK4, CsTRAF6* and *CsIKKα* among various tissues were evaluated by quantitative real-time PCR (qRT-PCR).

Moreover, to explore the expression levels of *CsIRAK4*, *CsTRAF6* and *CsIKK* in *C. sinensis* in response to different pathogens, we used immune stimulation experiments. Seventy-two clams were randomly divided into three groups of 18 individuals and injected with 50 μl SSW, *V. anguillarum*, *M. luteus* or poly I:C, and haemocytes only were collected from five clams. Haemolymph from the above samples was collected at 3, 6, 12, 24, 48 and 96 h post-injection (hpi) and pooled for cDNA synthesis to detect of the expression of the three genes of interest by quantitative real-time PCR.

2.4. Synthesis of double-stranded RNA

The DNA templates of *CsTLR13* dsRNA of 560 bp in length (designated as dsCsTLR13) and an EGFP DNA fragment (dsEGFP; 657 bp) from the pEGFP vector (Clontech, USA) were prepared by PCR using specific primer pairs (Table S2). Products with a T7 promoter were confirmed via sequencing. Furthermore, the products were used as templates to produce the sense and antisense RNA strands, subjected to *in vitro* transcription, and then purified using the RiboMAXTM Large-Scale RNA production System-T7 (Promega, USA) according to the manufacturer's protocol. Finally, the dsRNA was dissolved in RNase-free water to a final concentration of 1 mg/mL.

2.5. The regulatory mechanism of TLR13 signalling pathway in C. sinensis by dsRNA interference

First, 24 h after injection with dsCsTLR13 and dsEGFP, haemocytes were extracted from 6 individuals to examine the gene silencing efficiency using qRT-PCR and western blot [27]. Furthermore, a total of 78 clams were randomly selected and 36 individuals were injected with dsCsTLR13 in their adductor muscles. Meanwhile, the other 36 individuals were injected with dsEGFP