Fish & Shellfish Immunology 36 (2014) 552–562

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Molecular cloning and characterization of a Toll receptor gene from *Macrobrachium rosenbergii*

Chutima Srisuk [a], Siwaporn Longyant [a], Saengchan Senapin [b,c], Paisarn Sithigorngul [a], Parin Chaivisuthangkura [a,d,*]

[a] Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand
[b] CENTEX Shrimp, Faculty of Science, Mahidol University, Bangkok, Thailand
[c] National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand
[d] Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 14 August 2013
Received in revised form
25 December 2013
Accepted 25 December 2013
Available online 5 January 2014

Keywords:
Toll receptor
Macrobrachium rosenbergii
Aeromonas caviae
RNA interference
Innate immunity

ABSTRACT

Toll receptors are cell surface molecules acting as pattern recognition receptors (PRRs) that have been implicated in the signaling pathway of innate immune responses. In this study, the full-length cDNA of a Toll receptor gene of *Macrobrachium rosenbergii*, designated *MrToll*, was successfully isolated using designed degenerate primers and the rapid amplification of cDNA ends (RACE). The *MrToll* gene sequence contained an open reading frame (ORF) of 2799 nucleotides encoding a protein of 932 amino acid residues. The protein contained distinct structural motifs of the Toll-like receptor (TLR) family, including an extracellular domain containing 15 leucine-rich repeats (LRRs), a transmembrane segment of 23 amino acids, and a cytoplasmic Toll/interleukin-1R (TIR) domain of 139 residues. Phylogenetic analysis revealed that MrToll and Toll receptor of *Marsupenaeus japonicus* (MjToll) evolved closely. However, the MrToll ORF demonstrated only 48–49% identity with shrimp Toll1, suggesting that MrToll isolated from a palaemonid shrimp might belong to a novel class of Toll receptors in shrimp. The transcripts of the *MrToll* gene were constitutively expressed in various tissues, with high levels in hemocytes, the stomach and muscle. A reverse transcriptase PCR assay demonstrated that the expression patterns of *MrToll* were distinctly modulated after *Aeromonas caviae* stimulation, with significant enhancement at 3–12 h post-challenge and a decline to basal levels at 24 h post-challenge. In addition, when *MrToll*-silenced shrimp were challenged with *A. caviae*, there was a significant increase in mortality and bacterial CFU counts. These results suggest that MrToll might be involved in host innate defense, especially against the pathogen *A. caviae*.

1. Introduction

The innate immune system is crucially important in the host defense against pathogens, especially in invertebrates. The non-self-recognizing immune response cascade is triggered by receptors that recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, peptidoglycan, lipoteichoic acid and unmethylated CpG DNA [1]. Different types of PAMPs can be directly recognized by several types of receptors in the host, pattern recognition receptors (PRRs) [2,3]. Tolls and Toll-like receptors (TLRs) have been regarded as important PRRs and have been implicated as a link between innate and acquired immunity [4,5]. The Toll receptor protein was originally identified in *Drosophila* and named dToll [6]. TLRs are evolutionarily conserved glycoproteins characterized by extracellular amino-terminal leucine-rich repeat (LRR) areas, one transmembrane domain (TM), and an intracellular carboxyl terminal Toll/interleukin-1 receptor domain (TIR), occasionally supplemented with an extracellular leucine-rich repeat C-terminal (LRR-CT) [7]. Compared with insects and mammals, shrimp defense mechanisms, including the Toll signaling system, are poorly understood, and information regarding Toll homologs in shrimp is relatively sparse. Although various Tolls have been identified in many shrimp species, including black tiger shrimp *Penaeus monodon* (PmToll) [8], kuruma shrimp *Marsupenaeus japonicus* (MjToll) [9], Chinese shrimp *Fenneropenaeus chinensis* (FcToll) [10] and whiteleg shrimp *Litopenaeus*

* Corresponding author. Department of Biology, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand. Tel.: +66 2 649 5000x8511; fax: +66 2 260 0128.
E-mail addresses: parin@swu.ac.th, parinc@yahoo.com (P. Chaivisuthangkura).

1050-4648/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.12.025

vannamei (LvToll) [11,12], their functions in shrimp innate immunity against foreign molecules are unknown. It has been shown that PmToll is not directly involved in the defense against white spot syndrome virus (WSSV) [8]. However, FcToll expression in the lymphoid organ was reduced immediately after WSSV exposure but significantly enhanced 5 h post-*Vibrio anguillarum* challenge [10]. Recently, it has been shown that in the gill of *L. vannamei*, LvToll1 is upregulated but LvToll2 and LvToll3 display no obvious changes after *Vibrio alginolyticus* challenge. However, 3 h post-WSSV challenge LvToll1, LvToll2 and LvToll3 were markedly upregulated [12]. MjToll expression is significantly increased in the lymphoid organ 12 h post-stimulation with peptidoglycan [9].

In contrast to penaeid shrimp, there is no information regarding Toll receptors in palaemonid shrimp, i.e., the giant freshwater prawn *M. rosenbergii*. Interestingly, the adult of *M. rosenbergii* exhibited resistance and clearance capability to *M. rosenbergii* nodavirus (*Mr*NV), extra small virus (XSV) and white spot syndrome virus (WSSV) despite the presence of these viruses [13,14]. However, the exact mechanism of immune-mediated resistance is not known. Therefore, it is in our interest to begin study innate immunity against pathogens regarding Toll receptors in this shrimp species. In this study, a full-length cDNA that encoded a putative Toll homolog from *M. rosenbergii*, designated *MrToll*, was isolated. The tissue localization of *MrToll* mRNA was investigated, and its expression profiles in hemocytes following challenge with the pathogen *Aeromonas caviae* at high-intensity sampling points (0, 3, 6, 12, 24, 36, 48 h post-injection) were investigated using RT-PCR. The role of MrToll in innate immunity against *A. caviae* was also studied using RNA interference.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

M. rosenbergii shrimp, ranging in weight from 40 to 45 g, were purchased at a local market in Bangkok, Thailand. Total RNA was extracted from gill tissues using TRIzol Reagent (Invitrogen). A total of 2 μg of total RNA and 50 μM oligo(dT)$_{15}$ were used to synthesize cDNA with SuperScript III First-Strand Synthesis (Invitrogen).

2.2. Cloning and sequencing of the MrToll cDNA fragment

A pair of degenerate primers, TIR-F and Toll-R1.5 (Table 1), designed on the basis of the conserved regions of the TIR domain of Toll receptors in *L. vannamei* (Protein ID: ABK58729, from GenBank database), *F. chinensis* (Protein ID: ABQ59330), *M. japonicus* (Protein ID: BAG68890 and BAF99007) and *P. monodon* (Protein ID: ABO43764.1), was used to isolate a partial sequence of the target gene. The PCR conditions were 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 42 °C for 30 s and 72 °C for 45 s; and an extension at 72 °C for 10 min. The products were analyzed with a 1% agarose gel, and the target band was purified with a MinElute Gel Extraction Kit (Qiagen) and then cloned into the pCR 2.1 vector (Invitrogen) for sequencing.

To further isolate the partial cDNA fragment toward the 5′ end, a degenerate primer, Toll-F6, and a gene-specific primer called Nested 5′-RACE (Table 1), designed from the obtained cDNA sequence described above, was used for PCR amplification. The PCR conditions and the analysis of the PCR product were described earlier.

2.3. Rapid amplification of 5′ and 3′ cDNA ends

Based on the partial sequence data of MrToll, the 5′ and 3′ ends were obtained with the SMARTer RACE cDNA Amplification kit (Clontech). The 5′ and 3′ RACE-Ready cDNAs were synthesized using an RNA template from gill tissues using 5′-RACE CDS Primer A and 3′-RACE CDS Primer A, respectively. To isolate the 5′ end of MrToll cDNA, 5′-RACE touchdown PCR was performed with the gene-specific primer Nested2Toll 5′-RACE and an adaptor primer UPM (Table 1) with a program of 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; and 30 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min. The second nested PCR was performed with the primer NToll-600R and a nested adaptor primer NUP (Table 1) with a program of 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 3 min.

To isolate the 3′ end of MrToll cDNA, 3′-RACE touchdown PCR was performed using the gene-specific primer N2Toll 3′-RACE and an adaptor primer UPM (Table 1) with the PCR conditions described above, except for the extension at 72 °C for 2 min at each step. The PCR products were cloned into the pCR 2.1 vector (Invitrogen) and sequenced.

Table 1
PCR primers used for cloning of *MrToll*, RT-PCR analysis, *Aeromonas caviae* verification and dsRNA production.

Primers	Primer sequence (5′–3′)	Amplicon size (bp)
Amplification of the partial *MrToll* cDNA		
TIR-F	TGY CTY CAC TAY CGY GAC TG	96
Toll-R1.5	TGA RAG CAC SAC AAT RGT	
Toll-F6	AAY CTR ACM ATT CCM WAC AA	627
Nested 5′-RACE	CTG ATT TTG AAT GTA CTC TCC AGG TAC C	
5′-RACE		
Nested2Toll 5′-RACE	ACA GTC ACA GTC CCA TGG ATT TCC AC	3000
UPM	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT	
NToll-600R	CCT CTG ACA TCT AGA ACC	2500
NUP	AAG CAG TGG TAT CAA CGC AGA GT	
3′-RACE		
N2Toll 3′-RACE	CAC AGT TTT GGT CCC TGG GCT TGA A	1500
UPM	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT	
RT-PCR analysis		
MrTollexp-F	AGT GGA AAT CCA TGG GAC TG	200
MrTollexp-R	AAT GCA CCA ATT GCA ACA AG	
β-actin-F	CCC AGA GCA AGA GAG GTA	337
β-actin-R	GCG TAT CCT TCG TAG ATG GG	
Aeromonas caviae verification		
ACF	GGC GAG CCG CAG GCA CCC	237
ACR	CTC GAC GAA GGC CTT GAT GCC C	
dsRNA production for *MrToll*		
RNAiTollF	AAC ACA GTT TTG GTC CCT GG	381
RNAiTollR	CAG CTT TTT CGG TGG ATG AG	

2.4. Sequence analysis of MrToll

The nucleotide sequence and deduced amino acid sequence of *MrToll* cDNA were analyzed using the BLAST algorithm (NCBI: http://www.ncbi.nlm.nih.gov/BLAST/). The signal peptide was predicted with the SignalP program (http://www.cbs.dtu.dk/services/SignalP). The extracellular domain, transmembrane domain, cytoplasmic domains and other characteristic structures of MrToll were predicted with the simple modular architecture research tool (SMART) program (http://smart.embl-heidelberg.de/). Potential N-linked glycosylation sites were predicted with NetNGlyc 1.0 Serve (http://www.cbs.dtu.dk/services/NetNGlyc/). Regulatory motifs in the 5′-untranslated region were predicted with the NSITE program (http://linux1.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter).