Toll-like receptor 4 signaling pathway can be triggered by grass carp reovirus and Aeromonas hydrophila infection in rare minnow Gobiocypris rarus

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A B S T R A C T

Toll-like receptor 4 (TLR4) is critical for LPS recognition and cellular responses. It also recognizes some viral envelope proteins. Detection mostly results in the inflammation rather than specific antiviral responses. However, it’s unclear in fish. In this report, a TLR4 gene (named as GrTLR4b) was cloned and characterized from rare minnow Gobiocypris rarus. The full length of GrTLR4b cDNA consists of 2766 nucleotides and encodes a polypeptide of 818 amino acids with an estimated molecular mass of 94,518 Da and a predicted isoelectric point of 8.41. The predicted amino acid sequence comprises a signal peptide, six leucine-rich repeat (LRR) motifs, one leucine-rich repeat C-terminal (LRRCT) motif, followed by a transmembrane segment of 23 amino acids, and a cytoplasmic region of 167 amino acids containing one Toll – interleukin 1 – receptor (TIR) motif. It’s closely similar to the zebrafish (Danio rerio) TLR4b amino acid sequence with an identity of 77%. Quantitative RT–PCR analysis showed GrTLR4b mRNA was constitutive expression in gill, heart, intestine, kidney, liver, muscle and spleen tissues in healthy animals and up-regulated by viruses and bacteria. After being infected by grass carp reovirus or Aeromonas hydrophila, GrTLR4b expressions were up-regulated from 24 h post-injection and lasted until the fish became moribund (P < 0.05). These data implied that TLR4 signaling pathway could be activated by both viral and bacterial infection in rare minnow.

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1. Introduction

Considerable interest in understanding how signaling by TLRs, NLRs, and RLRs — critical innate immune receptors that signal the presence of pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents — is regulated. Many data published to date focuses on regulation of TLR signaling. TLR signaling is controlled at a variety of levels [1]. At least 11 TLRs have been described in mammals [2], and 17 TLRs have been predicted in fish [3]. In the most heavily studied TLR, TLR4 is the most robustly signaling TLR — something attributable to its ability to recruit all four known TIR-containing signaling adaptor molecules [4]. TLR4 receptor recognizes not only lipopolysaccharide (LPS) from gram-negative bacteria [5] but also viral envelope proteins [6,7]. TLR4 signaling pathway is likely to require activation of both the MyD88 dependent and independent pathways to induce inflammatory cytokines [8]. TLR4 also stands out for another reason. TLRs signal the presence, not only of microbe-associated molecular patterns but also of a variety of structures generated or unmasked during tissue injury and inflammation — damage-associated molecular patterns [9].

The TLR4 gene was first found in human in 1997 [10]. Up to now, some TLR4 protein sequences have been isolated from mammals and birds, just a 152 amino acid short partial sequence has been identified in amphibian (Xenopus laevis, BAF57489) and two sequences have been reported in fish (Danio rerio, NP_997978 and NP_001124523) in GenBank (searched by blastp, January, 2009). However, it remains unclear whether piscine TLR4 gene responds to viruses and gram-negative bacterial infections.

Grass carp (Ctenopharyngodon idellus) is an important aquaculture species in China, but tremendous economic loss is often caused by grass carp reovirus (GCRV). Better understanding of the immune defence mechanisms of grass carp may contribute to the development of management strategies for disease control and long-term sustainability of grass carp farming. The rare minnow Gobiocypris rarus is a small cyprinid species and very sensitive to GCRV [11]. Moreover, Aeromonas hydrophila, a gram-negative bacterium of the family Aeromonadaceae, is often found in association with hemorrhagic septicemia in cold-blooded animals including fish, reptiles and amphibians [12].

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In this paper, we employed the rare minnow as a model fish to study the mechanism of GCRV disease. A full-length cDNA sequence (GrTLR4b) encoding a TLR4 was identified and characterized in rare minnow. We showed GrTLR4b mRNA expression profiles in different tissues and showed both GCRV and A. hydrophila infection.

2. Materials and methods

2.1. Animals, immune challenge and sample collection

Rare minnows with body weight of 2–3 g were obtained from a laboratory-breeding stock and acclimatized to new laboratory conditions for one week in a quarantine area. They were maintained in 25 L aerated aquaria at 28 °C and fed twice a day with commercial diet (feed composition: protein 32%, starch 63%, fat 3%, additive 2%). Before experiments, they are obviously healthy.

For challenge experiments with GCRV (991 strain) or A. hydrophila (C1 strain), fish were injected with 10 μl PBS per gram body weight, 10 μl GCRV suspended in PBS (2 × 10^8 PFU ml⁻¹) or 10 μl A. hydrophila resuspended in PBS (OD₂₆₀ = 0.1) intraperitoneally. The non-injected animals were used as blank group. Three individuals were killed and tissues including gill, heart, intestine, kidney, liver, muscle, and spleen were collected at 0, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h after injection. Approximate 50–100 mg of fresh tissue was homogenized using a glass–teflon in 800 μl of TRIZOL® LS reagent (Invitrogen) and total RNA was extracted according to the manufacturer’s instruction. Total RNA was incubated with RNase-free DNase I (Roche) to remove contaminated genomic DNA before being reverse transcribed into cDNA using random hexamer primers with SuperScript™ III Reverse Transcriptase (Invitrogen).

2.2. Amplification of cDNA and nucleotide sequence analysis

To obtain TLR4 cDNA from rare minnow, degenerate primers were designed based on the multiple alignment of some known TLR4 sequences including D. rerio (accession Nos. AY388400 and XM_001335971), Canis familiaris (accession No., NM_001002950), Rattus norvegicus (accession No., NM_019178), Mus musculus (accession No., NM_021297). PCR was performed with primers F112a and R113a (Table 1) using the cDNA generated from liver. The PCR programme was: one cycle of 94 °C/1 min, 5 cycles of 94 °C/30 s, 56 °C/30 s, 72 °C/45 s; one cycle of 72 °C/5 min. The PCR product was ligated into pMD18-T easy vector, transformed into the competent TOP10 cells, and plated on the LB agar plate for colony PCR and plasmid preparation using an Axygen (Shanghai Invitrogen Biotechnology Co., Ltd., China).

2.3. Rapid amplification of cDNA ends (RACE)

Using the BD SMART™ RACE cDNA amplification kit (BD Biosciences Clontech), first strand cDNA synthesis and RACE were performed on liver-derived RNA. To obtain the 3’ unknown region, primer pairs, F145/adaptor primer UPM and F146/adaptor primer NUP (Table 1), were used for the primary PCR and the nested PCR, respectively. The amplified PCR products were cloned and sequenced as described above. Similarly, the 5’ end of GrTLR4b was obtained by nested PCR using primer pairs R117/UPM and R168/NUP (Table 1). The full-length cDNA sequences were confirmed by sequencing the PCR product amplified by primers F168a and R168b (Table 1) within the predicted 5’ and 3’ untranslated regions, respectively.

2.4. Sequence analysis and phylogeny

Sequences homology was obtained using BLAST program (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and the protein domain features were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) [13]. Intra-domain features were predicted by a scan of the sequence against the PROSITE database (http://us.expasy.org/tools/scanprosite) [14]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [15] and optimized manually.

2.5. Quantification of gene expression

Expression of GrTLR4b mRNA was assessed in different tissues and infection states using quantitative real-time RT–PCR (qRT–PCR) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The expression of putative house keeping gene β-actin was utilized as an internal control for cDNA normalization. The gene specific primers for β-actin were forward primer F86 and reverse primer R87 (Table 1). qRT–PCR mixture consisted of 1 μl of cDNA sample, 8 μl nuclease-free water, 10 μl of SYBR Green PCR master mix (Toyobo), and 0.5 μl of gene specific primers (5 μM). The PCR cycling conditions were: one cycle of 95 °C/2 min, 40 cycles of 95 °C/25 s, 60 °C/30 s, 72 °C/1 min, followed by dissociation curve analysis to verify the amplification of a single product. The threshold cycle (CT) value was determined using the manual setting on the ABI Sequence Detection System and exported into a Microsoft Excel Sheet for subsequent data analysis where the PCR efficiency and relative expression ratio of target gene in treated groups versus that in control were calculated by Pfaffl equation [17]. The expression data obtained from three independent biological replicates were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed t-test. P < 0.05 was considered statistically significant.

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GrTLR4b</td>
<td></td>
</tr>
<tr>
<td>F112a (forward)</td>
<td>GAAWGTYWTAGYGWCSATTT</td>
</tr>
<tr>
<td>R113a (reverse)</td>
<td>CWCMTTYRAGYCKTMTCCACGAA</td>
</tr>
<tr>
<td>F145 (forward)</td>
<td>GAAWGTYATAGGCGAAACTGGA</td>
</tr>
<tr>
<td>F146 (forward)</td>
<td>ATGGAACGCAATGCCAACATGCTC</td>
</tr>
<tr>
<td>R167 (reverse)</td>
<td>GCAGCACAAGTACCTGCTGAGCC</td>
</tr>
<tr>
<td>R168 (reverse)</td>
<td>GCAGTGCAGAAAGCAGATGAGCCATCTG</td>
</tr>
<tr>
<td>F168a (forward)</td>
<td>TTTTATGGTCTGCTGGAA</td>
</tr>
<tr>
<td>R168b (reverse)</td>
<td>CGCGATGGATTTCGTTT</td>
</tr>
<tr>
<td>F124 (forward)</td>
<td>GTTCCACCTATTCATTTGCC</td>
</tr>
<tr>
<td>R125 (reverse)</td>
<td>GAAGTGTGAGACGACCAGCACA</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
</tr>
<tr>
<td>F86 (forward)</td>
<td>GATGATGAAATTGCCGCACTG</td>
</tr>
<tr>
<td>R87 (reverse)</td>
<td>ACCAACATGACACCCCTGATG</td>
</tr>
<tr>
<td>Universal adaptor primer</td>
<td></td>
</tr>
<tr>
<td>UPm</td>
<td>Long: CTAAATGCTACTATGCTGCGACAG</td>
</tr>
<tr>
<td>Short: CTAAATGCTACTGGGCGCC</td>
<td></td>
</tr>
<tr>
<td>NUP</td>
<td>AACGATGCGTGTCACCAGCAGCAG</td>
</tr>
<tr>
<td>5’-RACE adaptor OligodG</td>
<td>AACGATGCGTGTCACCAGCAGCAGG</td>
</tr>
<tr>
<td>3’-RACE primer 3’-CD5</td>
<td>AACGATGCGTGTCACCAGCAGCAGT</td>
</tr>
</tbody>
</table>

Note: Y = C/T; M = A/C; R = A/G; K = G/T; W = A/T; V = A/G/C; and N = A/G/C/T.
2.6. Nucleotide sequence deposition

The GrTLR4b cDNA sequence was submitted to GenBank and has been designated accession number EF636802.

3. Results

3.1. Characterization of GrTLR4b mRNA and the encoded protein

Initial amplification of a 431 bp product (trimmed vector sequence) was shown by blastx analysis to have significant homology (E-value = 3e−74) to the C-terminal region of D. rerio TLR4b gene (accession No. AAQ90475). 3′-RACE sequence aligned with the first fragment yielded a 518 bp consensus sequence. The clone included a poly(A) tail, suggesting that the expressed sequence tag (EST) represented the 3′ region of the TLR4b gene. The 5′ region of the gene was amplified by using 5′-RACE. A product of 2366 bp from nested PCR perfectly overlapped the above 518 bp consensus sequence. To confirm that the resulting 2766 bp consensus sequence represented a single mRNA expressed in the liver of adult G. rarus, primers were designed within the 5′ and 3′ UTRs of the sequence. Using these primers, a single 2552 bp product was amplified from liver CDNA. Sequence analysis of the product confirmed that the full-length transcript in G. rarus comprises 2766 nucleotides including the 5′ untranslated region, coding sequence, 3′ untranslated region and poly(A) tail (Fig. 1). The longest open reading frame commences at nucleotide 236 and terminates at nucleotide 2692, encoding a putative 818 amino acid polypeptide. Its 3′ UTR contains an ARE (AU-rich element) (ATTTA) mRNA instability sequence, a motif possibly involved in rapid message degradation [18]. Blastp analysis indicated that the translated sequence has significant homology to previously characterized TLR4 proteins.

3.2. Analysis of the deduced amino acid sequence of GrTLR4b

The 818 amino acid polypeptide encoded in the amplified gene has a calculated molecular weight of 94,518 Daltons and isoelectric point of 8.41. Blastp search showed that this gene was most similar to zebrafish TLR4b protein (Identities = 77%; E-value = 0.0), indicating that it might be a rare minnow homolog of TLR4b (named GrTLR4b). The predicted amino acid sequence has a signal peptide, six leucine-rich repeat (LRR) motifs, one leucine-rich repeat C-terminal (LRRCT) motif, followed by a transmembrane segment of 23 amino acids, and a cytoplasmic region of 167 amino acids containing one Toll – interleukin 1 – receptor (TIR) motif (Fig. 1). We selected some representative TLR4 homologous proteins from mammals, birds and fishes to compare their conserved domains and other sequence features. All the sequences contain several LRR motifs, one LRRCT motif, one transmembrane region and one TIR motif. These motifs present different relative loci in the corresponding sequence. These representative TLR4 protein sequences had signal peptide sequence and were greater than 810 amino acids in length except D. rerio TLR4a (DdTIR4a) sequence. DdTIR4a sequence is 750 amino acids in length and doesn't hold signal peptide (Fig. 2). To study the molecular evolutionary aspects, all the representative TLR4 homologous protein sequences, including all known fish TLR4 homologous protein sequences, human and mouse TLR4s standing for mammal, and red jungle fowl and zebra finch TLR4s representing bird, were used to construct a phylogenetic tree (Fig. 3). GrTLR4b firstly clustered with DdTIR4b, then with DdTIR4a, at last with mammal and bird TLR4s.

3.3. Expression analysis of GrTLR4b mRNA in different tissues

RNA was extracted from gill, heart, intestine, kidney, liver, muscle and spleen tissues at 36 h post-injection and the levels of GrTLR4b transcripts were assessed using qRT–PCR. GrTLR4b mRNA was detected in all fish tissues tested at low levels, and was significantly up-regulated by viral or bacterial infection (P < 0.05) (Fig. 4). In the virally or bacterially infected groups, the GrTLR4b expression in spleen was most up-regulated 5.4-fold or 9.3-fold. For GCRV disease, liver is one of the main affected organs, and GrTLR4b was the second most up-regulated 4.9-fold by viral infection or 6.8-fold by bacterial infection in liver. Therefore, liver tissue was selected to investigate the temporary expression of GrTLR4b gene after immune stimulation.

3.4. GrTLR4b mRNA expression profile stimulated by viral infection

To determine the effects of viral or bacterial infection on GrTLR4b gene expression in liver tissue, qRT–PCR assays were conducted. No significant differences were detected in control and blank groups at any of the time points (P > 0.05) (data not shown). In infected groups, the expressions were significantly up-regulated at 24 h post-injection, reached a peak at 36 h post-injection (P < 0.05), and then gradually decreased, but they still kept high levels until the fish became moribund (P < 0.05) (Fig. 5). The data clearly demonstrated that GrTLR4b expression levels were stimulated in infected fish, for both viral and bacterial infection (P < 0.05). The response of GrTLR4b was more drastic to bacterial infection than that to viral infection.

4. Discussion

TLRs recognize microbial structures (from gram-positive and gram-negative bacteria, mycobacteria, RNA and DNA viruses, fungi and protozoans) in the earliest phase of the host defence response, and induce hundreds of host genes through a complex network of signaling that allows for an appropriate response for eliminating the invading pathogen [19,20]. TLRs are comprised of an ectodomain with LRRs, a transmembrane region, and a cytoplasmic TIR domain. LRRs form a single continuous structure and adopt an arc or horseshoe shape, and have been inferred to be responsible for molecular recognition [21]. Upon receptor activation, it is believed that a TIR domain signaling complex is formed between the receptor and the adaptor TIR domains in order for signal transduction to occur [20].

TLR4 diverged from other TLRs during early vertebrate evolution, more than 400 million years ago [22]. According to the draft of the pufferfish Fugu rubripes genome project, it lacks TLR4 [23]. The apparent absence of TLR4 in F. rubripes may be explained by a specific loss of this gene in the F. rubripes lineage or by incompleteness of the F. rubripes genome shotgun sequence [3]. In another model fish, zebrafish D. rerio contains two copies of the TLR4 homolog in a tandem duplication, DtTLR4a and DtTLR4b [3,24]. DtTLR4a lacks signal peptide and consists of 750 amino acids, however, DtTLR4b contains signal peptide as other TLR4 and comprises 817 amino acids (Fig. 2). The TLR4 that we identified from G. rarus shares the similar characteristic structure with DtTLR4b, so we named the gene GrTLR4b, which doesn’t mean there is TLR4a in G. rarus.

GrTLR4b nucleotide sequence contained an ARE motif (ATTTA). ARE is frequently found in the 3′ UTR of the mRNAs of transiently expressed genes [18]. It plays a critical role in the regulation of gene expression during cell growth and differentiation, immune responses, signal transduction, transcriptional and translational control, hemapoiesis, apoptosis, nutrient transport, and metabolism [25,26].
Fig. 1. Nucleotide sequence and deduced amino acid sequence of GrTLR4b cDNA. The “stop codon” in front of start codon is marked by dot underline. The start codon (ATG) was boxed and the stop codon (TAA) was indicated with an asterisk. The motif associated with mRNA instability (ATTTA) was underlined and in bold. In the deduced amino acid sequence, signal peptide is shaded (1–24aa). The LRR motifs were underlined (74–97aa, 98–121aa, 145–170aa, 197–220aa, 490–513aa, 538–561aa). The LRRCT motif was also underlined (574–624aa). The transmembrane domain was marked by double underline (629–651aa). The TIR domain was indicated by wavy underline (674–817aa).
Fig. 2. Schematic representation of the predicted domain organization of the TLR4b protein and comparison with the structures of TLR4 proteins of the human (Homo sapiens), mouse (Mus musculus), red jungle fowl (Gallus gallus) and a zebra finch (Taeniopygia guttata) and the TLR4a and TLR4b proteins of zebrafish (D. rerio). The approximate locations of the conserved functional domains were shown. The sequences were as follows: GrTLR4b, G. rarus TLR4b (AB223263); DrTLR4b, D. rerio TLR4b (NP_597978); DrTLR4a, D. rerio TLR4a (NP_00124523); GgTLR4, G. gallus TLR4 (NP_0011028964); TgTLR4, T. guttata TLR4 (NP_001135926); MmTLR4, M. musculus TLR4 (AAH268564); HsTLR4, H. sapiens TLR4 (NP_012564).
Dysregulation of regulatory signaling pathways and regulatory proteins affecting ARE mRNA stability can lead to abnormalities in many critical cellular processes and to specific disease conditions [26]. A better understanding of these processes may form the basis for the development of novel therapeutics to treat diseases [27]. The function and utilization of ARE motif need further study in fishes.

Up to now, the expression profile of TLR4 in fish was just reported in zebrafish. DrTLR4a could be detectable in blood, intestine, testis, skin, brain, liver, heart ect., however, DrTLR4b was only expressed in skin and heart. During development, the expression of DrTLR4a was detectable from 12 h post-fertilization, and DrTLR4b was expressed from 24 h post-fertilization [24]. No induction of DrTLR4a and DrTLR4b was observed in the pathogen Mycobacterium marinum (gram-positive bacterium) infection experiment in zebrafish [3]. The effects of LPS (major component of gram-negative bacterial cell walls) have not been studied as extensively in fish as in mammals, however, some reports indicate that teleost fish also display LPS responsiveness [28,29]. Therefore, it is possible that piscine TLR4 gene was already implicated in LPS sensing.

In the present study, GrTLR4b expression was constitutively at low levels in the tested tissues (Fig. 4), which was different from the expression of DrTLR4b [24]. GrTLR4b expression was induced after infection by bacteria or viruses (Fig. 4). Liver is one of the main target organs for GCRV, which prompted us to study GrTLR4b expression profiles in liver infected by bacteria or viruses (Fig. 5). Liver is one of the main target organs for GCRV, which prompted us to study GrTLR4b expression profiles in liver infected by bacteria or viruses. In mammals, TLR4 is critical for the recognition of LPS/endotoxin from gram-negative bacteria by different host cells initiating cell activation and the release of proinflammatory cytokines [22,30–32]. LPS is the main component of gram-negative bacterial cell walls. After infection with A. hydrophila, GrTLR4b transcription was induced at 24 h post-infection (P < 0.05), and lasted until the fish became moribund (Fig. 5). This feature inferred piscine TLR4 gene could sense gram-negative bacterial infection. Further work need be done to use purified LPS from A. hydrophila to more specifically trigger GrTLR4b. In mammals, respiratory syncytial virus (RSV) fusion (F) protein activates cells through TLR4 [6,33,34]. Infection with RSV results in increased expression of TLR4 mRNA and sensitizes airway epithelial cells to endotoxin [35]. Measles virus (MV) through TLR4 but not other TLRs mediates suppression of IL-12 synthesis [36]. After infection with GCRV, GrTLR4b expression profile was similar to that in A. hydrophila infection, just up-regulation extent was less (Fig. 5). The data implies that piscine TLR4 can initiate innate immune responses to both bacterial and viral pathogens.

The TLR4 gene which encodes the receptor recognizing bacterial LPS is highly polymorphic. TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide [34]. Whether GrTLR4b has polymorphisms and whether the polymorphisms relate GCRV disease need further research.

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