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Molecular characterization and functional analysis of MyD88 in Chinese soft-shelled turtle *Trionyx sinensis*

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ABSTRACT

Myeloid differentiation factor 88 (MyD88) is one of the key adaptor proteins to signal transduction that triggers downstream cascades involved in innate immunity. In this study, the MyD88 gene from Chinese soft-shelled turtle (*Trionyx sinensis*) (tMyD88) was identified, representing the fist example from reptile species. The tMyD88 has a 894-bp ORF and encodes a polypeptide of 297 amino acids including a typical death domain (DD) at the N-terminus and a conservative Toll/IL-1R (TIR) domain at the C-terminus. It was expressed at high levels in spleen, blood, lungs and liver, but marginal in kidneys and intestines of turtles challenged with live cells of *Aeromonas hydrophila*, as determined by real-time PCR. RAW 264.7 cells transfected with pcDNA-tMyD88 showed higher NF-κB activity than the vector control (673.78 vs 410.72, $P < 0.05$). Expression of proinflammatory cytokines IL-1β and TNF-α was also significantly higher in RAW 264.7 cells transfected with pcDNA-tMyD88 than those having pcDNA3.1 control vector ($P < 0.01$). These results indicate that tMyD88 might possess an important role in defense against microbial infection in Chinese soft-shelled turtles similar to that in mammals.

1. Introduction

Innate immunity is the first line of defense for vertebrates against invading microbial pathogens and provides crucial signals for activation of adaptive immune responses, leading to immunological memory [1–3]. Innate immune cells can trigger the release of inflammatory cytokines and expression of co-stimulatory molecules for host defense once the pathogen associated molecular patterns (PAMPs) are sensed by pattern recognition receptors (PRRs) [4]. Toll like receptors (TLRs), as a class of PRRs, can induce innate immune responses through association with downstream appropriate adaptor proteins [5,6]. Myeloid differentiation factor 88 (MyD88) is the universal adaptor for signals from all TLRs except TLR3, and play important roles in MyD88-dependent pathway of TLRs signalling transduction [5,7,8].

MyD88 was first found in 1990, but its functions involved in signalling by the type 1 IL-1 receptor (IL-1R1) remain unknown until 1997 [9,10]. MyD88 possesses a C-terminal Toll/interleukin-1 receptor (TIR) domain, an intermediary domain (ID) and a N-terminal death domain (DD) [7]. Upon stimulation, the TIR domain of TLRs associates with TIR domain–containing adaptor molecules, including MyD88. The complex recruits the IRAKs (IL-1 receptor-associated kinases) family members such as IRAK4, IRAK1 and IRAK2 through homophilic interaction with the death domain. The activated IRAKs bind to TRAF6 (TNF receptor-associated factor 6). The signaling cascade responses are then initiated, leading to the activation of two distinct signaling pathways including the canonical IKK (IκB kinase) complex, p38 MAPK (mitogen-activated protein kinases) and JNK (c-jun N-terminal kinase). Transcription factors AP-1 (activator protein-1) and NF-κB are then activated, and proinflammatory cytokines such as TNF-α, IL-1β and IL-12 are induced [1,11,12]. With an increasing number of MyD88s from such species as human, mice, chicken, xenopus, zebrafish, scallop and fruitfly being cloned and identified [13–15], it is possible to better understand not only their phylogenetic relationship, but also their functionality in the TLR-induced signaling pathways. However, there are still many MyD88 molecules in the animal kingdom, such as turtle, snake, etc that remain unidentified so far.

The Chinese soft-shelled turtle (*Trionyx sinensis*) belongs to reptile, living in fresh water and known as a health-promoting food in Asian countries including China, Japan and Korea [16]. Along with rapid development of large-scale farmed culture in south-eastern Chinese provinces Zhejiang, Jiangsu and Fujian, farms raising the Chinese soft-shelled turtles have suffered serious economic losses due to infectious diseases caused by microbial pathogens such as *Aeromonas hydrophila* [17]. Therefore, a better

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understanding of the immune responses of the turtles may contribute to developing strategies against infections and for sustainable farming.

In this study, the full-length cDNA of MyD88 from Chinese soft-shelled turtles was cloned and characterized, and its mRNA expression levels in different tissues in turtles stimulated with live cells of *A. hydrophila* were examined. Its function in signaling the NF-κB pathway in a model cell line RAW264.7 was further evaluated.

2. Materials and methods

2.1. Treatment of turtles and tissue sample collection

Chinese soft-shelled turtles (CSST) (*T. sinensis*) were from Hangzhou Zhongde Aquatic Culture Co., Ltd. (Hangzhou, China). Each turtle was raised separately at 29 ± 1 °C in 30 × 40 × 40-cm tanks. The turtles were acclimatized for one week before experiments. The animal experiments were approved by Zhejiang University Committee for Experimental Animal Management.

For sequence analysis of CSST MyD88 (tMyD88), healthy turtles (weight at about 550 g, 2 years old) were intraperitoneally challenged with 10^6 CFU of live cells of *A. hydrophila* strain AS 1.927 (China General Microbiological Culture Collection Center, Beijing, China) suspended in phosphate buffered saline (PBS, 0.1 mol/L pH 7.2). Spleen was collected 2 days post-challenge from euthanized turtles, and stored in liquid nitrogen immediately for total RNA extraction and cDNA synthesis.

For analysis of tMyD88 mRNA expression in different tissues, six healthy 2-year-old CSSTs (weight 550 ± 20 g) were randomly divided into two groups. Those in the experimental group were challenged with *A. hydrophila* as above, and those in the control group received 1 mL of PBS. Samples of blood, kidney, intestine, spleen, liver, heart and skeleton muscle were taken from the turtles 48 h post-challenge and put into liquid nitrogen immediately for total RNA extraction.

2.2. Total RNA extraction

Total RNA was extracted from tissue samples using the RNAprep pure kit Tissue Kit (Tiangen Biotech. Co Ltd, Beijing, China) and treated with DNase I (Takara Biotech. Co Ltd, Dalian, China) at 37 °C for 30 min. Purity and quantity of RNA were measured on NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Integrity of RNA was tested by agarose gel electrophoresis. Only intact RNA samples with 260:280 nm ratio between 2.0 and 2.2 were used for cDNA synthesis.

2.3. Amplification and cloning of tMyD88 cDNA

For cloning of the tMyD88 gene fragment, cDNA was synthesized from 1 μg total RNA by SuperScript® III Reverse Transcriptase (Invitrogen Biotech. Co Ltd, Shanghai, China) following the manufacturer's protocol with random primers (Takara Biotech. Co Ltd, Dalian, China). A partial fragment of tMyD88 gene was amplified using the degenerate primer pair MyD88-dF and MyD88-dR (Table 1) based on the conserved region of MyD88 genes from mammals, avian species, amphibian and fishes available in the public database. PCR was performed using 2 μL of cDNA as template in a 30-μL reaction mixture with a denaturation step of 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and completed with a 10-min extension at 72 °C.

For 3′-end RACE amplification, Q_T, Q_O and Q_I primers (Table 1) were designed and nested PCR protocol performed according to Scotto-Lavino et al. [18]. The first-strand cDNA was synthesized from 1 μg total RNA by SuperScript® III Reverse Transcriptase (Invitrogen) with Q_T primer. The first round PCR was carried out using 2 μL of synthesized cDNA and the primer pair Q_O and MyD88-3_O in a 30-μL reaction mixture. The cycling conditions included a denaturation step of 98 °C 3 min, followed by 60 °C for 2 min, 72 °C for 40 min, and then 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. The reaction was completed with a 10-min extension at 72 °C. The first round PCR products were further amplified using primer pair Q_I and MyD88-3_I under the following conditions: a denaturation step of 95 °C 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and completed with a 10-min extension at 72 °C.

Table 1
Primers for PCR and real-time PCR in this study.

Primers information	Sequence(5′–3′)
MyD88-dF	GTTTGTSCABGAGATGATC
MyD88-dR	GCVAGNCKDDYCCARAACCA
Q_T	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC$(T)_{17}$
Q_O	CCAGTGAGCAGAGTGACG
Q_I	GAGGACTCGAGCTCAAGC
MyD88-3_O	TTCAAGCTGAAGCTGTGTGTGTTT
MyD88-3_I	GAAGCTGTGTGTGTTTGATCGGGATGTC
MyD88-5_O	GGGATTAGTGTAGTCGCAGAC
MyD88-5_I	CTCTATGAGCTGGCTAGTGAT
5′ RACE outer primer	GCTGATGGCGATGAATGAACACTG
5′ RACE inner primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
MyD88-wF1	AGTTGCGAAAGCGGCAGG
MyD88-wF2	CGGCGAACCATGGACCTGA
MyD88-wR1	CCTCTGCACAGGAGGAGGCA
MyD88-wR2	GGCTCAGGAAGCTTTGCCTCA
MyD88-ORF-F[a]	AA*GGATCC*ATGGACCTGACCCCGGC
MyD88-ORF-R[a]	CC*CTCGAG*TCATGGGAGCAAGAGGGATTT
β-actin-F	AACTGGGATGACATGGAGAAGA
β-actin-R	AACATGATCTGGGTCATCTT
MyD88-qF	CGAGAGCTGGAGCAAACGGAGTTCAAG
MyD88-qR	GCTGGCTAGTGATGGACCACACGCA

[a] Underline means *BamH*I or *Xho*I restriction endonuclease sites.

For 5′-end RACE amplification, the cDNA for 5′ RACE was synthesized from total RNA using 5′ FirstChoice® RLM-RACE Kit (Ambion, USA) according to the manufacturer's instruction. cDNA fragments of tMyD88 were amplified by two rounds of PCR with gene-specific primers and adapter primers (Table 1) provided in the 5′-end RACE Kit. The first round PCR was carried out with MyD88-5_O and 5′-RACE Outer Primer. The reaction conditions were as follows: a denaturation step of 95 °C 3 min, followed by 35 cycles of 95 °C for 30s, 62 °C for 30 s and 72 °C for 90 s, and completed with a-10 min extension at 72 °C. The first round PCR products were further amplified using the primer pair MyD88-5_I and 5′ RACE Inner Primer with the cycling conditions as for the first round PCR.

Nested PCR was used to amplify the full-length MyD88 ORF, first using primer pair MyD88-wF1 and MyD88-wR1 and then with MyD88-wF2 and MyD88-wR2 (Table 1). The PCR products were gel-purified, cloned into pMD18-T vector and sequenced in Invitrogen Corp. (Shanghai, China). The GenBank accession number is given as HM991902 for the tMyD88 cDNA.

2.4. Sequence analysis of tMyd88

The nucleotide and deduced amino acid sequence of tMyD88 were analyzed via blastn and blastp at http://blast.ncbi.nlm.nih.gov/. Its functional domains were analyzed with the SMART search program (http://smart.embl-heidelberg.de). A multiple sequence alignment was done with Clustal W and the phylogenetic tree of MyD88 constructed by MEGA 4.0.