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Identification, expression pattern and functional characterization of *As-MyD88* in bacteria challenge and during different developmental stages of *Artemia sinica*

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ABSTRACT

Myeloid differentiation factor 88 (MYD88), a key adapter protein in Toll-like receptor signaling, affects the immune response and the formation of the dorsal–ventral axis. Here, the 1555bp full-length cDNA of *MyD88* from *Artemia sinica* (*As-MyD88*) was obtained. Molecular characterization revealed that the sequence includes an 1182bp open reading frame encoding a predicted protein of 393 amino acids. The predicted protein contains a death domain in the N-terminus, and box1 and 2 motifs of the TIR domain in the C-terminus. Real-time quantitative PCR, Western blotting and immunohistochemistry were used to determine the expression level, protein production and location of *As*-MYD88 during embryonic development and bacterial challenge. The highest expression level during embryonic development was at the 0h and 5h stages of *A. sinica*. *As*-MYD88 was remarkably upregulated after bacterial challenge. Our results suggested that *As*-MYD88 plays a vital role in response to bacterial challenge, and during post-diapause embryonic development of *A. sinica*.

1. Introduction

Myeloid differentiation factor 88 (*MyD88*) is a key adapter protein associated with the intracellular roles of IL-1R and Toll. It has been proposed that MYD88 should be classified in the signal transduction molecule family, which has an immunomodulatory function (Hultmark, 1994; Lord et al., 1990). Wesche et al. (1997) showed that MYD88 mediated the combination of IRAK and receptors by combining with the intracellular region of IRAK and IL-1R. MYD88 has also been linked to the Toll-like receptor (TLR) signaling pathway (Medzhitov et al., 1998; Muzio et al., 1998). During innate immunity, the only defense system in invertebrates, the process of identifying the highly conserved structure of an intrusive antigen, which is known as pathogen-associated molecular patterns (PAMPs), is heavily dependent on specific receptors on the cell membrane termed pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). Among these PRRs, Toll receptors and/or TLRs are considered as canonical pathogen-recognition molecules in metazoans (Li et al., 2012; Medzhitov et al., 1997). Toll was initially recognized as a protein closely related to the *Drosophila* embryo anterior–posterior axis development (Belvin and Anderson, 1996). The Toll signaling pathway is also necessary for antimicrobial peptide expression in organisms resistant to fungi and Gram-positive bacteria (Hoffmann et al., 1999; Lemaitre et al., 1997).

The MYD88 protein comprises three functional regions: the N-terminal death domain (DD), the intermediate domain and the C-terminal Toll/interleukin-1 receptor (TIR) homology domain. The TIR domain includes three typical box motifs, namely box1, box2 and box3 (Qiu et al., 2007). The TIR may trigger a series of signal responses through interactions with other proteins having TIR domains (Janssens et al., 2002; Luke et al., 2007). The DD domain's main responsibility is the recruitment of downstream signaling molecules that have a death domain to enable signal transduction. The intermediate region's function remains unknown; NF-κB was activated when MYD88's DD sequence and intermediate domain were expressed simultaneously (Linehan et al., 2000; Xu and Shen, 2007).

Abbreviations: MyD88, myeloid differentiation factor 88; *As-MyD88*, myeloid differentiation factor 88 gene from *Artemia sinica*; *As*-MYD88, myeloid differentiation factor 88 protein from *Artemia sinica*; ORF, open reading frame; DD, death domain; PCR, polymerase chain reaction; RT-qPCR, real-time quantitative PCR; UTR, untranslated region; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; IL-1R, interleukin-1 receptor; TLR, Toll-like receptor; TIR, Toll/Il-1 receptor homologous region; IRAK, IL-1 receptor-associated kinase; IMD, immune deficiency; PBS, phosphate buffer saline.

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Although the Toll pathway is more sensitive to the amount of MYD88 during the immune response than during dorsoventral development, MYD88 plays a crucial role in the dorsoventral patterning of the embryo through the Toll pathway (Charatsi et al., 2003). In insects like *Drosophila melanogaster*, the formation of the embryonic dorsoventral axis is regulated by the Toll/Dorsal pathway (Morisato and Anderson, 1995), which is homologous to the vertebrate Toll/IL-1 receptor signaling pathway (Belvin and Anderson, 1996). *MyD88* is a new member of the dorsal group of genes. Prior to initiating signaling, MYD88 is stably associated via death domain interactions. In the pre-signaling state, the intracellular TIR domain of Toll must be inaccessible to MYD88 binding. Absence of MYD88 disrupts ventral or lateral cuticle formation in *Drosophila* (Sun et al., 2004). In addition, MYD88 activity is required for normal Spemann organizer formation, implying an essential role for maternal Toll/IL-1 receptors in Xenopus axis formation (Prothmann et al., 2000).

Artemia sinica (Phylum Arthropoda, Class Crustacea, Subclass Branchiopoda, Order Anostaca, Family Artemiidae, Genus *Artemia*) is distributed widely in the hyperosmotic environment of salt pools and salt lakes in China (Jiang et al., 2007). It is a commercially important crustacean because of its use as a main food resource to feed newborn fish in aquaculture. Its resistance to high salinity, drying, low temperature, pressure and other adverse environments stress has led to *Artemia* being widely used in various fields, ranging from developmental biology to evolution and ecology (Janeway and Medzhitov, 2002), especially in innate immune research, where it is widely studied as an animal model.

The role of the *MyD88* gene during early embryonic development and immune response of *A. sinica* remains unknown. We investigated its expression pattern, expression location and potential roles during different developmental stages of *A. sinica*, and during the immune response bacterial challenge. Therefore, in the present study, the *As-MyD88* cDNA from *A. sinica* was cloned and its expression level during early embryonic development and in response to bacterial challenge was analyzed by real-time qPCR. In addition *As*-MYD88 was expressed in *E. coli* by a prokaryotic expression plasmid, pET-28a. Meanwhile, the protein yield of *As*-MYD88 and the location of its protein expression were investigated using Western blotting and whole mount immunohistochemistry, respectively. Our aim was to further understand the role of MYD88 during early embryonic development and during the immune response of *A. sinica.*

2. Materials and methods

2.1. Animal preparation

A. sinica cysts were harvested from the salt lake of Yuncheng in Shanxi Province, China, and stored at –20 °C in the dark. The cysts were hatched in axenic seawater and allowed to propagate under these conditions: a temperature of 28 °C, salinity of 28‰, and light intensity of 1000 lx.

A. sinica has five main developmental stages: the gastrula stage of *Artemia sinica* cysts (0h), umbrella stage (5~10h), the nauplius stage (15h~20h), the metanauplius stage (40h~3d), the pseudoadult stage (5d~7d) and the adult stage (10d). Animal samples (about 50 mg) were collected at different periods of development (0, 5, 10, 15, 20 and 40 h, and 3, 5, 7 and 10 d) for subsequent experiments. For the bacteria stimulation assay, nauplius stage *A. sinica* (20h) cultured in axenic sea water for 24h were used as the control group, and nauplius stage *A. Sinica* (20h) in the experimental groups were maintained at seawater with Halophilic Gram-negative bacterium *Vibrio harveyi* and Gram-positive bacteria *Micrococcus lysodeikticus* for 24h respectively. The bacterium concentrations were 10^4cellsL^{-1}, 10^5 cellsL^{-} and 10^6 cellsL^{-1}.

A

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1    ACATGGGGATCTGGAAAAAGTCTAACTATATAACAGCCCAAAAATCCCCTAAAAGGAACT   60
61   TGCAACATAAGAAGCCTAGATTGTATCTATACATATGTTTGTACAACAAGCAGTTGAGAA  120
121  AAA                                                            123
124  ATGAAAACTAACCCGAAGTTGGATGCTCTTCGTAGCTCTGTACCAAGTCCTCCAGACATA  183
1    M  K  T  N  P  K  L  D  A  L  R  S  S  V  P  S  P  P  D  I    20
184  ACACCTATGTATGAGAGGTGGTTAAAATCTGGCGAAAATGTTCTCTGGGCGCGCTGTCTT  243
21   T  P  M  Y  E  R  W  L  K  S  G  E  N  V  L  W  A  R  C  L    40
244  GGATATAAGCCTGGCTGGCGAGCAAACAAGTCCATCTGCCGTTATATAGATGCCAGTAAC  303
41   G  Y  K  P  G  W  R  A  N  K  S  I  C  R  Y  I  D  A  S  N    60
304  CCTGGTCAAGGGGATTGGAAGGACTTTGCATCAGAACTTGGTATACATCATATTGACATG  363
61   P  G  Q  G  D  W  K  D  F  A  S  E  L  G  I  H  H  I  D  M    80
364  AAGAGAATTGAAAACACGTACGGGAGAGAGGGTCCTACATGCCGAGTACTTGAAGAATAT  423
81   K  R  I  E  N  T  Y  G  R  E  G  P  T  C  R  V  L  E  E  Y   100
424  CTCAACATCAAAGAAGCAACCGTTTCACATTTGCTTCAATCATTACAAAAACTGGGAAGA  483
101  L  N  I  K  E  A  T  V  S  H  L  L  Q  S  L  Q  K  L  G  R   120
484  GAGGATATCCTATTTTCTATCTCCCCATATTTAGGTGAGCTTTTTCAACTCTACAACTCC  543
121  E  D  I  L  F  S  I  S  P  Y  L  G  E  L  F  Q  L  Y  N  S   140
544  GGATACCCTCCGCTCCCTATTGAGCCGGATGAAACACCTGAAGATAACAATTCCGACTCG  603
141  G  Y  P  P  L  P  I  E  P  D  E  T  P  E  D  N  N  S  D  S   160
604  GGAGTATCAAGTGTTCGCAGTATTTCAATGTTTTCCACTGGACAAGCTGAATCTCGACCC  663
161  G  V  S  S  V  R  S  I  S  M  F  S  T  G  Q  A  E  S  R  P   180
664  GTTGAAAGTGTCCCTTTGCAGCTGAAAGCAGCGCCAGAGACAGAAAAAGACGAAGAGAAC  723
181  V  E  S  V  P  L  Q  L  K  A  A  P  E  T  E  K  D  E  E  N   200
744  GGATGTAAAGGCATAGAAAACAACGATTTTGAAGACGCAAAGAGTTGCGACTCAAAAGGA  783
201  G  C  K  G  I  E  N  N  D  F  E  D  A  K  S  C  D  S  K  G   220
754  GAAATTCAAGAAAAAAAAAGGAGTGGATTTTAAAGTTGTTCTGTTAACATATGCAAATGAT  843
221  E  I  Q  E  K  K  G  V  D  F  K  V  V  L  L  T  Y  A  N  D   240
844  GGTCGAGATTTGGCTAAAGACGTTGCAAAACAGTTCCGCAAACATCGACCAGGTTTGCCA  903
241  G  R  D  L  A  K  D  V  A  K  Q  F  R  K  H  R  P  G  L  P   260
889  AGGTTAGGTGTTGTCACTTTGGAAGAAAATGAAGAATTTTTAAGTGTTGATCCTTGGGGA  963
261  R  L  G  V  V  T  L  E  E  N  E  E  F  L  S  V  D  P  W  G   280
964  ATTATTCAAAAGTGGTTTTATGAGGTTGATTATATTGTCCCGATACTGACAGAAGAATAT 1023
281  I  I  Q  K  W  F  Y  E  V  D  Y  I  V  P  I  L  T  E  E  Y   300
1024 TTAGAAAGGATATCCAGCCAATTCGTCCAGTCAATTGATTCTGACAGTTGCTTCGATGCT 1083
281  L  E  R  I  S  S  Q  F  V  Q  S  I  D  S  D  S  C  F  D  A   320
1084 CGTTACGTACGTCTGATTTATACAATGATGTGCAACGAGTTTATGCAGAGAGGATGTCTT 1143
321  R  Y  V  R  L  I  Y  T  M  M  C  N  E  F  M  Q  R  G  C  L   340
1144 AACTATCGCGTTCGTCCCCTTATGTCAAACGAAATATTGGCCAAAATTGATCAGAGGGCT 1203
341  N  Y  R  V  R  P  L  M  S  N  E  I  L  A  K  I  D  Q  R  A   360
1204 AGTATGAAAAATCCGATTTTCATGGCATGGAAGAAAGTTAATGATTGTGACGCGTTGGCG 1263
361  S  M  K  N  P  I  F  M  A  W  K  K  V  N  D  C  D  A  L  A   380
1264 GGGAATATGTTGAAACCTGCGCCTCGATCGCCTGCATTTTAA                    1305
381  G  N  M  L  K  P  A  P  R  S  P  A  F  *                     393
1306 TAGAATGATTTGTTTTCGCACTTATTCGCCTTTTGAGTGTTTTTTTCGTTTCAACGACAA 1365
1366 ATTAGTTGCCTAAAATTAAAATACATTGCATGAGCGAAATCTTTTTCATGAATTTGAAAG 1425
1426 TTTGGAAACTCTAAGAAGCTGATATATGCCTATATTCGAACAGTAGAATAATTTATTATT 1485
1486 CTATTTTATATGACTCTCTAGATAAACTTTTATTTTCTAGATAAATTATTGTACAACGAA 1545
1546 AAAAAAAAAA                                                     1555
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B

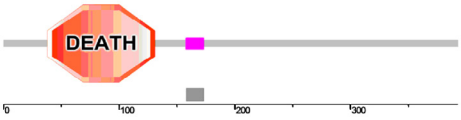

Fig. 1. (A) Nucleotide sequences and deduced amino acid sequences of the *MyD88* gene in *A. sinica*. The numbering of the nucleotide and amino acid sequences is shown to the left and right, respectively. The green letters represent the start codon; the pink letters represent the end codon; the red line represent the death domain (DD); the green line represents the box2 sequence motif of the TIR; and the blue line represent the box1sequence motif of the TIR. (B) Domain analysis of the putative *As*-MYD88 protein. The mature protein includes a death domain (DD) and a low complexity region in the N-terminus.

2.2. Cloning of As-MyD88 cDNA

Total RNA from *A. sinica* cysts (0h) was extracted using TRIzol-A$^+$ (Tiangen, Beijing, China) in accordance with the manufacturer's instructions. An oligo (dT) primer and MLV reverse transcriptase (Takara, Dalian, China) then reverse transcribed the RNA into cDNA. We obtained the gene library of *A. franciscana* from the GenBank, and related homologous sequence of *MyD88* gene (GenBank Sequence Number: ES523270.1) was identified by bioinformatics