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Short communication

Characterization of MyD88 in Japanese eel, *Anguilla japonica*

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ABSTRACT

Myeloid differentiation factor 88 (MyD88) is a key adaptor protein required for the signaling of all Toll-like receptors except TLR3, which results to the interaction of activated TLR complexes via C-terminal TIR domain and the binding of downstream kinase via N-terminal death domain. In this study, the MyD88 gene from the Japanese eel (*Anguilla japonica*) was identified. The open reading frame of AjMyD88 was 918 bp in length, encoding a protein composed of conserved N-terminal death domain and C-terminal TIR domain, respectively. Multiple alignment revealed highly conserved sites across all examined vertebrate lineages in death and TIR domains. Site-directed mutagenesis and luciferase analysis revealed that the W78A, L91A and L95A mutations in death domain had modest impairment of their ability in activating NF-κB promoter. The expression level of AjMyD88 was investigated by real-time PCR in response to poly I:C stimulation and *Edwardsiella tarda* infection. Significantly increased MyD88 expression was observed at early phase in all tested tissues/organs in response to *E. tarda* infection and slight increase was detected in intestine and gill at 16 hpi and in head kidney, spleen and liver at 24 hpi after poly I:C stimulation. Immunofluorescence staining revealed that AjMyD88 is present as condensed forms in the cytoplasm. Taken together, sequence characterization, gene expression and cellular distribution data obtained in this study suggest that AjMyD88, similar to its mammalian ortholog, plays an important role in eel immune response against bacteria.

1. Introduction

Toll-like receptors (TLRs) represent the first line of host defense against invading pathogens by recognizing conserved microbial features called pathogen associated molecular patterns (PAMPs) [1,2]. TLRs are located on cell surface, endosomes, lysosomes and endolysosomes, recognizing a variety of endogenous and exogenous ligands such as lipids, lipoproteins, proteins and nucleic acids which are derived from a wide range of microbes including bacteria, viruses, parasites and fungi [3]. Upon ligand binding, the signal transmitted from TLRs is undertaken by a family of adaptor proteins bearing cytosolic Toll/IL-1 receptor (TIR) domain, which couple to downstream protein kinases to trigger further signaling events through the activation of transcription factors [4]. The TIR domain containing adaptors signaling by TLRs involves five adaptor proteins known as Myeloid differentiation primary response protein (MyD88), MyD88-adaptor-like (MAL, also termed TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF, also termed TICAM1), TRIF-related adaptor molecule (TRAM, also termed TICAM2) and sterile α- and armadillo-motif-containing protein (SARM) [5]. Of these, MyD88, originally identified as a myeloid differentiation primary response gene from activated M1D$^+$ myeloid precursors in response to IL-6, has drawn particularly attentions because it is recruited in signaling of all TLRs except TLR3 [6,7]. In human, the MyD88 protein contains 296 amino acids in length and an N-terminal death domain (DD), and a C-terminal TIR domain (TIR) that are separated by an intermediated segment or intermediary domain (ID) [8]. The TIR domain interacts with the cytoplasmic TIR domain of TLRs, while the death domain is responsible for the interaction with the IL-1R-associated kinases (IRAK1 or IRAK4) and for propagation of further downstream signals [9]. Studies in knockout mice revealed that MyD88-deficient mice showed a high resistance to LPS-induced shock and failed to produce proinflammatory cytokines including IL-6 and

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TNFα in vivo [10,11]. The expression of IL12, IFN-γ, nitric oxide synthase 2 were also impaired in MyD88-deficient mice, which displayed significantly enhanced susceptibility to the infection of a number of pathogens, such as *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Toxoplasma gondii* [11–15].

In general, fish PRRs, together with their downstream components in signaling cascade, are functionally equivalent to their mammalian counterparts [16–18]. However, the increasing knowledge in fish immune recognition revealed some distinct features. For example, mammals possess 10 to 15 functional TLRs, whereas more TLR genes, at least 17 TLRs were present in teleosts [18–20]. TLR4 in mammals, known to recognize the major component of the outer membrane of Gram-negative bacteria, i.e. LPS, has been proved to be irrelevant in sensing LPS in zebrafish [21,22]. As for adaptors, gene duplication (i.e. Tollip in salmonids) or gene loss (i.e. IRAK2 in teleosts) events have occurred in teleost lineage [18,23–25]. In addition, MyD88 was found duplicated in common carp although it exists typically as a single form in mammals and most teleosts [26].

The Japanese eel, *Anguilla japonica*, is one of the most important aquaculture and traded commodities in eastern Asia, including China, Japan and Korea. According to the global aquaculture production statistics of FAO, the production of this specie increased dramatically from 339 tons in 1950 to 278 177 tons in 2016. At present, the intensive culture of eels has resulted in outbreak of various diseases. The most frequently bacterial pathogens in anguilliculture include *Edwardsiella tarda*, *Aeromonas hydrophila*, *Citrobacter freundii* [27,28]. In this paper, we describe the AjMyD88 cDNA from *A. japonica* and compare the sequence with other vertebrate MyD88. Site-directed mutagenesis of conserved residues within the dead domain was performed to investigate their effects on MyD88-mediated NF-κB activation. The expression of AjMyD88 was analyzed at mRNA level in response to poly I:C treatment and to *E. tarda* infection. The protein localization of AjMyD88 was observed using immunofluorescence confocal microscopy.

2. Materials and methods

2.1. Sample collection

Japanese eels, with average weight of 100 g, were maintained in a laboratory re-circulating water (28 ± 2 °C) for 2 weeks before being used in experiments, with the permission from College of Fisheries, Jimei University. For expression analysis, fish were challenged with poly I:C or *Edwardsiella tarda*. Fish were divided into three groups, each with twenty-four eels. Two groups of eels were injected intraperitoneally with 0.2 mg poly I:C (Sigma) in phosphate buffered saline (PBS; 2 mg/ml, 100 μl per fish) or 200 μl bacterial suspension in PBS at a concentration of 1×10^6 cfu/ml, respectively. Control group was injected with the same volume of PBS. At 8, 16, 24 and 72 h post injection (hpi), six fish from each group were anaesthetized in 0.05% 2-phenoxyethanol and immediately killed for the dissection of head kidney, spleen, liver, intestine, skin and gill for RNA isolation.

2.2. RNA preparation and cDNA synthesis

The total RNA from each sample was extracted using Trizol (Invitrogen Corp) according to the manufacturer's instruction. Quality and quantity of extracted RNA were assessed by electrophoresis and Nanodrop 2000 spectrophotometry (Thermo Scientific, USA). For cloning open reading frame, 2 μg total RNA from spleen was reverse-transcribed using SMART™ RACE cDNA Amplification Kit (Takara) with oligo dT primer following the manufacturer' s instruction. For expression analysis, the equal amount of total RNA of each sampled tissues/organs were treated with gDNA Eraser (Takara) and the first stand cDNA was synthesized by using GoScript™ Reverse Transcription System (Promega).

Table 1
Sequences used for blast search and phylogenetic tree construction.

Gene	Species	Abbreviation	Accession
MyD88	*Petromyzon marinus*	*P. marinus*	ENSPMAG00000000756
	Lepisosteus oculatus	*L. oculatus*	ENSLOCG00000009811
	Danio rerio	*D. rerio*	ENSDARG00000010169
	Astyanax mexicanus	*A. mexicanus*	ENSAMXG00000002362
	Gadus morhua	*G. morhua*	ENSGMOG00000010938
	Takifugu rubripes	*T. rubripes*	ENSTRUG00000017474
	Tetraodon nigroviridis	*T. nigroviridis*	ENSTNIG00000012746
	Oreochromis niloticus	*O. niloticus*	ENSONIG00000005835
	Gasterosteus aculeatus	*G. aculeatus*	ENSGACG00000003543
	Oryzias latipes	*O. latipes*	ENSORLG00000009788
	Xiphophorus maculatus	*X. maculatus*	ENSXMAG00000009277
	Poecilia formosa	*P. formosa*	ENSPFOG00000013420
	Latimeria chalumnae	*L. chalumnae*	ENSLACG00000016801
	Xenopus tropicalis	*X. tropicalis*	ENSXETG00000018434
	Anolis carolinensis	*A. carolinensis*	ENSACAG00000009971
	Gallus gallus	*G. gallus*	ENSGALG00000005947
	Homo sapiens	*H. sapiens*	ENSG00000172936
TIRAP	*Lepisosteus oculatus*	*L. oculatus*	ENSLOCG00000002669
	Danio rerio	*D. rerio*	ENSDARG00000074371
	Takifugu rubripes	*T. rubripes*	ENSTRUG00000007154
	Oreochromis niloticus	*O. niloticus*	ENSONIG00000005330
	Oryzias latipes	*O. latipes*	ENSORLG00000014682
	Xiphophorus maculatus	*X. maculatus*	ENSXMAG00000001314
	Poecilia formosa	*P. formosa*	ENSPFOG00000003099
	Latimeria chalumnae	*L. chalumnae*	ENSLACG00000017866
	Xenopus tropicalis	*X. tropicalis*	ENSXETG00000031764
	Xenopus tropicalis	*X. tropicalis*	ENSXETG00000017919
	Anolis carolinensis	*A. carolinensis*	ENSACAG00000000103
	Gallus gallus	*G. gallus*	ENSGALG00000040224
	Homo sapiens	*H. sapiens*	ENSG00000150455
TICAM1	*Lepisosteus oculatus*	*L. oculatus*	ENSLOCG00000000094
	Danio rerio	*D. rerio*	ENSDARG00000102493
	Takifugu rubripes	*T. rubripes*	ENSTRUG00000014679
	Tetraodon nigroviridis	*T. nigroviridis*	ENSTNIG00000001099
	Oreochromis niloticus	*O. niloticus*	ENSONIG00000010091
	Xiphophorus maculatus	*X. maculatus*	ENSXMAG00000008608
	Latimeria chalumnae	*L. chalumnae*	ENSLACG00000017601
	Anolis carolinensis	*A. carolinensis*	ENSACAG00000008683
	Gallus gallus	*G. gallus*	ENSGALG00000026850
	Homo sapiens	*H. sapiens*	ENSG00000127666
TICAM2	*Pelodiscus sinensis*	*P. sinensis*	ENSPSIG00000017006
	Anolis carolinensis	*A. carolinensis*	ENSACAG00000028497
	Homo sapiens	*H. sapiens*	ENSG00000243414
SARM	*Petromyzon marinus*	*P. marinus*	ENSPMAG00000003311
	Lepisosteus oculatus	*L. oculatus*	ENSLOCG00000005448
	Gadus morhua	*G. morhua*	ENSGMOG00000002197
	Oreochromis niloticus	*O. niloticus*	ENSONIG00000008177
	Oryzias latipes	*O. latipes*	ENSORLG00000008096
	Xiphophorus maculatus	*X. maculatus*	ENSXMAG00000008493
	Poecilia formosa	*P. formosa*	ENSPFOG00000006701
	Takifugu rubripes	*T. rubripes*	ENSTRUG00000016046
	Tetraodon nigroviridis	*T. nigroviridis*	ENSTNIG00000005561
	Gasterosteus aculeatus	*G. aculeatus*	ENSGACG00000008602
	Danio rerio	*D. rerio*	ENSDARG00000010610
	Astyanax mexicanus	*A. mexicanus*	ENSAMXG00000006542
	Latimeria chalumnae	*L. chalumnae*	ENSLACG00000006649
	Anolis carolinensis	*A. carolinensis*	ENSACAG00000012024
	Gallus gallus	*G. gallus*	ENSGALG00000003595
	Homo sapiens	*H. sapiens*	ENST00000579593
TLR1	*Lepisosteus oculatus*	*L. oculatus*	ENSLOCG00000012910
	Oryzias latipes	*O. latipes*	ENSORLG00000004420
	Poecilia formosa	*P. formosa*	ENSPFOG00000020047
	Takifugu rubripes	*T. rubripes*	ENSTRUG00000009990
	Tetraodon nigroviridis	*T. nigroviridis*	ENSTNIG00000014211
	Gasterosteus aculeatus	*G. aculeatus*	ENSGACG00000017958
	Danio rerio	*D. rerio*	ENSDARG00000100649
	Astyanax mexicanus	*A. mexicanus*	ENSAMXG00000025563
	Latimeria chalumnae	*L. chalumnae*	ENSLACG00000010038
	Homo sapiens	*H. sapiens*	ENST00000308979

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