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Short communication

Molecular cloning and expression studies of the adapter molecule myeloid differentiation factor 88 (MyD88) in turbot (*Scophthalmus maximus*)

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ABSTRACT

Myeloid differentiation factor 88 (MyD88) is an adapter protein involved in the interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR)-mediated activation of nuclear factor-kappaB (NF-κB). In this study, a full length cDNA of MyD88 was cloned from turbot, *Scophthalmus maximus*. It is 1619 bp in length and contains an 858-bp open reading frame that encodes a peptide of 285 amino acid residues. The putative turbot (*Sm*)MyD88 protein possesses a N-terminal death domain and a C-terminal Toll/IL-1 receptor (TIR) domain known to be important for the functions of MyD88 in mammals. Phylogenetic analysis grouped *Sm*MyD88 with other fish MyD88s. *SmMyD88* mRNA was ubiquitously expressed in all examined tissues of healthy turbots, with higher levels observed in immune-relevant organs. To explore the role of *SmMyD88*, its gene expression profile in response to stimulation of lipopolysaccharide (LPS), CpG oligodeoxynucleotide (CpG-ODN) or turbot reddish body iridovirus (TRBIV) was studied in the head kidney, spleen, gills and muscle over a 7-day time course. The results showed an up-regulation of *SmMyD88* transcript levels by the three immunostimulants in all four examined tissues, with the induction by CpG-ODN strongest and initiated earliest and inducibility in the muscle very weak. Additionally, TRBIV challenge resulted in a quite high level of *SmMyD88* expression in the spleen, whereas the two synthetic immunostimulants induced the higher levels in the head kidney. These data provide insights into the roles of *Sm*MyD88 in the TLR/IL-1R signaling pathway of the innate immune system in turbot.

1. Introduction

The innate immunity in fish, as in all vertebrates, is the first line of defense and provides crucial signals for activation of adaptive immune responses (Akira et al., 2001). Detection and clearance of invading pathogens by the innate immune system are associated with plenty of signaling pathways that are evolutionarily conserved throughout vertebrates. It works through the way of being triggered when pathogen-associated molecular patterns (PAMPs) come into contact with host-expressed pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000). One of the well-characterized PRRs is the family of Toll-like receptors (TLRs) that detects microbial PAMPs such as bacterial lipopolysaccharides (LPS), peptidoglycans (PGN) and flagellin, viral RNA, unmethylated CpG DNA of viruses, bacteria and protozoa, β-glycan of fungi and lipoproteins of various pathogens (Akira et al., 2006; Mogensen, 2009). The signaling pathways mediated by TLRs are broadly classified into the myeloid differentiation factor 88 (MyD88)-dependent and -independent ones. The former uses MyD88 as an adapter molecule to activate the signaling cascades and produces inflammatory mediators.

Although MyD88 was first found in mice in 1990 as a myeloid differentiation primary response gene induced during terminal differentiation of M1D$^+$ myeloid precursor cells in response to interleukin (IL)-6 treatment (Lord et al., 1990), its function as a key adapter molecule in the interleukin-1 receptor (IL-1R)/TLR-mediated signaling remains unknown until 1997 (Wesche et al., 1997). MyD88 has a bipartite structure composed of an N-terminal death domain and a C-terminal Toll/IL-1 receptor (TIR) domain with a short intervening linker segment. Upon activation by PAMPs, all of TLRs except TLR3 recruit MyD88 through the TIR domain; the death domain interacts with the corresponding domain in IL-1R-associated kinases (IRAKs), leading to recruitment of downstream tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6); these events eventually result in activation of NF-κB and interferon

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regulator factors (IRFs) and induction of pro-inflammatory cytokines or antiviral genes that play an important role in combating pathogens (Deepika et al., 2014). To date, MyD88 has been identified in mammals, birds, reptiles, amphibians, fishes and invertebrates (Bonnert et al., 1997; Deepika et al., 2014; Li et al., 2011; Prothmann et al., 2000; Wheaton et al., 2007). The fishes with MyD88 identified include zebrafish (van der Sar et al., 2006), Japanese flounder (Takano et al., 2006), half-smooth tongue sole (Yu et al., 2009), large yellow croaker (Yao et al., 2009), Atlantic salmon (Skjæveland et al., 2009), rainbow trout (Rebl et al., 2009), rock bream (Whang et al., 2011), common carp (Kongchum et al., 2011), grass carp (Su et al., 2011), orange-spotted grouper (Yan et al., 2012), miiuy croaker (Tang et al., 2012), etc. Fish MyD88 exhibits structural and functional homologies with its mammalian counterpart.

Turbot, *Scophthalmus maximus*, is an important commercial marine species cultured widely in the world. However, the knowledge about turbot (*Sm*)MyD88 is scarce. The aim of this study is to improve understanding of the innate immune system in turbot by studies of a *MyD88* gene, which will help in development of strategies of microbial infectious disease control for this species. Herein, we report the cDNA sequence, mRNA tissue distribution and transcriptional modulation of *SmMyD88*. The last study was performed *in vivo* upon stimulation of turbots with LPS, synthetic CpG oligodeoxynucleotide (CpG-ODN) or turbot reddish body iridovirus (TRBIV). We demonstrated the involvement of *Sm*MyD88 in immune responses of turbots to these three immunostimulants.

2. Materials and methods

2.1. Fish, stimulants and immunostimulation experiments

Turbot (*S. maximus*) juveniles (68.4 ± 4.5 g, n = 170) were purchased from a local fish farm. Fish were kept in aerated seawater tanks at 16 °C for 1 week before use. LPS (L2880, *Escherichia coli* 055:B5; Sigma, St. Louis, MO, USA) was diluted in phosphate buffer saline (PBS, pH 7.4) to make stock. CpG-ODN 2395 (class C, 5′-T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G-3′, phorothioate modifications are marked with *; Coley Pharmaceutical Group, Ottawa, CA) was dissolved in TE buffer (10 mM Tris, 1 nM EDTA, pH 8.0). TRBIV was isolated from cultured turbots with TRBIV disease as previously described (Shi et al., 2004). The viral titers were measured by a 50% tissue culture infective dose ($TCID_{50}$) assay according to the method of Reed and Muench (1938). Three groups of turbots were intraperitoneally (i.p.) injected with LPS (2.5 mg/ml, 112 μl per fish), CpG-ODN 2395 (0.15 mg/ml, 95 μl per fish) or TRBIV (2×10^6 $TCID_{50}$/ml, 120 μl per fish), respectively. Control fish for LPS- or TRBIV-treated group were injected with PBS, while those for CpG-ODN 2395-treated group were injected with TE buffer. The volume of PBS or TE buffer used was same with that of corresponding stimulant. The spleen, head kidney, gills and muscle of injected fish were collected for gene expression assay at various time points post injection (0, 3, 6 and 12 hours and 1, 2, 3, 4, 5 and 7 days after LPS or CpG-ODN 2395 injections, or 0, 3 and 6 hours and 1, 2, 3, 4, 5 and 7 days after TRBIV injection), while the untreated healthy fish were used for tissue distribution analysis.

2.2. RNA extraction

Fish were sacrificed and various tissues (including brain, gills, stomach, intestine, heart, head kidney, kidney, liver, spleen, gonad, muscle and skin) were excised, immediately snap-frozen in liquid nitrogen and stored at −80 °C until used. Total RNA was extracted from each tissue using Isogen reagent (Nippon Gene, Tokyo, Japan). RNA samples were incubated with DNase I to remove genomic DNA contamination using Turbo DNA-free Kit (Ambion, Austin, TX, USA). The RNA concentration was determined by measuring the absorbance at 260 nm, and its quality was monitored by A_{260} nm/A_{280} nm ratios >1.8.

2.3. Cloning of SmMyD88 cDNA

The SMART cDNAs were generated from 1 μg of total RNA extracted from head kidney of a turbot using a cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Based on the conserved sequences of known fish MyD88s, degenerate primers (Supplementary Table S1) were designed. A 539-bp partial cDNA of *SmMyD88* was obtained by homology cloning, while the 5′- and 3′-end fragments, with lengths of 305 bp and 1008 bp, respectively, were obtained by a rapid amplifications of cDNA ends (RACE). The full length cDNA sequence was assembled and its continuity was confirmed by sequencing the cloned PCR product amplified with a pair of terminal primers. PCRs across this group were carried out with Ex Taq DNA polymerase (TaKaRa, Dalian, Liaoning, China) under the following condition: initial denaturation at 94 °C for 4 min, then 25–40 cycles of 94 °C for 40 s, 48.9–61 °C for 30 s and 72 °C for 40 s–2 min, and final extension at 72 °C for 7 min. The PCR products were isolated using an E.Z.N.A Gel Extraction Kit (Omega Bio-tek, Doraville, GA, USA), cloned into pMD18-T vector (TaKaRa) and sequenced with an ABI PRISM 3100 DNA sequencer (Applied Biosystem, Foster City, CA).

2.4. Sequence analysis

Sequence result of *SmMyD88* was compared with the GenBank/EMBL database by using the BLASTX and BLASTP search programs (http://blast.genome.ad.Jp). The nucleotide sequence was translated to protein sequence using Translate Tool DNAman. The multiple alignment of protein sequences was produced by the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic tree was created using the neighbor-joining (NJ) method by MEGA version 5.0. Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches.

2.5. Quantitative real time PCR (qPCR)

qPCR analysis was employed to study *SmMyD88* mRNA tissue distribution and gene expression in response to TRBIV, CpG-ODN 2395 or LPS stimulation in specific organs. Five individuals were studied for tissue distribution and, also, five individuals for each time point of gene expression assay. One microgram of total RNA from each tissue was reverse-transcribed into cDNA by random primers using Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). qPCR was conducted in 20 μl volume containing 1 × SYBR Green Real Time PCR Master Mix (Toyobo, Osaka, Japan), 0.2 μM each of gene-specific forward and reverse primers (Supplementary Table S1) and 1.0 μl diluted cDNA (50 ng/μl). PCR conditions were 94 °C for 4 min, followed by 40 cycles of 94 °C for 40 s, 61.5 °C for 30 s, 72 °C for 20 s, and final elongation at 72 °C for 7 min. Turbot *18S rRNA* (GenBank accession number: EF126038) was used as endogenous control. All samples were amplified in triplicates. Fluorescent detection was performed after each extension step. A dissociation protocol was performed after thermo cycling to verify that a single amplicon of expected size was amplified. The expression levels of the target gene were normalized to *18S rRNA*, and further expressed as fold change relative to the expression level in control according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) in the gene expression assay upon immune stimulation.

2.6. Statistical analysis

Statistical analysis was performed using SPSS13.0 software (SPSS Inc., Chicago, IL, USA). Differences in the data were compared by one-way analysis of variance (ANOVA) followed by Duncan's post hoc