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Short communication

Molecular characterization and functional analysis of MyD88 from the tropical sea cucumber, *Holothuria leucospilota*

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ABSTRACT

In this study, a myeloid differentiation factor 88 (MyD88) named as HLMyD88 was identified from the sea cucumber *Holothuria leucospilota*. The full-length cDNA of *HLMyD88* is 4797 bp in size, containing a 227 bp 5′-untranslated region (UTR), a 3721 bp 3′-UTR and an 849 bp open reading frame (ORF) encoding a protein of 282 amino acids with a deduced molecular weight of 32.25 kDa HLMyD88 contains an N-terminal death domain and a C-terminal Toll/interluekin-1 receptor (TIR) domain with three highly conserved sequence motifs named as Box 1, Box 2 and Box 3. The results of luciferase reporter assay showed that over-expressed HLMyD88 in HEK293T cells could activate the transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). Additionally, the secretion of proinflammatory cytokines IL-1β and TNF-α in the HEK293T cells was increased by over-expressed HLMyD88, indicating the potential role of HLMyD88 in the innate immunity of sea cucumber. Moreover, we further confirmed that over-expressed HLMyD88 could also induce apoptosis.

1. Introduction

Myeloid differentiation factor 88 (MyD88) is a common signaling adaptor shared by all the Toll-like receptors (TLRs) except TLR3 in the TLR/interleukin-1 receptor (TLR/IL-1R) signaling pathways and plays a critical role in initiating and activating the innate immune system [1–5]. In addition, MyD88 also mediates TLR2-induced apoptosis via an apoptotic mechanism including Fas-associated death domain protein (FADD) and caspase 8 [6].

TLRs are a class of germline-encoded pattern-recognition receptors (PRRs) that can specifically recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) produced exclusively by the pathogenic microbes [7]. The signaling pathways mediated by TLRs are broadly classified into two distinct ways: the MyD88-dependent pathway and the MyD88-independent/TRIF-dependent way [8]. In the MyD88-dependent pathway, MyD88 was used as an adapter molecule to activate the signaling cascades of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK) [1,9,10]. Once the PAMPs were recognized and combined by TLRs, MyD88 was recruited by TLRs through the homophilic interaction between the Toll/interluekin-1 receptor (TIR) domains in TLR and MyD88 [11]. Then, MyD88 in turn recruits interleukin-1 receptor-associated kinase (IRAK) 4 and IRAK2 via its death domain, forming a signal transduction myddosome complex, which further phosphorylates and recruits IRAK1 [12]. The activated IRAKs then bind to tumor necrosis factor receptor-associated factor 6 (TRAF6) and the cascaded responses belong to two distinct signaling pathways are initiated, leading to the activation of transcription factors NF-κB and activator protein 1 (AP-1), followed by the secretion of proinflammatory cytokines, such as TNF-α, IL-1β and IL-12 [13–15].

MyD88 orthologs are widely found in vertebrates and invertebrates, and have been proven to be essential adapters for the TLR/IL-1R signaling from *Drosophila* to mammals [16–19]. To date, the TLR signaling pathways involved in the innate immune defense against pathogen invasion have been identified in many invertebrates including crustaceans, insects and molluscs [19–24]. In echinoderm, the highest group of invertebrates, the homologues of TLRs and an MyD88 ortholog have

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been predicted from the purple sea urchin (*Strongylocentrotus purpuratus*) genome data [25]. In addition, two TLRs and two key adaptor molecules (MyD88 and TRAF6) have been cloned from sea cucumber (*Apostichopus japonicus*) with descriptions of their expression profiles after immune challenge [26,27]. However, the roles of echinoderm MyD88 in TLR/IL-1R signaling pathways and apoptosis remain unclear. In order to verify the existence of MyD88-dependent signaling pathway in echinoderm and further understand its potential role in apoptosis, the tropical sea cucumber *Holothuria leucospilota*, a start-up aquaculture species in Southern China [28], was used as the experimental object in this study.

H. leucospilota is a widespread tropical sea cucumber species in the Indo-Pacific region, playing an important role in the marine ecosystem as a sediment transporter and a seabed scavenger [29,30]. Compared to other sea cucumber species, *H. leucospilota* has broader environmental adaptation [29,30]. In the present study, we first cloned the cDNA sequence of *MyD88* from the tropical sea cucumber *H. leucospilota*. The functions of *H. leucospilota* MyD88 (HLMyD88) in activating NF-κB and AP-1 were investigated, and the involvement of HLMyD88 in apoptosis was also examined.

2. Materials and methods

2.1. Molecular cloning of HLMyD88 full-length cDNA

Total RNA was isolated from the intestine of sea cucumber *H. leucospilota* using TRIzol reagent and reverse transcribed into first strand cDNA by using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). To clone the full-length cDNA sequence of *HLMyD88*, the 3′- and 5′-rapid amplification of cDNA ends (RACE) were carried out using 3′ Full Race Core Set Ver. 2.0 and 5′ Full Race Kit (TaKaRa), respectively. The specific primers (3′ RACE1/3′ RACE2 and 5′ RACE1/5′ RACE2, Table 1) for *HLMyD88* used in the 3′- and 5′-RACE were designed based on a verified expression sequence tag (EST) of 4356 bp (Suppl. 1), which was obtained from a cDNA library of *H. leucospilota* constructed previously in our lab and shared high sequence similarity to the known MyD88 in other species. The amplification conditions for 3′- and 5′-RACE were as follows: 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 120 s. The obtained amplicons were sub-cloned into the pMD-18 vector (TaKaRa), and three positive clones for each amplicon were sequenced.

2.2. Bioinformatics analysis

Structural domains of HLMyD88 were predicted using the SMART program (http://smart.embl-heidelberg.de/). The three-dimensional (3-D) model of HLMyD88 was deduced with Swiss modeling software provided by the SWISS-MODEL server (https://swissmodel.expasy.org/). Alignment of MyD88 among various species was performed with ClustalX (http://wwwigbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and demonstrated by using the GeneDoc program. A phylogenetic tree was constructed based on amino acid difference (*p*-distance) with the Neighbor-joining method (pairwise deletion) with 1000 bootstrap replicates using MEGA 6.0.

Table 1
Primers used in this study.

Primers	Primer sequences (5′-3′)
For cDNA cloning	
3′ RACE1	TGAATAATTTGAAAGAGATTCTAG
3′ RACE2	AAAAAGTTGGGGAAAGTAATGTTG
5′ RACE1	TAAATAATGTGACAGCTTCATCCT
5′ RACE2	AAGATCCATGCTGTTTGTTGCCAT
For protein expression	
P*HLMyD88*-F	GGTACCATGGCAACAAACAGCATGGATCTTTTTTC
P*HLMyD88*-R	GAATTCAAGAGGAGCAACAATGGCTCTGTAAAGTC

2.3. Construction of the recombinant plasmid pcDNA3.0/Flag/HLMyD88

The coding region of *HLMyD88* was amplified by PCR using the gene-specific primers P*HLMyD88*-F and P*HLMyD88*-R (Table 1) with a restriction enzyme site for *Kpn* I or *Eco*R I at their 5′-ends, respectively. The amplified DNA fragment and pcDNA3.0/Flag plasmid (Invitrogen, USA) were digested with *Kpn* I and *EcoR* I (TaKaRa) and ligated with T4 DNA ligase (TaKaRa) at 16 °C for 2 h. Then, the cloned product was transformed into XL1-blue competent cells. The transformant confirmed by double-enzyme (*Kpn* I and *Eco*R I) digestion and sequencing was cultured in lysogen broth (LB) medium with ampicillin (100 μg/mL) at 37 °C with shaking for overnight, and the pcDNA3.0/Flag/HLMyD88 recombinant plasmid (rHLMyD88) was purified using the QIAGEN Plasmid Midi Kit (QIAGEN, Germany).

2.4. Cell culture and transfection

HEK293T cells, a specific cell line originally derived from human embryonic kidney cells, were used for transfection in this study due to their high transfection efficiency [31]. HEK293T cell suspension was cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS), penicillin (100 μg/mL) and streptomycin (100 μg/mL) at 37 °C with 5% CO_2. Before transfection, HEK293T cells (2.0×10^5 cells/well) were inoculated into a 24-well tissue culture plate and cultured at 37 °C with 5% CO_2 for 24 h. Then, 1 μg pcDNA3.0/Flag/HLMyD88 plasmid was transfected into the HEK293T cells by using 1 μL lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. As a control, the pcDNA3.0/Flag blank plasmid was transfected into HEK293T cells by using the same method.

2.5. Western blot

After transfection for 4 h, HEK293T cells that were transfected with pcDNA3.0/Flag/HLMyD88 (experimental group) or pcDNA3.0/Flag (control group), or untransfected (blank group), were cultured in DMEM containing 10% FBS at 37 °C with 5% CO_2 for 48 h before harvest. Then, the cells in the above three groups were respectively lysed and loaded onto a 10% (w/v) sodium dodecyl sulfate polyacrylamide gel for electrophoresis (SDS-PAGE), followed by western blot detection as described previously [32]. In this case, the rabbit anti-Flag antibody (Sigma, Germany) at 1:400 dilution and rabbit anti-β-tublin antibody (Saier Biotech Inc, China) at 1:300 dilution were used as the primary antibodies. The horseradish peroxidase conjugated goat anti-rabbit IgG (Abcam, UK) at 1:1000 dilution was used as the secondary antibody.

2.6. Detection of apoptosis

After transfection for 4 h, HEK293T cells in the three groups mentioned above were respectively cultured in 10 mL DMEM containing 10% FBS at 37 °C with 5% CO_2 for 48 h. Then, the apoptotic cells were detected by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay as described previously [32].

2.7. Luciferase reporter assay

In order to investigate the functional properties of HLMyD88 in the TLR/IL-1R signaling pathways, luciferase reporter gene assay was performed as described before [33]. The HEK293T cells cultured in a 24-well plate were transfected with 500 ng of pcDNA3.0/Flag/HLMyD88, 245 ng of NF-κB-luc or AP-1-luc reporter plasmid and 5 ng pRL-TK reference plasmid (Promega, USA). In this case, pcDNA3.0/Flag was used as the negative control. After transfection for 48 h, luciferase reporter activity was detected using the Dual-Luciferase Kit (Promega).