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Short communication

Cloning and characterization of apoptosis-associated speck-like protein containing a CARD domain (ASC) gene from Japanese flounder *Paralichthys olivaceus*

Shuo Li [a, 1], Xiaoli Chen [a], Weijiao Peng [a], Gaixiang Hao [a], Xuyun Geng [b], Wenbin Zhan [c], Jinsheng Sun [a, *]

[a] Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Sciences, Tianjin Normal University, 393 West Binshui Road, Xiqing District, Tianjin 300387, China
[b] Tianjin Center for Control and Prevention of Aquatic Animal Infectious Disease, 442 South Jiefang Road, Hexi District, Tianjin 300221, China
[c] Laboratory of Pathology and Immunology of Aquatic Animals, LMMEC, Ocean University of China, Qingdao 266003, China

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ABSTRACT

Apoptosis-associated speck-like protein containing a CARD domain (ASC) is a critical adaptor molecule in multiple inflammasome protein complexes that mediate inflammation and host defense. However, few studies have been performed in lower vertebrates such as in teleost. Here we identified and characterized a novel *ASC* gene (namely *PoASC*) from Japanese flounder *Paralichthys olivaceus*. The complete cDNA sequence of *PoASC* contains a 22 bp 5′-untranslated sequence, a 612 bp open reading frame, and a 438 bp 3′-untranslated sequence. The deduced PoASC protein is comprised of 203 amino acids with a conserved N-terminal PYD domain and a C-terminal CARD domain and shows 35–62% sequence identity with other vertebrate ASC proteins. *PoASC* mRNA transcripts was detected in various Japanese flounder tissues and is dominantly expressed in hepatopancreas. Oligomeric speck-like structures were observed when PoASC was exogenously expressed in Japanese flounder FG-9307 cells. Immune challenge experiments revealed that *PoASC* gene expression was significantly induced in the Japanese flounder head kidney macrophages and peripheral blood leukocytes by the canonical TLR ligands LPS, Poly(I:C) and zymosan stimulations. In addition, the induction of *PoASC* was also observed in *Edwardsiella tarda* challenged head kidney and gill tissues. Furthermore, we for the first time showed that extracellular ATP, an important signaling molecule in triggering innate immune response and activation of NLR inflammasome, significantly up-regulates *PoASC* expression in the Japanese flounder head kidney macrophages in a dose-dependent manner. Together, these findings addressed the involvement of *PoASC* in TLR and extracellular ATP-mediated innate immune signaling in the Japanese flounders.

1. Introduction

The inflammasome, an innate immune signaling platform sensing pathogens and danger signals, is comprised of inflammasome sensors (member of host pattern recognition receptors) such as NLRP3 and AIM2, adaptor molecule and the effector caspases [1–3]. The adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), initially isolated from human Leukemia HL-60 cells by immunoscreening approach using an anti-ASC monoclonal antibody [4], is a key component of several identified inflammasomes including NLRP3, NLRC4 and AIM2 [5]. The cytosolic adaptor protein ASC bridges the upstream inflammasome sensor proteins and downstream caspase-1 to form multi-protein inflammasome complexes, achieved through pyrin domain (PYD) interactions between sensors and ASC and recruitment domain (CARD) interactions between ASC and caspase-1 [6]. By formation of highly oligomeric structures (called “specks”), ASC activate cysteine protease caspases, resulting in proteolytic cleavage of pro-IL-1β and pro-IL-18 to their respective biological active forms [7,8]. The mature cytokines then recruit and activate immune cells and induce pyroptotic cell death [3]. In contrast, knockdown of ASC

* Corresponding author. College of Life Sciences, Tianjin Normal University, 393 West Binshui Road, Xiqing District, Tianjin 300387, China.
E-mail addresses: shuo76@yahoo.com (S. Li), jssun1965@aliyun.com (J. Sun).
[1] S. Li is a senior author.

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inhibited inflammatory caspase-1 activity and IL-1β secretion [9]. Studies also have demonstrated that ASC is critical for the immune response and survival in West Nile Virus Encephalitis [10], host defense against infection with *Mycobacterium tuberculosis* [11], *Brucella abortus* [12] and *Streptococcus pneumonia* [13]. In addition, ASC has been identified as an activating adaptor for NF-κB and caspase-8-dependent apoptosis [14]. In some cases, ASC also shows caspase-1-independent innate immune activities [15], pointing out an uncanonical inflammasome pathway ASC may contribute in innate immune response. Furthermore, in addition to serve as an intracellular adaptor protein, ASC also shows important extracellular function in promoting maturation of IL-1β and cell-to-cell communications [16]. Despite the important roles in innate immune system, emerging evidence also highlights the importance of ASC in facilitating adaptive immune responses. It has been suggested that ASC may influence the development and functioning of Treg cells [17]. Recent studies also revealed that ASC can regulate the function of adaptive immune cells by modulating Dock2-mediated Rac activation and actin polymerization in an inflammasome-independent manner [18]. In teleost, however, the immune significance of ASC remains largely unexplored and so far only one *ASC* gene has been reported [19].

In this study we have cloned and characterized a *ASC* gene, namely *PoASC*, from Japanese flounder *Paralichthys olivaceus*. We revealed that PoASC is a cytosolic protein that appears as speck-like structures when exogenously expressed in Japanese flounder FG-9307 cells. In addition, we showed that *PoASC* gene expression was regulated by immune challenges and extracellular ATP stimulations as well. These observations indicate that *PoASC* is an inducible immune response gene implicated in extracellular ATP-mediated immune signaling in fish.

2. Materials and methods

2.1. Fish and maintenance

Japanese flounder *P. olivaceus* were obtained from a local farm in Dagang, Tianjin, China. Fish were transported to the laboratory and maintained in an aerated running seawater system at 21 °C for two weeks before experimentation. Only healthy Japanese flounders without any pathological signs were selected in experiments. For tissue collection, *P. olivaceus* was euthanized with overdose of tricaine methane sulfonate (Sigma) as described in previous study [20] and the individual tissue was separated and collected aseptically.

2.2. RNA preparation and cDNA synthesis

Total RNA from tissues and cells was purified by TRIzol reagent and the PureLink® RNA Mini Kit (Invitrogen), respectively, according to the manufacturer's recommendations. RNA integrity was examined by electrophoresis on a 1.2% formaldehyde-denatured ethidium bromide agarose gel and documented by a GelDoc™ XR system (Bio-Rad). RNA concentration was measured using a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific) and treated with DNase I (Invitrogen, amplification grade) to digest possible genomic DNA contamination according to the manufacturer's procedure. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and oligo-deoxythymidine adaptor primer (5′-TCGAATTCGGATCCGAGCTCT$_{17}$V-3′) following the manufacturer's instructions. No PCR products were amplified from mock reverse transcribed samples (data not shown), confirming that there is no genomic DNA contamination.

2.3. Cloning of Japanese flounder ASC (PoASC) cDNA

The internal fragment of *PoASC* cDNA was cloned by RT-PCR with Taq2000 DNA polymerase (Stratagene) and the conserved degenerate primer pair F1/R1 listed in Table 1, using the cDNAs synthesized from Japanese flounder spleen tissue as templates. The obtained sequence was further used to design gene-specific primers to amplify the 5′- and 3′-untranslated region (UTR) of the Japanese flounder *PoASC* cDNA by rapid amplification of cDNA ends (RACE) strategy. Briefly, touch-down PCR was carried out to amplify the 3′-UTR of *PoASC* cDNA end with a gene-specific forward primer F2 designed based on the sequence obtained above and a universal reverse adaptor primer R2 (Table 1). The 5′-RACE reaction was performed by two rounds of nested-PCR amplification with a SMARTer™ RACE kit (Clontech) on a MyCycler™ gradient thermocycler (Bio-Rad) according to the manufacturer's instructions. The first round of PCR reaction was performed with a forward primer UPM (a mixture of primers UPM-L and UPM-S, Table 1) and a reverse gene specific primer GSP, followed by a nested amplification with primer pair NUP/GSP (Table 1). To amplify the entire open reading frame (ORF) of the Japanese flounder *PoASC* cDNA, RT-PCR was performed using the cDNAs generated from spleen tissue as templates and the gene-specific primer F3 and R3 (Table 1) targeted on the *PoASC* 5′-UTR and 3′-UTR sequences obtained from RACE PCR, respectively.

2.4. Bioinformatics analysis

Gene identities were determined by search against the GenBank database using BlastX algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The predicted molecular mass and isoelectric point of PoASC protein were computed using the EXPASY web tool (http://web.expasy.org/compute_pi/). Protein domain architectures were searched by the SMART web tool (http://smart.embl-heidelberg.de/). Phosphorylation prediction was performed by NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). Multiple sequence alignments were carried out using the ClustalW multiple alignment program at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis) software version 4.0 and the neighbor-joining phylogenetic trees were created based on the amino acid sequence alignments of the full-length proteins and tested for reliability using 1000 bootstrap replications.

Table 1
Sequence of primers used in this study.

Primer name	Sequence (5′→3′)
F1	WGAYGYKYTGGTBTCYACYTT
R1	YKRTRTYTRTCCACRAAGTG
F2	GTGGAGCAAATGATGGAAG
R2	CGAATTCGGATCCGAGCTC
GSP	CCTCCACAGCCACAGACACCGCTCCTTT
UMP-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UMP-S	CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
F3	GGAGACAGTCTGTGAGCCAT
R3	CTGATGAGGACAAAGAAGAAACT
F4	GGAAAGGCAGAGGGTGGAT
R4	ACTGGCTTTCAGGGATGTAGAGA
F5	AGGTTCCGTTGTCCCG
R5	TGGTTCCTCCAGATAGCAC
FLAG-f	CCG GAATTCA GCGCCAAAGACTGTCAGGA
FLAG-r	CGG GGTACC CTATTGTCTCTGCAGGTCA