Fish & Shellfish Immunology 60 (2017) 411–419

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Identification and functional analysis of dual-specificity MAP kinase phosphatase 6 gene (*dusp6*) in response to immune challenges in Japanese flounder *Paralichthys olivaceus*

Shuo Li [a, 1], Weijiao Peng [a], Gaixiang Hao [a], Jiafang Li [a], Xuyun Geng [b], 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

ARTICLE INFO

Article history:
Received 8 September 2016
Received in revised form
29 November 2016
Accepted 5 December 2016
Available online 8 December 2016

Keywords:
Mitogen-activated protein kinase phosphatases
Dusp6
Immune challenge
Proinflammatory cytokine
Paralichthys olivaceus

ABSTRACT

Dual-specificity phosphatase 6 (Dusp6) is a member of mitogen-activated protein kinase (MAPK) phosphatases that play crucial roles in regulating MAPK signaling and immune response. The immunological relevance of Dusp6 in fish, however, remains largely uncharacterized. In the present study, a full-length Japanese flounder *dusp6* cDNA ortholog, termed *PoDusp6*, was identified and characterized from *Paralichthys olivaceus*. The deduced PoDusp6 protein is comprised of 383 amino acids with a conserved N-terminal regulatory rhodanese homology domain and a C-terminal catalytic domain. Immunofluorescence microscopy revealed that PoDusp6 protein is mainly localized in cytoplasm. Sequence analysis indicates that PoDusp6 is highly conserved (>70% identity) throughout the evolution from teleost to mammals. In unstimulated conditions, *PoDusp6* mRNA was present in all examined tissues and showed the highest expression in Japanese flounder head kidney macrophages (HKMs). Immune challenge experiments revealed that the expression of *PoDusp6* was down-regulated at the early stage after LPS and poly(I:C) stimulations but significantly up-regulated at the later stage in the HKMs. The similar expression pattern was also observed in the Japanese flounder immune-related tissues including head kidney, gill and spleen upon bacterial challenge with *Edwardsiella tarda*. Overexpression of PoDusp6 in Japanese flounder FG-9307 cells led to a significant down-regulation of proinflammatory cytokine genes *IL-1beta*, *TNF-alpha* and *IFN-gamma*, and antiviral gene *Mx*. Interestingly, inhibition of Dusp6 activity also down-regulated the LPS-induced *IL-beta* gene expression but did not affected on the LPS-induced *IFN-gamma* and *TNF-alpha* expression in the HKMs. Our findings suggest that the expression of *PoDusp6* is modulated by immune stimuli and PoDusp6 may act as an essential modulator in fish inflammatory response.

1. Introduction

Dual-specificity phosphatases (Dusps) are a heterogeneous group of protein phosphatases that can simultaneously dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within one substrate [1]. Mitogen-activated protein kinase (MAPK) phosphatases (MKPs) belong to the class I family of Dusps which can dephosphorylate phosphotyrosine and threonine residues located in the same MAPK [2]. MKPs play important negative regulatory roles in shape the duration, magnitude and spatiotemporal profile of MAP kinase activities and MAPK-dependent signaling in response to both physiological and pathological stimuli in a substrate specificity and phosphatase activity-dependent manner [2–4]. Based on their substrates and subcellular localization properties, the dual-specificity MAP kinase phosphatases can be divided into three subfamilies: the inducible nuclear phosphatases including Dusp1/MKP-1, Dusp2/PAC1, Dusp4/MKP-2 and Dusp5/hVH-3; the extracellular-signal-regulated kinase (ERK)-specific and cytoplasmic Dusp6/MKP-3, Dusp7/MKP-X and Dusp9/MKP-4; and the MKPs (Dusp8/hVH-5, Dusp10/MKP-5 and Dusp16/MKP-7) that preferentially inactivate the stress-activated p38 and JNK MAP kinases [5].

* Corresponding author.
E-mail addresses: shuo76@yahoo.com (S. Li), jssun1965@aliyun.com (J. Sun).
[1] S. Li is a senior author.

http://dx.doi.org/10.1016/j.fsi.2016.12.008
1050-4648/

Table 1
List of PCR primer pairs used in this study.

Primer name	Sequences (5'→3')	Purpose
F1	TVCYBYTGMAGCCRAGAARC	Gene cloning
F2	TCTBTGCKCTCYGACRRAGAGG	
R1	GGTTGGAGGACACTCTGTTGG	
R2	CCAACAGAGTGTCCTCCAACC	
F3	TACTTCACCACTCCGACCAA	3′RACE PCR
F4	GTGAGGTCAGGCGTTGCCATCC	
R3	CGAATTCGGATCCGAGCTC	
NUP	AAGCAGTGGTATCAACGCAGAGT	5′RACE PCR
UMP-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UMP-S	CTAATACGACTCACTATAGGGC	
R4	GCCTTCGTCCTTCATCCTCCTCAG	
R5	CGCACGAACTTCTCCCGCTCCTCTC	
F5	CAACCTCCTGCGTATAAATG	Confirmation PCR
R6	CAACAGGAGGCTCACAAAACT	
F6	CCCAAGCTTATAGAGAAACTCAAGCC	Plasmid construct
R7	CCGGAATTCTCACGTGGACTCCAGGG	
PoDusp6-f	CGAAATCAATCTGGACGGCT	qRT-PCR
PoDusp6-r	GGTAGAGGTGCGGCAGGAT	
IL-1beta-f	CCTGTCGTTCTGGGCATCAA	
IL-1beta-r	CACCCCGCTGTCCTGCTT	
TNF-f	CCGACTGGATGTGTAAGGTG	
TNF-r	GTTGTGGGGTTCTGTTTTCTC	
IFN-f	TGTCAGGTCAGAGGATCCACAT	
IFN-r	GCA GGA GGT TCT GGA TGG TTT	
Mx-f	TAAAATGGCTGGGGTCGGTGTG	
Mx-r	ACCCTGGTGATTCCAGGCAGGT	
beta-actin-f	AGGTTCCGTTGTCCCG	
beta-actin-r	TGGTTCCTCCAGATAGCAC	

Dusps are crucial components of the MAPK system and play important roles in regulating innate immunity in mammals [6]. Mice deficient *dusp6* show enhanced ERK1/2 phosphorylation associated with increased myocyte proliferation and reduced apoptosis rates in the heart affecting disease susceptibility [7]. The abnormal expression of Dusp6 is also associated with diseases such as Hepatitis C virus infection [8]. In addition, previous study has indicated that Dusp6 is a novel transcriptional target of p53 and regulates p53-mediated apoptosis by modulating ERK phosphorylation and the expression levels of Bcl-2 family proteins such as Bad, Bcl-2 and Bcl-$_{xL}$ [9]. Recently studies also showed that Dusp6 regulates CD4(+) T-cell activation and differentiation by inhibiting the TCR-dependent ERK1/2 activation [10].

Using a small-molecule inhibitor of Dusp6, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI), Molina et al. studied the biological role of Dusp6 in zebrafish embryonic development [11]. The immunological relevance of Dusp6 in fish, however, remains largely uncharacterized. In the current study, we identified a *dusp6* cDNA ortholog, termed *PoDusp6*, from Japanese flounder *Paralichthys olivaceus* and examined its expression changes in response to different immune challenges. By inhibition of the endogenous Dusp6 activity together with an overexpression system, we also examined the potential regulatory role of PoDusp6 in Japanese flounder innate immunity. Our findings indicate that PoDusp6 may play an essential role in regulating fish innate immunity.

2. Materials and methods

2.1. Reagents

TRIzol reagent, DNase I (amplification grade), PureLink® RNA Mini Kit, SuperScript III reverse transcriptase, Lipofectamine™ LTX and Plus transfection reagent, OPTI-MEM medium, DMEM-F12 medium, RPMI 1640 medium, MEM medium, fetal bovine serum (FBS), 1% penicillin–streptomycin, trypsine-EDTA, and goat anti-mouse secondary IgG labeled with Alexa Fluor 488 were obtained from Invitrogen. Anti-Flag monoclonal antibody, anti-beta tubulin antibody, LPS, poly(I:C) and (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI, inhibitor of Dusp6) were obtained from Sigma-Aldrich. KOD Taq DNA polymerase was ordered from TOYOBO. ExTaq DNA polymerase was purchased from TaKaRa. PfuUltra II fusion HS DNA polymerase was ordered from Stratagene. Transcriptor First Strand cDNA Synthesis kit and Fast-Start Universal SYBR Green Master (Rox) kit were obtained from Roche. SMARTerTM RACE amplification kit was ordered from Clontech. Fungizone was ordered from Solarbio Science & Technology Co. Ltd. (China). Restriction enzyme was ordered from NEB Biolabs. Peroxidase-conjugated goat anti-mouse secondary antibody was ordered from Pierce. QIAfilter Plasmid Maxi kit was ordered from Qiagen. PCR primers were synthetized from GENEWIZ Inc. (China).

2.2. Experimental animals

Experimental fish Japanese flounder *P. olivaceus* were purchased from a local fish farm in Dagang, Tianjin, China and cultured in an aerated recirculating seawater system in the laboratory at 21 °C for two weeks before experimentations. Only healthy animals without any pathological signs were selected for experiments.

2.3. RNA purification and cDNA preparation

Individual tissues form Japanese flounders were dissected and collected aseptically as described previously [12]. Total RNA from tissues and cells was purified by TRIzol reagent and the PureLink® RNA Mini kit, respectively, as per the manufacturer's instructions. The integrity of RNA was examined by electrophoresis on a 1.2%