Fish and Shellfish Immunology 77 (2018) 392–401

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

Cloning, characterization and functional analysis of *dctn5* in immune response of Chinese tongue sole (*Cynoglossus semilaevis*)

Min Wei[a,c,d,1], Wen-teng Xu[a,b,1], Kun-ming Li[a,b,e,1], Ya-dong Chen[a,b], Lei Wang[a,b], Liang Meng[a,b], Fa-zhen Zhao[a,b], Song-lin Chen[a,b,*]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS), Key Laboratory for Sustainable Development of Marine Fisheries, Ministry of Agriculture, Qingdao, 266071, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[c] Jiangsu Key Laboratory of Marine Biotechnology/College of Marine Science and Fisheries, Huaihai Institute of Technology, Lianyungang, 222005, China
[d] Wuxi Fisheries College, Nanjing Agricultural University, Wuxi, 214081, China
[e] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China

ARTICLE INFO

Keywords:
Dynactin subunit 5
Chinese tongue sole
Expression pattern
Immunity
Antimicrobial activity

ABSTRACT

In mammals, microtubule-dependent trafficking could participate the immune response, where the motor proteins are suggested to play an important role in this process, while the related study in fish was rare. In this study, *dctn5*, a subunit of dyactin complex for docking motor protein, was obtained by previous immune QTL screening. The full-length cDNAs of two *dctn5* transcript variants were cloned and identified (named *dctn5_tv1* and *dctn5_tv2*, respectively). Tissue distribution showed that *dctn5_tv1* was widely distributed and high transcription was observed in immune tissue (skin), while *dctn5*_tv2 was predominantly detected in gonad and very low in other tissues. Time-course expression analysis revealed that *dctn5_tv1* could be up-regulated in gill, intestine, skin, spleen, and kidney after *Vibrio harveyi* challenge. Moreover, recombinant Dctn5_tv1 exhibited high antimicrobial activity against *Escherichia coli* and *Streptococcus agalactiae* due to binding to bacteria cells. Taken together, these data suggest Dctn5_tv1 is involved in immune response of bacterial invasion in Chinese tongue sole.

1. Introduction

Microtubule-based transport is critically important for the protein localization to endomembranes by the cooperation of motor proteins, which consist of dynein, kinesin and myosin. Dynactin is multi-subunit protein complex which is essential for dynein activity [1,2] and can be thought of “dynein receptor” [3]. Impaired dynein-dynactin complex was reported to disrupt the normal immune function in mammals [4,5]. Dynactin is first reported in eukaryotic cells, and aids in bidirectional intracellular transport via binding to dynein and kinesin-2 and linking them to the destinated organelle or vesicle [1,6]. 10 subunits are detected in three major structural domains of this dynactin complex (sidearm-shoulder, the Arp 1 rod, and the pointed end complex), e.g. DCTN1-6, Arp1, actin, CapZ, Actr10 [7–16].

Dynactin 5 (DCTN5), the encoded protein is also called p25, is one of the smallest dynactin subunits [7], and involved in minus-end-directed transport. It contains isoleucine-patch motif and adopts the left-handed parallel β-helix fold (LβH), which may achieve its multiplicity of functions via establishing the complex interactions with other dynactin subunits, microtubules or cargo particles [17]. Moreover, in contrast to other core subunits of dynactin, DCTN5 is detected exist in a freely soluble pool in cells, suggesting that it is possible to participate other molecular function besides structural subunit of dynactin [18]. For example, recent study demonstrates that DCTN5 combined with modification by ubiquitination regulates the transmembrane prostate androgen-induced protein (TMEPAI) intracellular trafficking to the lysosome [19]. TMEPAI has been reported to be widely expressed in various tumor cells [20–22] and down-regulates TGF-β signaling via localizing to the lysosome for promoting lysosomal degradation of TGF-β receptor (TβR) [23]. Thus DCTN5 may display an indirect function in regulation of TGF-β signaling pathway. However, most reports of DCTN5 are concentrated in its function as structural subunit of dynactin

Abbreviations: DTT, DL-Dithiothreitol; GssG, L-Glutathione (Oxidized); GsH, L-Glutathione (Reduced); PMSF, Phenylmethanesulfonyl fluoride

* Corresponding author. Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS), Key Laboratory for Sustainable Development of Marine Fisheries, Ministry of Agriculture, Qingdao, 266071, China.
E-mail address: chensl@ysfri.ac.cn (S.-l. Chen).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2018.04.007
Received 7 February 2018; Received in revised form 21 March 2018; Accepted 2 April 2018
Available online 07 April 2018

in mammals, while few were reported in fishes.

dctn5 in Chinese tongue sole (*Cynoglossus semilaevis*) was previously screened as immune-related gene by quantitative trait locus (QTL) mapping and in the present study, the full-length cDNAs of two *dctn5* transcript variants were cloned and identified. Quantitative RT-PCR revealed that *dctn5_tv1* was widely expressed in the immune-related tissues, while *dctn5_tv2* predominately expressed in gonads. Based on these, the role of *dctn5_tv1* in immune response was then focused. Expression of *dctn5_tv1* in varying time-course after *Vibrio harveyi* challenge was examined. Furthermore, the antimicrobial activity of recombinant Dctn5_tv1 was evaluated. We postulate that Dctn5_tv1 may be involved in immune response of Chinese tongue sole.

2. Materials and methods

2.1. Experimental animals

Healthy Chinese tongue sole of about one year (body weight, 31.0 ± 6.5 g; length, 17.1 ± 1.0 cm) were purchased from Haiyang Huanghai Aquatic Ltd., Shandong Province, China. 12 tissues (brain, heart, gill, skin, stomach, intestine, gonad, liver, spleen, kidney, muscle, and fin) were collected from four untreated individuals, and immediately transferred to liquid nitrogen and stored at −80 °C for RNA extraction. Moreover, 60 individuals were cultured in circulating seawater at 22 °C for one week, and then challenged with *V. harveyi* as previously reported [24], and the final concentration of bacterium for intraperitoneal injection was set as 1.0×10^4 colony-forming units (cfu). The immune-related tissues (liver, spleen, intestine, kidney, gill and skin) were collected at 0, 12, 24, 48, and 72 h post injection for total RNA extraction (three individuals for each group at each time point).

All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals at the Chinese Academy of Fishery Sciences, and approved by the Yellow Sea Fisheries Research Institute's animal care and use committee.

2.2. RNA extraction, cDNA synthesis, and RACE PCR

The total RNA was extracted using RNA isolation kit (Omega, USA) according to the manufacturer's instructions, and stored at −80 °C until usage, and the cDNA was synthesized using the PrimerScript RT reagent Kit (TaKaRa Bio Inc., Otsu, Japan). To obtain the full-length cDNA of two *dctn5* transcripts (*dctn5_tv1* and *dctn5_tv2*), the 5′-RACE and 3′-RACE were carried out using a Smart RACE cDNA amplification kit (Clontech Inc., CA, USA) according to the manufacturer's instructions, and the nested PCR was conducted. The specific primers of 5′-RACE and 3′-RACE were designed based the sequences difference region between *dctn5_tv1* and *dctn5_tv2* downloaded from the data of whole-genome sequencing of Chinese tongue sole (Genbank accession No. XM_008320820). The nested PCR was conducted with the universal primer (UPM) and the universal primer (NUP), and the information of all primers used in this study was displayed in Table 1. To ensure the specificity of amplification, the touchdown PCR was conducted, and its program was performed described as before [25].

Note: The start and stop codons are marked with red, and restriction sites are underlined in the sequences of primers for PCR amplification of Dctn5_tv1 ORF region.

2.3. Phylogenetic analysis

The phylogenetic tree was constructed with MEGA v6.0 software using neighbor-joining (NJ) method [26]. All the sequence used in this phylogenetic analysis were downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov/) and aligned with ClustalX v2.0 using the MUSCLE method. The information of sequences was listed in Table 2. The value of bootstrap replicates was set to 1000, and the phylogenetic tree was edited with FigTree v1.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Real-time quantitative PCR

Quantitative real-time PCR (qRT-PCR) was performed in a 20 μl reaction volume described as before [27]. To distinguish the two transcripts of *dctn5* in tongue sole, the primer of qRT-PCR was also designed based on the sequences difference region between *dctn5_tv1* and *dctn5_tv2*. The amplification procedure of the qRT-PCR was described as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and then 60 °C for 34 s *β-actin* was used as suitable endogenous control in Chinese tongue sole [28]. All the samples were amplified in triplicate, and the gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [29]. The differences of expression level were analyzed using one-way ANOVA with the SPSS v18.0 software (IBM, New York, USA).

2.5. Recombinant expression of Dctn5_tv1 and its purification and refolding

The cDNA fragment encoding the full-length CDS of Dctn5_tv1 was amplified using specific primers Dctn5-Exp-F/R (Table 1) and inserted into pETM11 expression vector with His-tag, and then transformed into DH5α (Transgen, Beijing, China). After the positive clones were confirmed using sequencing, the recombinant expression plasmid was extracted and transformed *E. coli* Transetta (DE3) (Transgen, Beijing, China). The positive clones were incubated in LB (Luria Bertani) medium with 50 μg/ml antibiotic at 37 °C with shaking at 200 rpm until the culture reached an optical density at OD_{600} of 0.6. The isopropyl-b-D-thiogalactopyranoside (IPTG) was then added into culture to a final concentration of 0.5 mmol/l and incubated at 20 °C and 200 rpm for another 6 h. Bacteria were collected by centrifugation at 4000 rpm at 4 °C for 30 min, re-suspended in lysis buffer (25 mmol/l Tris, 5 mmol/l EDTA, 50 mmol/l NaCl, 0.3 mg/ml lysozyme, 1 mmol/l PMSF; pH7.5) and placed on ice for 2 h, and then disrupted by sonication. After the sonication, the supernatant and sediment were separately collected by centrifugation. The sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) was used to detect the existence of recombinant protein described before [24]. The sediment was dissolved by Pre-cooled solution (100 mmol/l Tris, 8 mol/l Urea, 10 mmol/l imidazole, 500 mmol/l NaCl; pH 7.4), and purified using Ni-NTA Purification System (Invitrogen, Shanghai, China). The denatured Dctn5_tv1 was refolded by the following six steps of dialysis (each dialysis step for 12 h): Dialysate I(25 mmol/l Tris-HCl, 200 mmol/l NaCl, 1 mmol/l EDTA, 5 mmol/l DTT, 6 mol/l Urea; PH 7.4); Dialysate II (25 mmol/l Tris-HCl, 50 mmol/l NaCl, 1 mmol/l EDTA, 4 mol/l Urea, 1 mmol/l GssG, 1 mmol/l GsH; PH 7.4); Dialysate III (25 mmol/l Tris-HCl, 20 mmol/l NaCl, 1 mmol/l EDTA, 2 mol/l urea, 1 mmol/l GssG, 1 mmol/l GsH; PH7.4); Dialysate IV (25 mmol/l Tris-HCl, 10 mmol/l NaCl, 1 mmol/l EDTA, 1 mol/l urea, 1 mmol/l DTT, 0.01% L-Cysteine, 1 mmol/l GssG, 1 mmol/l GsH; PH 7.4); Dialysate V (25 mmol/l Tris-HCl, 10 mmol/l NaCl, 1 mmol/l EDTA, 0.5 mol/l urea, 1 mmol/l DTT, 0.01% L-Cysteine, 1 mmol/l GssG, 1 mmol/l GsH; PH7.4); Dialysate VI (25 mmol/l Tris-HCl, 10 mmol/l NaCl; PH7.4). After dialysis, the supernatant was collected by centrifugation and stored in −80 °C, and the quantification of the purified protein was conducted with the Bradford Protein Assay Kit (Beyotime Biothechnology). All the reagents used in this study were purchased from Solarbio Life Sciences (Beijing, China).

2.6. Western blot analysis

To evaluate the recombinant expression of Dctn5_tv1, the western blot analysis was conducted. Purified protein samples were separated by SDS-PAGE in a 12% polyacrylamide gel and then transferred to the nitrocellulose filter membrane (300 mA for 40 min). The membrane was blocked with 5% (w/v) nonfat milk for 2 h at room temperature and washed three times with TBST (Tris-buffered saline (TBS)