Fish & Shellfish Immunology 35 (2013) 249–261

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

A tailless Dscam from *Eriocheir sinensis* diversified by alternative splicing

Jingjing Wang [a,b], Lingling Wang [a], Yang Gao [a,b], Qiufen Jiang [a,b], Qilin Yi [a,b], Huan Zhang [a], Zhi Zhou [a], Limei Qiu [a], Linsheng Song [a,*]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 13 January 2013
Received in revised form
18 March 2013
Accepted 17 April 2013
Available online 10 May 2013

Keywords:
Dscam
Eriocheir sinensis
Alternative splicing
Immune defense

ABSTRACT

Dscam (Down syndrome cell adhesion molecule), a member of the immunoglobulin superfamily (IgSF), plays an essential role in pathogen recognition and further involves in the innate defense of invertebrates. In the present study, the cDNA of a Dscam from Chinese mitten crab *Eriocheir sinensis* (designated EsDscam) was cloned and characterized. It contained a 5-terminal untranslated region (UTR) of 60 bp, a 3-UTR of 216 bp with a poly (A) tail, and an open reading frame (ORF) of 4848 bp encoding a polypeptide of 1615 amino acids with the putative molecular mass of 178.4 kDa and theoretical isoelectric point of 6.31. The EsDscam protein shared higher sequence identities and similar domain architecture with Dscams from other invertebrate, including typical 10 immunoglobulin (Ig) domains, 6 fibronectin type 3 domains (FNIII) and one cell attachment sequence (RGD) in extracellular region, while it lacked the expected transmembrane domain and cytoplasmic tail compared with other members of Dscam family. After sequencing 80 separate clones of Ig2, 3 and Ig7 regions from pooled cDNA libraries constructed from normal and bacterial-infected crabs, 44 alternative sequences were detected in the N-terminal of Ig2, 39 ones in Ig3, and 31 ones in Ig7 domain, suggesting that EsDscam could potentially encode at least 53196 unique isoforms. Furthermore, two 3′UTR isoforms and two 5′UTR isoforms of EsDscam were also identified by RACE strategy. EsDscam mRNA was most abundantly expressed in the tissues of nerve, muscle, hepatopancreas and gill, and weakly expressed in heart, gonad and hemocytes. Western blotting and immunofluorescence analysis revealed that EsDscam protein was mainly distributed in serum, and few on the membrane of crab hemocytes. These results suggested that this tailless EsDscam was one member of crustacean Dscam family, and the generation of diverse isoforms through alternative splicing allowed it to recognize various pathogens and play an active role in immune defense of crabs.

1. Introduction

The immunoglobulin superfamily (IgSF) is a large group of proteins containing at least one immunoglobulin (Ig) domain [1], which can bind diverse ligands and contribute to a variety of cellular activities [2]. As one IgSF member, Down syndrome cell adhesion molecule (Dscam) have attracted much attention, since they can produce a broad repertoire of molecules containing variable Ig domain combinations with specificity in recognition and binding via alternative splicing [3,4]. They are proposed to be important in pathogen recognition and play extensive roles in innate immune defenses in invertebrate.

Dscam was first identified from human, and it was supposed to be associated with neuronal wiring [5]. Subsequently, many orthologues of Dscam were identified in various invertebrate species with novel functions in innate immunity [3,6]. The typical Dscam contains an extracellular domain, a transmembrane domain (TM) and a cytoplasmic tail, among which extracellular domain can produce diverse isoforms through mutually exclusive (ME) splicing [7]. For instance, in Drosophila and mosquito, Dscam could produce more than 30,000 diverse sets by alternative splicing of three Ig-like domains [8]. The diversity of Dscam is important for its role in pattern recognition [3,9], which probably allows Dscam to recognize pathogens specially in an analogous way as vertebrate antibody to recognize and bind different antigens [10,11]. Different Dscam isoforms were generated in mosquito to response against different pathogen challenge, with varying pathogen interaction specificities [12]. Although it has been accepted that the Dscam in arthropods is involved in pathogen recognition through alternative splicing of Ig domain, the functions and regulation mechanism of Dscam in crustacea, especially crabs, are still not well unknown.

* Corresponding author. Tel.: +86 532 82898552.
E-mail address: lshsong@ms.qdio.ac.cn (L. Song).

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.04.029

The extracellular region of Dscam is responsible for the specific binding to homophilic isoforms or pathogens, while the cytoplasmic tail region usually mediates subsequent signal transduction through related motifs on the tail [13]. Due to exon inclusion or exclusion, several isoforms could be produced in cytoplasmic tail region of Dscam, and the isoform without cytoplasmic tail occasionally existed. For example, in crustacean, *Daphnia magna* Dscam produced four distinct isoforms via exon inclusion and exclusion in the cytoplasmic tail [14]. While Dscam from shrimp *Litopenaeus vannamei* (LvDscam) was lack of both transmembrane domain (TM) and cytoplasmic tail [15], which was suggested not to be shed from the membrane-bound but to be directly expressed as a secreted protein [16]. Although Dscams was implied to exist in different forms with or without cytoplasmic tail, the knowledge of their existing forms in cells and the possible pathways to trigger the downstream reactions in crustacean was quite meagre.

Chinese mitten crab *Eriocheir sinensis* (Arthropoda, Crustacea, Varunidae) is economically important for aquaculture in China. Study on immune-related genes, such as Dscam from Chinese mitten crab (designated EsDscam), may give reference to the pathogen recognition in crustacea and diseases management strategies in crab farming. The main objectives of the present study are (1) to clone the full-length cDNA of Dscam from *E. sinensis*, (2) to analyze the phylogenesis and the evolutional status of EsDscam, (3) to investigate tissue-specific expression of EsDscam transcripts and the location of EsDscam protein in hemolymphs, and (4) to detect the variants of EsDscam in different sources of hemocytes, hopefully providing more information to understand roles of Dscam in the pathogen recognition and extensive involvement in immune defense of crustacean.

2. Materials and methods

2.1. Crabs and tissue collection

Crabs (50 ± 5 g) were collected from a local farm in Qingdao, Shandong province, China, and maintained in fresh water for one week before processing. Seven healthy adult crabs were employed to investigate the tissue distribution of EsDscam mRNA as parallel samples, and seven tissues including hepatopancreas, gill, gonad, muscle, heart, nerve and hemocytes were collected.

In order to obtain as many as isoforms containing hypervariable regions of the extracellular region and UTRs of EsDscam, three microorganisms, *Aeromonas hydrophilia*, *Listonella anguillarum* and *Pichia pastoris GS115* (10^7 CFU mL^{-1}) were employed as immune elicitors to stimulate ten crabs, respectively. Among which the first two gram negative bacteria were reported to be pathogenic to crabs [17]. And the fungi *P. pastoris GS115* was selected in order to induce different EsDscam isoforms from that of bacteria. Hemocytes of six stimulated crabs from each group were collected at 12 h post treatment.

2.2. RNA isolation

Hemolymph was collected from the cheliped using a syringe with an equal volume of anticoagulant (510 mM NaCl, 100 mM glucose, 200 mM citric acid, 30 mM sodium citrate, 10 mM EDTA·2Na, pH 7.3). Samples were immediately centrifuged at 800 g, 4 °C for 10 min to harvest the hemocytes, and then stored at −80 °C after addition of 1 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction. Total RNA from hemocytes was isolated according to the manufacturer's protocol. The concentrations and quality of RNAs were measured by spectrophotometry (Genequant, Amersham Biosciences, USA), and their integrity was checked by electrophoresis in 1% agarose gel.

2.3. Cloning of full-length EsDscam cDNA

cDNAs were synthesized by using reverse transcription kit (Promega) with AP-dT primer. The reaction was carried out at 42 °C for 60 min and inactivated at 95 °C for 5 min. The full-length cDNA sequences of EsDscam gene was generated by using reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) strategies. The PCR reactions were performed to obtain core cDNA fragments of EsDscam by using the cDNAs templates from blood and heart, and two primer sets based on the conserved sequences of *L. vannamei* Dscam (GenBank accession no.: GQ154653) and *Pacifastacus leniusculus* Dscam (GenBank accession no.: HQ596367). Due to the large size of EsDscam mRNA, 5 rounds of 5′ RACE were employed to amplify overlapping fragments of EsDscam according to the Usage Information of 5′RACE system (invitrogen). The PCR amplification was carried out using sense primer Oligo(dG)-adaptor (AP-dG) and antisense primer 5R1, 5R2, 5R3, 5R4, or 5R5 to obtain the 5′ end of EsDscam. Sense primer 3R1 and antisense primer Oligo(dT)-adaptor (AP-dT) were used to get the 3′ end of EsDscam. The sequences and their relative positions of the primer sets are shown in Table 1 and Fig. 1. The PCR products were cloned into pMD 18-T vector (TAKARA) and transformed into competent cells of *Escherichia coli* top 10. At least 3 positive colonies of each PCR product were sequenced to verify. To assemble the full-length cDNA of EsDscam by overlapping the cDNA fragments, sequence alignments were performed using Seqman (DNAstar) software. To confirm the assembled EsDscam sequence, two PCR reactions were performed using the primer sets F109/5R-3 and RCF/RCR to obtain the extracellular region of EsDscam (Fig. 1 and Table 1).

2.4. Sequence analysis

The homology searches of the cDNA and protein sequence of EsDscam were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org), and Signal P 3.0 program was utilized to predict the presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP). The protein domains were predicted with the simple modular architecture research tool (SMART) version 5.1 (http://smart.embl-heidelberg.de/). Multiple sequences alignment of the EsDscam and some other Dscams was performed with the Clustal W multiple alignment program (http://www.ebi.ac.uk/Tools/clustalw2/) and multiple sequences alignment show program (http://www.biosoft.net/sms/index.html). An unrooted phylogenic tree was constructed based on the deduced amino acid sequence of EsDscam and some other Dscams by the neighbor-joining (NJ) algorithm using the MEGA 4.1 beta software (http://www.megasoftware.net). To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.5. Identification of diversity in the extracellular hypervariable regions and UTRs of EsDscam

To obtain as many as possible isoforms of EsDscam, two cDNA libraries constructed from seven tissues (hepatopancreas, gill, gonad, muscle, heart, nerve and hemocytes) collected from the normal crabs and hemocytes collected from eighteen bacterial-infected crabs (six each in *A. hydrophilia*, *L. anguillarum* and *P. pastoris GS115* group) were pooled together as PCR templates [18].