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Identification and characterization of *pro-interleukin-16* from mud crab *Scylla paramamosain*: The first evidence of proinflammatory cytokine in crab species

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A B S T R A C T

IL-16 is a pro-inflammatory cytokine originally designated as a lymphocyte chemoattractant factor. In mammal and avian, it has been characterized as an essential regulator of various cellular processes including cell recruitment and activation against pathogen invasion. So far, neither of the full-length of IL-16 homologue nor the response mechanism against pathogen was reported in crab species. In the present study, the *pro-IL-16* homologue was firstly cloned and characterized from mud crab *Scylla paramamosain*. The full-length *Sp-pro-IL-16* consisted of 4107 bp with an opening reading frame encoding 1369 amino acids. Multiple alignment analysis showed the putative amino acid sequence of *Sp-pro-IL-16* had about 73.86% identity with *Litopenaeus vannamei* pro-IL-16. Additionally, two conserved PDZ domains and protein binding sites were found in *Sp*-pro-IL-16 and showed high similarities about 94.19% and 51.14% with their *Litopenaeus vannamei* and *Mus musculus* counterparts. RT-PCR analysis indicated that *Sp-pro-IL-16* transcripts were constitutively expressed in all tissues examined with an extreme high level in hepatopancreas. Moreover, *Sp-pro-IL-16* transcripts in hepatopancreas were significantly up-regulated 15-fold at 72 h after *Vibrio alginolyticus* challenge and 3.5-fold at 12 h after virus-analog Poly (I:C) challenge. The Western blot analysis revealed that *Sp*-pro-IL-16 can be cleaved to its bioactive form, an approximately 35 kDa mature IL-16, and the protein levels of both pro-IL-16 and mature IL-16 increased after *Vibrio alginolyticus* challenge. It is the first experimental identification of pro-inflammatory cytokine *IL-16* in arthropods. This study could shed new light on further understanding of the response mechanism of pro-inflammatory cytokine IL-16 in *Scylla paramamosain* against pathogens. Meanwhile, it brought new insight into the origin and evolution of IL-16 in crab species.

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1. Introduction

IL-16 is a pro-inflammatory cytokine originally designated as a lymphocyte chemoattractant factor [1,2]. Since its discovery in 1982 in human, it has been characterized as an essential regulator of various cellular processes including cell recruitment and activation [3]. Generally, IL-16 is generated as a precursor molecule (pro-IL-16), which appears to be predominantly produced by $CD8^+$ and $CD4^+$ T lymphocytes [3]. However, other cells including eosinophils, dendritic cells, mast cells, macrophages, B cells, and monocytes have been shown to also produce high levels of IL-16 under certain conditions [4–10]. The pro-IL-16 is cleaved by caspase-3 following cell activation into an N-terminal prodomain and a C-terminal peptide [11]. The former can function as a transcriptional repressor with regulatory effects on cell cycle progression, whereas the latter is secreted from the cell to form mature IL-16 to exhibit lymphocyte chemoattractant activity [12]. IL-16 contains PDZ domain in the C-terminal region, which is a common structural domain of 80–90 amino-acids found in the signaling proteins [13]. PDZ is an initialism combining the first letters of the first three proteins discovered to share the domain including post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) [14]. PDZ domain plays a key role in the formation and function of signal

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transduction complexes such as chemotactic activity [15]. In addition to its chemotactic activity, IL-16 had typically been associated with recruitment of $CD4^+$ or $CD9^+$ immune cells to sites of inflammation [16,17]. The *IL-16* homologues had been well characterized in vertebrates [18–23], but to our knowledge, few reports have clarified the existence and function in arthropods. Partial sequence of *IL-16* with a 115 amino acid polypeptide was reported and determined may be implicated in microglia recruitment in *Hirudo medicinalis* [24]. Meanwhile, an *IL-16-like* gene was characterized and revealed to response to bacteria and virus infection in shrimp *Litopenaeus vannamei* [25]. However, little is known about the existence and immune function of pro-IL-16 and mature IL-16 in crab species.

The mud crab *Scylla paramamosain* is one of the most precious marine species for aquaculture in China. Unfortunately, the mud crab is prone to be infected by microorganisms such as *Vibrio parahaemolyticus*, which will bring a huge commercial loss [26]. Therefore, researches have become increasing paying close attention to address the immune-related factors in the mud crab. In the present study, the full-length cDNA of *pro-IL-16* homologue was firstly cloned and characterized in *Scylla paramamosain*. The expression profiles of *Sp-pro-IL-16* in different tissues and at various time points after Poly (I:C) and *Vibrio alginolyticus* challenge were also analyzed. To the best of our knowledge, this is the first report of *pro-IL-16* in crabs. This is helpful to further understand its important role in the immunity of invertebrates. It also brought new insight into the origin and evolution of *pro-IL-16* in crabs.

2. Materials and methods

2.1. Experimental animal and pathogen

Healthy *S. paramamosain*, weighing 50.3 ± 8.4 g, were obtained from local vendors in Sanmen Bay of Zhejiang province, China. Before the beginning of experiments, crabs were acclimated for one week in rounded plastic container with running fresh seawater with a salinity of 20‰ and water temperature at 22 ± 0.4 °C. One crab per container was maintained during the whole time. During the acclimatization, crabs were fed with fresh manila clam *Ruditapes philippinarum* one-time daily.

Poly (I:C) (#P0913, Sigma) was dissolved in PBS at a concentration of 1 mg/mL, aliquoted and stored at −70 °C. *Vibrio alginolyticus*, isolated on thiosulfate-citrate-bile-sucrose (TCBS) agar plates, was cultured overnight in Luria-Bertani (LB) liquid medium at 28 °C, and then collected by centrifugation at 3000*g* for 5 min, washed in PBS and adjusted to 10^7 CFU/mL.

2.2. RNA extraction and cDNA synthesis

The tissues (hepatopancreas, heart, gill, haemocytes, basilemma, intestine, ganglion and muscle) were collected, rinsed with 0.1% diethylpyrocarbonate (DEPC)-treated water and then put into liquid nitrogen immediately. Each tissue was collected from at least five crabs. Total RNA was extracted from different tissues, using Trizol reagent (Invitrogen). First-stranded cDNA, which served as the template for Rapid Amplification of cDNA Ends PCR (RACE-PCR), was synthesized using a SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. The template cDNA used for quantitative real-time PCR (qRT-PCR) were synthesized using PrimeScript RT reagent Kit (Takara) according to the manufacturer's protocol. The mRNAs were incubated with gDNA Eraser (Takara) at 42 °C for 5 min to eliminate genomic DNA.

2.3. Cloning of Sp-pro-IL-16

The primers of IL-16-F and IL-16-R (Table 1) were designed to amplify partial cDNA sequence based on the alignment of conserved domains of *pro-IL-16s* in different species. PCRs were performed as follows: 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 90 s, followed by a 10 min extension at 72 °C. The full-length cDNA sequence of the *Sp-pro-IL-16* was obtained by using the SMART™ RACE cDNA amplification kit (Takara). Six gene-specific primers, as listed in Table 1, were designed for 5′- and 3′ -RACE based on the partial cDNA sequence. Reaction conditions were adjusted according to the manufacturer's recommendations. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized by UV-transillumination. Then the products were cloned into the pMD-19T vector (Takara) followed by transformation of competent *E. coli* TOP10 cells (Invitrogen) and sequenced using the Applied Biosystems ABI Prism 3730 DNA Analyzer.

2.4. Sequence analysis

The nucleotide and deduced amino acid sequence of *pro-IL-16* cDNA were analyzed and compared using the BLAST search programs (http://blast.Ncbi.nlm.nih.gov/Blast/). EdiSeq program in DNAstar software was used to find the open reading frame (ORF) and translate the nucleotide sequence into amino acid sequence. The potential active site was predicted using ScanProsite (http://prosite.expasy.org/scanpro site/), multiple sequence alignment was generated using the CLUSTALW program (http://www.genome.jp/tools/clustalw/), and phylogenetic tree was constructed with MEGA5.0.

2.5. Tissue expression pattern analysis of Sp-pro-IL-16

The relative expression levels of *Sp-pro-IL-16* in different tissues were analyzed by qRT-PCR. Total RNAs were isolated from various tissues, including hepatopancreas, heart, gill, haemocytes, basilemma, intestine, ganglion and muscle. All PCR reactions were performed in a total volume of 10 μL by using a SYBR Premix Ex Taq kit (Takara). The amplification conditions of qRT-PCR were as follows: denatured at 95 °C for 30 s, then 95 °C for 5 s, 60 °C for 20 s followed by 45 cycles, and a final single cycle of 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method with *β-actin* for normalization. Each PCR trial was performed in triplicate and repeated independently at least thrice. The gene-specific primers sequences IL-16-qF and IL-16-qR were listed in Table 1.

Table 1
Primers for cloning and expression analysis of *Sp-pro-IL-16*.

Primers	Sequences (5′→3′)	Purpose
IL-16-F	TTGTCACAGCCGTCTCCTAC	Cloning
IL-16-R	CGCCCCAATCAAACCCTCAT	Cloning
IL-16-3′-1	AAGAACGCAAGTCAAGGGAACAAACT	3′RACE
IL-16-3′-2	GCTCACAGTCTCTGATTGACATAGGT	3′RACE
IL-16-3′-3	CGGACTATGAAACAAAGGAGATTACG	3′RACE
IL-16-5′-1	TGTGGAGGAGGCGAGAGAGGTGATAGAG	5′RACE
IL-16-5′-2	ACCGTAATCTCCTTTGTTTCATAGTCCG	5′RACE
IL-16-5′-3	TGGATAAAGTTCCCATCGGATTCTGTCA	5′RACE
IL-16-qF	TGGCAGAGGTTACAGGTCACGGTTAT	qRT-PCR
IL-16-qR	GGAGTCTGGTGTTCGTCACTGTTTCT	qRT-PCR
β-actin-F	GCCCTTCCTCACGCTATCCT	qRT-PCR
β-actin-R	GCGGCAGTGGTCATCTCCT	qRT-PCR

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