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A *Cg*IFNLP receptor from *Crassostrea gigas* and its activation of the related genes in human JAK/STAT signaling pathway

Ran Zhang [a, d], Rui Liu [a], Lusheng Xin [a, c], Hao Chen [a, c], Chenghua Li [d], Lingling Wang [b], Linsheng Song [b, *]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture, Dalian Ocean University, Dalian 116023, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] School of Marine Sciences, Ningbo University, Ningbo, Zhejiang Province 315211, China

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ABSTRACT

Interferon is a highly pleiotropic cytokine, once binding to its receptors, can activate JAK kinases and STAT transcription factors to initiate the transcription of genes downstream from interferon-stimulated response elements. In the present study, a cytokine receptor-like 3 molecule was selected and cloned from oyster *Crassostrea gigas*, which contained a fibronectin type III domain (designed *Cg*IFNR-3). The expression pattern of *Cg*IFNR-3 mRNA was detected in all the tested tissues including mantle, gills, hepatopancreas, muscle, and hemocytes, with the highest expression level in hemocytes. After poly (I: C) stimulation, the expression level of *Cg*IFNR-3 in hemocytes was observed to significantly increase at 3 h (13.06-fold, $p < 0.01$). *Cg*IFNR-3 was indicated to interact with *Cg*IFNLP by *in vitro* GST pull-down assay, and to activate the expression of transcription factors including ISRE, STAT3 and GAS, in human Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway after co-transfection in HEK-293T cells in the reporter luciferase activity assay. These results suggested that *Cg*IFNR-3 could bind to *Cg*IFNLP as an interferon receptor and participate in the activation of JAK/STAT pathway in human, which will benefit for intensive studies of interferon signaling pathway in mollusc.

1. Introduction

In mammals, interferons (IFNs) are potent cellular mediators essential for pleiotropic effects, which are triggered by many viruses and could induce the transcription of interferon stimulated genes (ISGs) (Kalvakolanu, 2003; Platanias, 2005). After pathogen infection, IFN signaling cascade is initiated by the Janus kinase (JAK), signal transducer and activator in JAK/signal transducer and activator of transcription (STAT) pathway through the binding effect of cell surface receptors (Stark and Darnell, 2012; Schneider et al., 2014), which plays an important role in regulating the adaptive immune response (Schoenborn and Wilson, 2007; Gonzalez-Navajas et al., 2012).

As for the IFN signaling pathway, many cell receptors for IFNs and ISGs have been identified and characterized (Hemmi et al., 1989, 1994; Noisakran and Carr, 2001), and these receptors can be divided into various sorts to specifically recognize different types of IFNs. For instance, the specific cell surface receptors of type I IFNs (IFNAR1 and IFNAR2 subunits) (Pestka et al., 1987; Uze et al., 1990) induce tyrosine phosphorylation in transcription of the IFNAR-associated JAK kinases (such as tyrosine kinase 2 associated with IFNAR1 and JAK1 associated with IFNAR2) (Platanias, 2005), which could activate several members in STAT family (Matikainen et al., 1999; Su, 2000; Nguyen et al., 2002). IFN-stimulated gene factor 3 (ISGF3, a trimetric transcription factor complex) is an interferon-dependent positive-acting transcriptional factor, which migrates to the nucleus and binds to the *cis* element ISRE (IFN-stimulated response element) in the promoters of ISGs to initiate their transcription after the activation of IFN signaling pathway (Schindler et al., 1992). For type II IFNs, IFNGR1 and IFNGR2 can bind to the IFN-γ by a mediation of the STAT1-containing transcription factor GAF (gamma-activated factor), which activates the tyrosine kinases JAK1 and JAK2 (Gao et al., 1999) and then binds to the promoter of GAS (gamma interferon activation site) to induce the activation of GAS elements (Platanias and Fish, 1999; Aaronson and Horvath,

* Corresponding author.
E-mail address: lshsong@qdio.ac.cn (L. Song).

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2002). The type III IFN (IFN-λ), including IFN-λ1 (IL-29), IFN-λ2, and IFN-λ3 (IL-28A/B) (Kotenko et al., 2003; Sheppard et al., 2003), is a kind of novel cytokine. Its receptor can induce the phosphorylation of STAT1, STAT2 and the generation of the ISGF3 transcription factor after binding to the IFN-λ (Dumoutier et al., 2004; Zhou et al., 2007), and further activate the typical IFN-induced genes like oligoadenylate synthetase (OAS) and myxovirus resistance (Mx) (Kotenko et al., 2003; Sheppard et al., 2003).

Some important elements of the IFN-signaling system, such as IFN I system against the VHSV infection in juvenile *Solea senegalensis* (Alvarez-Torres et al., 2016), JAK1, TYK2 and STAT1 had also been identified in pufferfish *Tetraodon nigrovidis* (Leu et al., 1998, 2000), zebrafish *Danio rerio* (Oates et al., 1999) and crucian carp *Carassius carassius* (L.) (Zhang and Gui, 2004), respectively, suggesting that fish IFN-signaling system could mediate the JAK/STAT pathway. In addition, zebrafish STAT1 was able to rescue type I IFN-signaling functions in a STAT1-deficient human cell line, indicating that cytokine-signaling molecules were likely to be conserved between fish and tetrapods (Oates et al., 1999).

In invertebrates, the main elements of IFN signaling pathway had been discovered, and the vertebrate IFNs were also reported to influence some invertebrate cellular immune functions (Ottaviani et al., 2004; Wang et al., 2015), suggesting the conservation of IFN receptors between vertebrates and invertebrates. For example, a homologous protein of human IFN-γ receptor was identified in starfish *Asterias rubens* (Legac et al., 1996). The specific binding sites for human IFN-γ were found on the surface of hemocyte and protocerebrum of the tobacco hornworm larvae *Manduca sexta*, indicating the existence of a conserved IFN-γ receptor in insect (Parker and Ourth, 1999). In *Pinctada fucata* martensii, the recombinant mammalian IFN-ω could specially bind on the surface of hemocytes, suggesting the presence of a presumptive and functionally conserved IFN-ω receptor in oyster (Miyazaki et al., 2002). It has been reported that the JAK/STAT pathway is evolutionarily conserved between vertebrate and invertebrate with functions of cellular signal transduction (Arbouzova and Zeidler, 2006; Chen et al., 2014). For instance, few critical STATs were also identified in some invertebrates, including *Drosophila* (Hou et al., 1996; Yan et al., 1996) and *Anopheles gambiae* (Barillas-Mury et al., 1999). In *Mytilus galloprovincialis Lam*., the bacteria-induced STAT1 phosphorylation in hemocytes increased markedly after treated with human IFN-γ, providing the first indication for the existence of STAT-like proteins in mollusc (Canesi et al., 2003). In *Caenorhabditis elegans*, a novel STAT protein (STAT-1) was identified with significantly structural and functional homology with other vertebrate STATs (Wang and Levy, 2006). Remarkably, an IFN regulatory factor (IRF)-like gene and IRF-Vago-JAK/STAT regulatory axis were firstly identified in shrimp *Litopenaeus vannamei*, indicating that shrimps might also possess an IFN system-like antiviral mechanism (Li et al., 2015).

Recently, a novel IFN-like protein (*Cg*IFNLP) identified from oyster *Crassostrea gigas* (Zhang et al., 2015) shared similar structure and functional characteristics with vertebrate IFNs, but its receptor and the mechanism of signaling pathway are still poorly characterized. In the present study, the genome sequence of oyster *C. gigas* was surveyed with the objectives to (1) identify the novel molecule containing cytokine receptor domain (designed *Cg*IFNR-3) and confirm the existence of IFNR in mollusc, (2) examine its mRNA expression in different tissues and the temporal expression pattern after poly (I: C) stimulation, (3) investigate the interaction with its ligand *Cg*IFNLP, and (4) *in vitro* detect the expression alternations of ISGs in human embryonic kidney (HEK-293T) cells activating by *Cg*IFNLP. The results would provide an essential perspective into the immune regulation of IFN in invertebrates and further lay the foundation for recognizing the evolution of IFN signaling pathway.

2. Materials and methods

2.1. Oysters

Adult pacific oyster *C. gigas* (about two years old) were purchased from a farm in Qingdao, Shandong Province, China. Before the experiment, oysters were temporary reared in tanks with continuously aerated seawater at 18 °C for one week.

2.2. Immune stimulation and samples collection

Before the experiment, ninety oysters were randomly distributed into three groups (each group including 30 individuals) and kept in aerated tanks. Oysters injected with 100 μL poly (I: C) (1 mg mL^{-1} in sea water, Sigma, USA) or seawater were employed as the treatment group and the control group, respectively, while the untreated oysters were regarded as the blank group. After the injection, oysters were returned to aerated tanks and sampled at 3, 6, 9, 12, and 24 h from each group. Hemocytes of these oysters were harvested from the hemolymph by centrifugation at 800 × *g*, 4 °C for 10 min (Zhang et al., 2014). And other tissues, including mantle, gills, hepatopancreas, and muscle, were obtained randomly from six oysters as parallel samples. All samples were stored at −80 °C by using Trizol reagent (Invitrogen, USA) for RNA extraction.

2.3. RNA extraction, cDNA synthesis and gene cloning

Total RNA was extracted from samples by using a modified method with Trizol reagent (Invitrogen, USA). Based on Promega M-MLV RT Usage information, total RNA was used to synthesize the first-strand cDNA with oligo (dT)-adaptor as primer P1 (Table 1) after treated by the DNase I (Promega, USA). The synthesis reaction was performed at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min. Then cDNA mix was diluted 50 times and stored at −80 °C for subsequent gene cloning and quantitative real-time PCR (qRT-PCR).

Sequence information of *Cg*IFNR-3 (Cytokine receptor-like factor 3, CGI_10027657) was obtained from the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). And the full cDNA sequence of *Cg*IFNR-3 gene was amplified by a pair of specific primers, P2 and P3 (Table 1). After gel-purified, the PCR product was cloned into the pMD19-T simple vector (TaKaRa, Japan) and sequenced by primers P4 and P5 (Table 1), then the resulting sequences were subjected to cluster analysis.

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
Clone primers	
P1 (oligo (dT)-adaptor)	GGCCACGCGTCGACTAGTACT_{17}
P2 (forward)	ATGGCTCAGCAGTTGATACAGA
P3 (reverse)	TTATTGCACTCCAACCTTCCA
Sequence primers	
P4 (M13-47)	CGCCAGGGTTTTCCCAGTCACGAC
P5 (RV-M)	GAGCGGATAACAATTTCACACAGG
RT primers	
P6 (RT-F)	CTTCCAACTTGGGCAGAGGTAGAGGC
P7 (RT-R)	CCAAATGCTCCACTTCTCCT
P8 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG
P9 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT
Recombination primers	
P10 (forward)	CGCGGATCCATGGCTCAGCAGTTGATACAGA
P11 (reverse)	AAGGAAAAAAGCGGCCGCTTATTGCACTCCAAC
***Cg*IFNLP recombination primers**	
P12 (forward)	CGGGATCCATGGAGAGGAAAAAGGAT
P13 (reverse)	CCCAAGCTTCTAAGTCATTAATTTTCTTTC