Fish & Shellfish Immunology 47 (2015) 485–499

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

A novel innexin2 forming membrane hemichannel exhibits immune responses and cell apoptosis in *Scylla paramamosain*

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ARTICLE INFO

Article history:
Received 22 June 2015
Received in revised form
6 September 2015
Accepted 14 September 2015
Available online 15 September 2015

Keywords:
Scylla paramamosain
Innexin
Hemichannel
Immune response
Apoptosis

ABSTRACT

Innexins are a class of transmembrane proteins that are important for embryonic development, morphogenesis and electrical synapse formation. In the present study, a novel innexin2 gene from *Scylla paramamosain* was named Sp-inx2 and characterized. The complete cDNA and genomic DNA sequences of Sp-inx2 were revealed. Sp-inx2 mRNA transcripts were distributed in various tissues of *S. paramamosain* and were most abundant in the hemocytes. The Sp-inx2 was significantly upregulated in hemocyte, gill and hepatopancreas tissues with the challenge of either *Vibrio alginolyticus*, *Vibrio parahaemolyticus* or lipopolysaccharides (LPSs) when analyzed at 3 and 6 h using quantitative real-time PCR, suggesting that it could activate an immune response against the challenge of LPSs or *Vibrio* species. Using the chemical inhibitors carbenoxolone and probenecid, the absorption of the fluorescent dye Lucifer yellow decreased in the primary cultured hemocytes of crabs, thus confirming that hemichannels composed of Sp-inx2 existed in the crab hemocytes. With LPS stimulation, the level of mRNA transcripts and protein expression of Sp-inx2 in the same cultured hemocytes gradually increased from 6 to 48 h, while the activity of hemichannels was down-regulated at 6 and 12 h, demonstrating that LPSs could modulate the absorption activity of hemichannels in addition to its upregulation of Sp-inx2 gene expression. Furthermore, the dye uptake rate in HeLa cells in which Sp-inx2 was ectopically expressed increased dramatically but the increase was significantly down-regulated with the addition of 50 μg mL^{-1} LPS, suggesting that the LPS stimulation could effectively reduce the activity of hemichannels. Interestingly, with the ectopic expression of Sp-inx2 in HeLa and EPC cells, apoptosis spontaneously occurred in both cultured cell lines when detected using TUNEL assay. In summary, a new Sp-inx2 gene was first characterized in a marine animal *S. paramamosain* and it had a function associated with immune response and cell apoptosis.

1. Introduction

Gap junctions (GJs) are found in most cells and tissues in all metazoan organisms and exert a function to promote intercellular communication [1,2]. GJs are composed of a pair of six polymer hemichannels. Each hemichannel consists of six connexin (in vertebrates) or innexin (in invertebrates) subunits. Hemichannels of adjacent cells interact with each other, and form a functional dodecameric GJ channel directly linking the cytoplasm of neighboring cells [3].

In vertebrates, there are not only connexins but also pannexins. Pannexins (not connexins) are homologous to invertebrate innexin proteins, and they all share a similar protein topology. Evolutionarily speaking, pannexins might be the chordate members of the innexin superfamily. Innexins in invertebrates might have a dual role, possibly providing the partial functions of both connexins and pannexins. Innexin, connexin and pannexin all have four transmembrane regions, two extracellular cycles, one intracellular cycle and intracellular N- and C-terminal tails. At least 20 connexin genes and three pannexin genes have been identified so far, likely encoding GJ proteins or hemichannel proteins in mice and humans

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http://dx.doi.org/10.1016/j.fsi.2015.09.028
1050-4648/

[2]. Multiple members of the innexin gene family are also found in invertebrates, and at least eight innexin genes are identified in *Drosophila melanogaster* [4], 25 in *Caenorhabditis elegans* [5], and 21 in the annelid *Hirudo verbana* [6]. In crustaceans, there are six innexin members which have been identified in the crab *Cancer borealis* [7]. However, there is no research on innexins in *Scylla paramamosain*.

The functions of connexins have been extensively studied, especially in embryogenesis, neurophysiology and endocrinology, however, much less is known about the function of the innexin/pannexin superfamily [8]. Some studies report that innexins are important for embryonic development, and morphogenetic and physiological regulation in invertebrates [9–11].

Previous studies reveal that innexins function mainly by forming GJs or hemichannels [12]. Dm-Inx2 GJs mediate intercellular transfer of GDP-L-fucose, a substrate for O-fucose modification, in the wing imaginal disc [11]. GJs are relatively non-selective in comparison to ligand-gated or voltage-gated channels and allow ions and small molecules such as cAMP to pass through, thus enabling electrical and metabolic signaling [8]. Some of the innexins in the *C. elegans* reproductive system do not create GJs at all, perhaps in favor of forming functional innexons which are called hemichannels [5]. The hemichannel is made of single (homomeric) or multiple (heteromeric) protein isotypes. It is possible that this hemichannel can help one cell dock with the adjacent cells under some circumstances in order to communicate with other cells. The channel activity might be very sensitive to various stimuli, such as the change of electrical potential inside and outside cells, the change of intracellular Ca^{2+} level, phosphorylation or dephosphorylation of innexin protein, immune response, and so on [13,14].

Hemichannels consisting of innexin/pannexin proteins can also regulate apoptosis. Previous studies show that insect innexons play a role in promoting apoptosis and inactivating the PI3K/Akt signaling pathway, a key cell survival pathway [15]. Moreover, the hemichannel can also participate in the process after the occurrence of apoptosis. Caspase-3 activation shears the C-termini of the pannexin1 protein, leading to the opening of the pannexin1 channel and increased ATP release [16]. This ATP release can induce phagocytes to swallow and digest the apoptotic cells and its components. In addition, gap–junction proteins act as tumor suppressors but the mechanism of action remains unclear [12].

It is noteworthy that the study on innexins is focused mainly on the fly *D. melanogaster* or the nematode *C. elegans* owing to their clear genetic background and advanced molecular techniques, whereas the relevant transmembrane proteins that function as barriers against pathogenic microorganisms in *S. paramamosain* have been less well reported. In our previous study, a novel transmembrane protein Sp-inx2 is identified by screening the suppression subtractive hybridization (SSH) cDNA library constructed in hemocytes of the mud crab *S. paramamosain* challenged with lipopolysaccharides (LPSs) [17]. This type of transmembrane protein, which was first found in *S. paramamosain*, greatly attracted us to further characterize it and evaluate its biological function. The present study was aimed to characterize the Sp-inx2 and further reveal its potential functions. The full length of the Sp-inx2 cDNA and the genomic DNA sequence were determined, and the tissue distribution plus expression patterns of Sp-inx2 in various tissues were also analyzed. Furthermore, the hemichannels in the hemocytes of *S. paramamosain* were elucidated. Considering that this channel protein itself or the signaling transduction pathways may be influenced by pathogen invasion and the hemichannel composed of Sp-inx2 may have a role involving apoptosis, we investigated the interaction between Sp-inx2 signaling, immune response and apoptosis. To our knowledge, this is the first characterization of the innexin2 gene in *S. paramamosain* and this work would much facilitate our understanding of the functional role of innexin2 in invertebrates.

2. Materials and methods

2.1. Experimental animals and hemocyte collection

Live healthy female *S. paramamosain* (with average body weight 300 ± 50 g) were purchased from a local commercial crab farm in Xiamen, China, and acclimated in seawater aquaria for one week before experiments were carried out. The haemolymph samples were prepared as previously described [17]. Briefly, 2 mL haemolymph was collected into an equal volume of anti-coagulant solution (NaCl 450 mM, glucose 100 mM, citric acid 26 mM, trisodium citrate 30 mM, EDTA 10 mM, pH 4.6) followed by centrifugation for 10 min at 4 °C, and 800 × g. The resulting hemocyte pellets were used for total RNA isolation.

2.2. Cloning of the full-length cDNA and genomic DNA sequences of Sp-inx2

Total RNA was extracted from hemocytes using TRIzol reagent (Qiagen) following the manufacturer's instructions, and treated with RQ1 RNase-free DNase (Promega) to remove the contaminated DNA. The integrity of the RNA was assessed using electrophoresis on a 1.2% formaldehyde-denatured agarose gel and visualized with ethidium bromide staining. The quantity of RNA was determined by OD260/280 measurement using a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific). To obtain the full-length cDNA of the Sp-inx2 gene, rapid amplification of the cDNA ends (RACE) was carried out using a SMART RACE cDNA amplification kit following the manufacturer's instructions (Clontech). Specific primers were designed based on the partial cDNA sequence obtained from an SSH cDNA library [17] (Table 1). The amplification reaction and polymerase chain reaction (PCR) temperature profiles were: 1 cycle of 94° C/5 min; 35 cycles of 94° C/30 s, 55° C/30 s, 72° C/90 s; and 7 min at 72 °C for the final extension. PCR products were gel-purified and cloned into the pMD18-T simple vector (Takara), then transformed into *Escherichia coli* DH5α competent cells. Potentially positive recombinant clones were identified using colony PCR. Positive recombinants were then selected for sequencing.

To analyze the Sp-inx2 genomic DNA organization, DNA was isolated from *S. paramamosain* muscle using a genomic DNA extraction kit (Takara). Based on the Sp-inx2 full-length cDNA

Table 1
Sequences of primers used in this study.

Primer	Sequence (5′-3′)
GSP 1	5′- CGCCCTTGAACTTCTTGC -3′
GSP2	5′- CAGCAGCATCTTCCTCACG- 3′
SMARTIIA Oligo	5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′
5′-CDS	5′- (T)25VN-3′
Genomic F1	5′-ATGTCGGACGTTTTCTCAGGTATCA- 3′
Genomic R1	5′-AACGAAGTTGAACATCTCGCAGGC- 3′
Genomic F2	5′-AAGGCGGCAAGGTGAAAATGC-3′
Genomic R2	5′-ATCATCCACGCCCTTGAACTTCTTG -3′
Sp-inx2CTF	5′-GCGCCATGGGCCTCACCCCCTCTGTGGCCGTGGATC-3′
Sp-inx2 CTR	5′- GGGCTCGAGATCATCCACGCCCTTGAACTTCTTG-3′
Innexin 2-q-F	5′ -ATGAGCAGCGGGTTGTG -3′
Innexin 2-q-R	5′ -TGCCTGGGTGAGCAGTTTCC -3′
β-actin F	5′ -GCCCTTCCTCACGCTATCCT- 3′
β-actin R	5′- GCGGCAGTGGTCATCTCCT -3′
pCMV-HA-inx2-F	5′- CGGAATTCCATCGGACGTTTTCTCAGGTATCA -3′
pCMV-HA-inx2-R	5′-ATAAGAATGCGGCCGCCTAATCATCCACGCCCTTGAACT-3