Fish and Shellfish Immunology 84 (2019) 213–222

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

A novel interleukin-1 receptor-associated kinase-4 from thick shell mussel *Mytilus coruscus* is involved in inflammatory response

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ARTICLE INFO

Keywords:
Mytilus coruscus
Interleukin-1 receptor-associated kinase-4
Inflammatory response
RNA interference
Subcellular localization

ABSTRACT

Interleukin-1 receptor-associated kinase-4 (IRAK4) is considered as the most upstream kinase of IRAKs and plays a vital role in Toll-like receptor/Interleukin-1 receptor (TLR/IL-1R) signal transduction. In the present study, IRAK4 from thick shell mussel *Mytilus coruscus* (*Mc*IRAK4) was identified and characterized. *Mc*IRAK4 showed the most similarity to its counterparts in bivalves. The conserved death domain (DD) and catalytic domain of serine/threonine kinases (STKc) were predicted in all examined IRAK4s. *Mc*IRAK4 transcripts were constitutively expressed in all examined tissues with the higher expression level in immune related tissues, and were significantly induced in haemocytes upon lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I:C) challenge. Further, the expression of *Mc*IRAK4 was obviously repressed by dsRNA mediated RNA interference (RNAi), meanwhile the proinflammatory cytokines TNF-alpha and IL17 were down-regulated while the anti-inflammatory cytokine TGF-β was up-regulated. Additionally, *Mc*IRAK4 showed a global cytoplasmic localization in HEK293T cells through fluorescence microscopy. These results collectively indicated that *Mc*IRAK4 is one member of IRAK4 subfamily and might play the potential signal transducer role in inflammatory response. The present study provides supplement for TLR-mediated signaling pathway triggered by pathogenic invasions in thick shell mussel, and contributes to the clarification of the innate immune response in molluscs.

1. Introduction

The thick shell mussel *Mytilus coruscus* represents one of the marine mussel species that mainly distributed in Chinese Yellow Sea, Korean Peninsula, and Japanese Hokkaido coastal areas. Due to its rapid growth, delicious taste and nutritional value, *M. coruscus* has developed into one of the most economically important aquaculture mussel species in China in recent years [1]. However, over the past decade, thick shell mussel aquaculture industry was suffering from seriously infectious pathogens accompanying with its rapid expansion of breeding scale as well as the deterioration of breeding environment, which was the same as the most marine breeding species facing, resulted in dramatic economic losses. Knowledge about the immune response to pathogenic invasions is helpful to develop the environmentally friendly strategies for disease prevention, such as disease-resistant breeding or development of vaccines [2]. Unfortunately, the information on immunity of thick shell mussel is still very scarce to date. This pitiful scenario has seriously hindered the sustainable development of mussel aquaculture industry.

Generally speaking, invertebrates lack of adaptive immune system, and mainly rely on the innate immune response to trigger diverse humoral and cellular activities to defense against microbial infections [3]. The innate immune system is the first defensive line against pathogens invasion, which is initiated by the recognition of a variety of pathogens via a limited number of pattern recognition receptors (PRRs) that recognize microbial components called pathogen associated molecular patterns (PAMPs) [4–6]. The Toll like receptor (TLR) family is one of the most well-studied PRRs, which can recognize various PAMPs of different microbial pathogens including bacteria, viruses, fungi and protozoa [7–11]. The mammalian TLR family consists of 13 members, sharing conserved functional characteristics containing C-terminal cytoplasmic toll-interleukin (IL)-1 receptor (TIR) domains, a transmembrane domain, and N-terminal leucine-rich repeats (LRRs) motifs [12]. Once PAMPs binding with their PRRs, most of TLRs trigger signaling cascades via applying their TIR domains to recruit downstream TIR domain-containing adaptor protein such as myeloid differentiation factor88 (MyD88) [13,14]. After that, MyD88 associate with IL-1R-associated kinase (IRAK) family members to form a complex through a homotypic interaction between their death domains (DD) [15–17], leading to the recruitment of tumor necrosis factor receptor-associated factor-6 (TRAF6). Subsequently, the complex induces the activation of downstream molecule transforming growth factor (TGF)-β-activated

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https://doi.org/10.1016/j.fsi.2018.10.018
Received 16 August 2018; Received in revised form 3 October 2018; Accepted 7 October 2018
Available online 09 October 2018

Table 1
PCR primer pairs used in the present study.

Primer pairs	Sequence (5'to 3')	Usage
IRAK4-ORF	ATGCCCACAAACAGTCGTGA CTAAATTTTATTTACAAGCT	For ORF cloning
IRAK4-5′	GCAAAATCTTCACAGAC GTTCTGCTTCAGTTAATGCCTT ATAAGTGTTTAACTCGCGAATT	For 5'RACE
IRAK4-3′	CTTAGATGAACAGCGATCAGAATGTGAC AGCAGGATCATGGGATATAGACCTTGCA	For 3′ RACE
Real-IRAK4	CCTTTTATGGCAGCAGCGTG AAAATCCAGTGCCCGATGGT	For *Mc*IRAK4 qPCR
Real-TNF-α	AACCAACCGGTGATTGTGGT TGGGATCAAGCAGCAACCAA	For *Mc*TNF-αqPCR
Real-IL-17	GGAGTTTGCGAAAATGGCGT AGCACCGATTGGAGGACTTG	For *Mc* IL-17 qPCR
Real-TGF-β	TGCGGGTAAAACCAAGACCA TCCCTGGCGGCTTCAATTAC	For *Mc*TGF-βqPCR
β-actin	GCTACGAATTACCTGACGGACAG TTCCCAAGAAAGATGGTTGTAACAT	Internal reference
Y- IRAK4	CAGAATTCATGCCCACAAACAGTCGTGA GAGGATCCAATTTTATTTACAAGCTTTT	For pEGFP-N1-*Mc*IRAK4 construction
*Mc*IRAK4-ds	TAATACGACTCACTATAGGGATGCCCACAAACAGTCGTGA TAATACGACTCACTATAGGGAATTTTATTTACAAGCTTTT	For *Mc*IRAK4 gene silencing
GFP-ds	TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA TAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA	Negative control in RNAi

kinase (TAK)1 and/or IκB kinase (IKK), which then leading to the activation of downstream signals, such as nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK), inducing the production of inflammatory cytokines and type I interferon [4,12,18].

Inflammation occurs after a pathogen infection, which is often characterized by the cell injury in host tissues [19]. In vertebrates, inflammation involves changes in local circulation and the recruitment of immune cells to injured foci to isolate or eliminate the causes of cell damage and eventually initiate tissue repair [20]. Although the complexity of the inflammatory response has increased during the differentiation of metazoan animals, its functional basis has not changed substantially during evolution [21]. Thus, similar functional events seem to occur in the inflamed tissue of molluscs as in vertebrates, though with obvious differences in some molecular features, the immune cells involved and their arrangement in injured tissues [22]. IRAK family contains four members: IRAK1, IRAK2, IRAK3 (also called IRAK-M) and IRAK4, which share general structural features containing an N-terminal DD domain and a conserved kinase domain [23]. Amongst, IRAK1 or IRAK2 are first recruited by IRAK4 and, in turn, they recruit TRAF6, and thus IRAK4 is considered as the most upstream kinase of IRAKs and plays crucial roles in TLR pathways [24,25]. Studies on mammals showed that IRAK4-deficient mice are completely resistant to lipopolysaccharide (LPS)- and CpG-induced shock, and severely impaired in responses to viral and bacterial challenges, which resulted from the impaired TLR/IL-1R-mediated induction of proinflammatory cytokines and chemokines [26,27]. Further studies have also revealed the essential role of IRAK4 in Tcell receptor (TCR) signaling [28], suggesting that IRAK4 may be involved in signal crosstalk between the innate and adaptive immune responses, although such hypotheses remain to be controversial in some other study [29].

Despite of the vital role of IRAK4 in TLR/IL-1R signaling pathway in mammals, the data about this molecule in lower vertebrates and invertebrates is still limited. Nevertheless, IRAK4 orthologs were identified in some teleost fishes, such as zebrafish [30], roughskin sculpin [31], half-smooth tongue sole [32], rainbow trout [33], grouper [34], rock bream [35], large yellow croaker [36] and golden pompano [2], as well as in some invertebrates, such as soft-shell clams [37], small abalone [38], pacific oyster [39] and brine shrimp [40]. In the present study, we focused on the molecular identification as well as its involvement in inflammatory response of IRAK4 in thick shell mussel (*Mc*IRAK4), in addition, its phylogenetic status, spatial and temporal expression profiles and subcellular localization were also assessed. The present research shed a new light on the functional role of IRAK4 in innate immunity of molluscs.

2. Material and methods

2.1. Animals

Thick shell mussel *Mytilus coruscus* healthy adults (shell length, 7.61 ± 0.43 cm; shell width, 3.53 ± 0.36 cm; wet weight, 60.3 ± 2.3 g) were bought from Donghe fish market, Zhoushan City, Zhejiang Province, China. These mussels were acclimated in 300 L aquaria under a laboratory condition with temperature of 24 ± 0.5 °C, salinity 28 ± 1‰ for one week before treated. Mussels were fed daily with spirulina powder, and filtered seawater was changed for half aquaria every day.

2.2. Challenge experiment

The LPS or polyinosinic-polycytidylic acid (poly I:C) challenge experiment was performed as described previously [41] with little modification. Briefly, 270 mussels were randomly divided into three groups, control group, LPS and poly I:C challenge groups, each group consisted of three biological duplications and 30 individuals in each duplication.

Ninety individuals of control group were adductor injected with 500 μL PBS (pH 7.4) while 90 individuals of each challenge group were injected with an equal volume of LPS (1 μg/mL) or poly I:C (1 mg/mL). These injected mussels were kept under the same conditions as above mentioned without food during the test. Haemolymph was collected at 0, 3, 6, 12, 24, and 36 h post induction (hpi) from the pericardial cavity through the adductor muscle and immediately centrifuged (700 × *g* for 10 min at 4 °C) to separate the haemocytes. Three individuals in each duplication were randomly sampled at every time point and pooled together to obtain enough blood cells and to reduce individual variation.

Seven tissues, including the gills, gonads, digestive glands, hepatopancreas, adductor, haemocytes and mantles were dissected from eight adult individuals to examine the tissue distribution of *Mc*IRAK4. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.