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Molecular characterization of complement component 3 (C3) in *Mytilus coruscus* improves our understanding of bivalve complement system

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ABSTRACT

Complement component 3 (C3) plays a central role in the complement system whose activation is essential for all the important functions performed by this system. Here, a novel C3 gene, termed *Mc-C3*, was identified from thick shell mussel (*Mytilus coruscus*). The deduced *Mc-C3* protein possessed the characteristic structure features present in its homologs and contained the A2M_N_2, ANATO, A2M, A2M_comp, A2M_recep, and C345C domains, as well as the C3 convertase cleavage site, thioester motif, and conserved Cys, His, and Glu residues. *Mc-C3* gene constitutively expressed in all examined tissues and predominantly expressed in immune-related tissues such as gills, hemocytes and hepatopancreas. After stimulation with lipopolysaccharide or Cu^{2+}, the expression of *Mc-C3* was significantly induced in gills. Further luciferase reporter assays showed the ability for activation of NF-κB signaling transduction of *Mc-C3a*. Taken together, these results show that C3 may play an essential role in the immune defense of *M. coruscus*. The present data therefore provide a more detailed insight into the functional activities of the bivalve complement system.

1. Introduction

The complement system, consisting of more than 40 plasma proteins that function either as enzymes or as binding proteins, is one of the major innate immune mechanisms that plays essential roles in both innate and adaptive immunity against infection, and participates in a wide array of physiological and pathological processes [1]. The complement system mediates the multiple functions including phagocytosis, cytolysis, inflammation, solubilization of immune complexes, clearance of apoptotic cells and promotion of humoral immune responses [2,3]. In addition, the activation of the complement system also contributes significantly to the adaptive immune responses [4]. The complement system is organized in three activation pathways in the vertebrates, named classical, alternative and lectin pathways. These three pathways merge at a common amplification step involving the central complement component 3 (C3), but they differ according to the nature of recognition [4,5]. Since its discovery as a critical system in microbial defense in the late 19th century [6], the complement system has been extensively studied in vertebrate. However, as an ancient immune defense mechanism, some components of the complement system have been recently identified in invertebrate [7–11].

Of all the components in complement system, C3 plays a central role in the three complement activation pathways [6]. The alternative pathway is initiated by the covalent binding of the complement component 3, C3, to the hydroxyl or amine groups on the surface molecules of pathogen. The lectin pathway is triggered by carbohydrate recognition through pattern-recognition receptors, such as mannose-binding lectin (MBL). Once the complement system is activated, a chain of reactions that involve proteolysis and assembly occur, and then the three activation pathways converge at formation of C3 convertases and cleavage of C3. Afterward, the lytic pathway is activated, during which the membrane-attack complex (MAC) is formed. C3, was first identified in 1912 [12], since that, this molecule has experienced the significant development and its role in immune response has been mainly studied in mammal and other higher vertebrate species. In past few years, the C3 homologs have been identified in invertebrate species, including cephalochordates, urochordates [9,13], echinoderms [8,11,14], and even cnidaria [10,15,16]. For the past few years, C3 homologs have also been identified in the molluscs, represented the second largest animal phyla, including oyster [1], razor clam and carpet-shell clam [17–19], common mussel [20], Hawaiian bobtail squid [21] and owl limpet [22]. Nevertheless, the information on the invertebrate complement C3 is still fragmentary, especially its physiological functions are poorly explored. Whether the C3 molecules in invertebrate species

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play roles similar to those of vertebrate C3s remains largely uncertain.

The thick shell mussel, *Mytilus coruscus*, which is widely distributed in Yellow Sea, Korean Peninsula, and Hokkaido waters of Japan, represents one of the most economically important mussel species in China. In recent years, this mussel is suffering from increasing sicknesses triggered by multiple environmental changes containing a wide range of biotic (bacterial and viral) and abiotic (heat or cold shock, hyperosmotic stress, food-deprivation, reduced oxygen level, heavy metal concentrations and organic or inorganic chemical substances) stresses, and resulted in a remarkable economic loss. In this context, to elucidate the immune modulation mechanism of thick shell mussel in response to external stimuli will contribute to the development of novel management strategies for disease control and long-term sustainability of the mussel culture industry. Here, we identified the C3 in *M. coruscus* for the first time, and analysed the tissue distribution and expression modulation of *Mc-C3* upon stimuli with lipopolysaccharide (LPS) or Cu^{2+}, and performed dual-luciferase reporter assays to evaluate the activation of NF-κB signaling transduction by *Mc-C3a.* These findings will lay the fundamental cornerstone for further research of bivalve C3 and expand the horizon for better understand the bivalve complement system.

2. Materials and methods

2.1. Mussel rearing, LPS and Cu^{2+} challenge, and sampling

Individuals of the thick shell mussel (weight mean 60.15 g, shell width mean 4.5 cm, shell height mean 9.1 cm) under healthy condition were obtained from the Zhou shan, Dongji Island aquaculture base (Zhejiang Province, China) and kept alive in a concrete tank filled with air-pumped filtered sea water and cultured at 24–25 °C, pH 8.0.

For LPS challenge, 80 adult individuals were randomly divided into two groups, 40 individuals of one group (control) were adductor injected with PBS (pH 7.4), and 40 individuals of another group were challenged with LPS. The challenged individuals were injected with 500 μL of LPS (1 mg/mL) in sterile PBS (pH 7.4). After injection, 3 mussels from each group were randomly selected for gill tissue collection at 0, 3, 6, 12, 24, and 36 h, respectively. Similar to the LPS challenge, 80 mussels were divided into two groups, the control and challenge groups for a Cu^{2+} challenge assay. In the challenge group, Cu^{2+} ($CuSO_4{\cdot}5H_2O$) was added to the seawater to a final concentration of 20 μg/L, whereas in the control group, nothing was added to the seawater. Three individuals were sacrificed for gill tissue collection at 0, 2, 5, 10, 16, 23 and 30 d post treatment. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. Cloning of the complete Mc-C3 cDNA

Partial cDNA sequences of the *Mc-C3* gene were obtained by scanning the *Mytilus coruscus* transcriptional database [23]. Based on the multiple alignments of these sequences with homologous nucleotide sequences, 10 primer pairs were designed to amplify the rest cDNA sequences of *Mc-C3* gene (Table 1). Following, specific and adaptor primers were designed to clone the 5′ and 3′ untranslated regions using rapid-amplification of cDNA ends (RACE).

2.3. Molecular characterization and phylogenetic analysis

We used the obtained C3 cDNA to search GenBank for homologous sequences using the BLASTX search tool at the National Center for Biotechnology Information (NCBI) website. We searched for the open reading frame of the target gene using ORF finder, and then the amino acid sequence was deduced. The functional domains in the deduced amino acid sequence were predicted using Conserved Domains at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Table 1
PCR primer sequences for *Mc-C3* cloning.

Primer	Sequences (5′–3′)	Usage
Mc-C3-F1	GACTCGATGCTCCAGAGACG	For gene cloning
Mc-C3-R1	TCCCACTGTCATCTGCTGTT	
Mc-C3-F2	GCTACCATCACGCCCATTCT	
Mc-C3-R2	AGGCTTAACGATTCGTCCCG	
Mc-C3-F3	GGAAAGCCAGCACCAAACTG	
Mc-C3-R3	CTGACAATCCAGCGTCCTCA	
Mc-C3-F4	GGTTGCAGTGTAGGTGGAGG	
Mc-C3-R4	ACACACACCCTCCGTGTTTT	
Mc-C3-F5	GCTGTCAGTCTGTCACCTCT	
Mc-C3-R5	TGGCAAGGCAAGGTCTACTG	
Mc-C3-F6	ACAAAACACGGAGGGTGTGT	
Mc-C3-R6	AATAGCCATTGCATCCCGGT	
Mc-C3-F7	CCATGGTTGTGGGGAACAGA	
Mc-C3-R7	GTTCCAGATGAGCGAGCAGA	
Mc-C3-F8	CCCACCACCCATATTCCCTG	
Mc-C3-R8	CGCACTGTTGGTCTCTCTGT	
Mc-C3-F9	CACAAGGAAGAGGTGTGGCT	
Mc-C3-R9	CTGTTGCCTACTGGCACCTC	
Mc-C3-F10	CCTCCAGATCGTGTCCAAGG	
Mc-C3-R10	CCAGCTATCCTTGCTCCACT	
B176-1	TTGTAACAGTTCTTTCAT	For 5′ race
B176-2	GCTGGATGGTCCTGTATGT	
B176-3	GCTGTATTGAGATATTGTCT	
C128-1	GAAAATCTTCAGTCGGCAAAAGTGGA	For 3′ race
C128-2	GAAGGTGAATGTGCAGAAATTTGGGA	
Mc-C3-F	TTTTGAGGACGCTGGATTGTTAG	For qPCR
Mc-C3-R	CTTCCCTTGGATTTATCGATCTTTT	
β-actin-F	GCTACGAATTACCTGACGGACAG	Internal reference
β-actin-R	TTCCCAAGAAAGATGGTTGTAACAT	
Y—F	CAGGATCCAGAAAAAGATCGATAAATCCAAGG	For expression plasmid construction
Y-R	GACCTCGAGAACTGTTCTGTCAAAATCAAA	

Phylogenetic relationships were investigated by the neighbour-joining (NJ) method using MEGA 5.0 [24].

2.4. qPCR

Various tissues, including the gills, mantle, gonads, digestive glands, hepatopancreas, adductor, and hemocytes, were dissected from 3 adult individuals to examine the tissue distribution of *Mc-C3*. The gills of 3 selected mussels were collected at specific time points from the control and LPS or Cu^{2+} treatment groups for real-time quantitative PCR (qPCR) assays. The qPCR was conducted on a 7500 Real Time PCR System (Applied Biosystems, USA) using the SYBRR® premix ExTaq Kit (TaKaRa). All samples were analysed in triplicate. The reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference [25]. In the tissue distribution analysis, the *Mc-C3* mRNA levels were expressed as the ratio of the C3 expression level in the gonads, and in the challenge assay, expressed as fold changes by comparing the normalized gene expression level of LPS or Cu^{2+} challenged mussel with that of the control mussel at 0 h.

2.5. Expression plasmid construction

The complete cDNA region encoding *Mc-C3a*, corresponding to amino acid residues 654 to 744 of *Mc-C3* was amplified by PCR with primers containing *Bam*H I and *Xho*I restriction enzyme cutting sites (Table 1). The PCR products were digested with *Bam*H I and *Xho*I, and subcloned into the plasmid expression vector pcDNA3.1 which was digested by corresponding enzymes [26]. The plasmid constructed was verified by sequencing, and designated *pcDNA3.1-Mc-C3a*.