Fish & Shellfish Immunology 32 (2012) 1171–1178

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Short communication

An unexpected loss of domains in the conservative evolution ninth complement component in a higher teleost, *Miichthys miiuy*

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ARTICLE INFO

Article history:
Received 14 November 2011
Received in revised form
3 February 2012
Accepted 7 February 2012
Available online 15 February 2012

Keywords:
Evolutionary analysis
Inductive expression
Miiuy croaker
Truncated C9

ABSTRACT

The complement systems of fish are well developed and play an important role in the innate immune response. C9 is the ninth member of complement components, involved in creating the membrane attack complex (MAC). In the present study, a truncated C9 cDNA sequence encoding 461 amino acids was cloned and characterized in the miiuy croaker (*Miichthys miiuy*). Typical fish C9 molecules have five characteristic modular domains, i.e. TSP1, LDLRA, MACPF, EGF, and a second TSP domain which is absent in mammalian counterparts. While in miiuy croaker, this truncated C9 presents only TSP1, LDLRA and MACPF domains. It is the first time of finding a truncated C9 in teleost components. RT-PCR analysis detected these C9 transcripts among all tissues examined, demonstrating its constitutive expression pattern in healthy fish. The highest levels of transcripts were detected in liver of both healthy and pathogen-infected miiuy croaker. Its constitutive and inducible expression pattern of this truncated C9 in liver is similar to most complement components which belong to the type of acute-phase proteins and are in general of hepatic origin. We cannot exclude the possibility that miiuy croaker presents the typical C9 although it has not yet been found. The molecular evolutionary analysis showed that this truncated C9 of miiuy croaker had a significantly higher omega value comparing with other fish and the positive selection pressure had happened on it after its divergence with other fish.

1. Introduction

The complement system was first identified as a heat-sensitive factor in fresh serum that 'complemented' the effects of specific antibody in the lysis of bacteria and red blood cells [1]. The functions of complement are numerous but it is most well known for its capacity to kill pathogens by creating pores in their surface membranes. The complement in teleost and cartilaginous fish can be activated by three overlapping pathways: the classical, alternative, and lectin pathways [2,3]. The activation of complement system results in the formation of the membrane attack complex (MAC) which is assembled by sequential addition of a single molecule of each complement component, C5b, C6, C7, and C8, plus variable numbers of C9 molecules [4–7]. Once assembled on the target surface it forms transmembrane channels causing membrane damage and cytolysis [8,9]. In teleost, the MAC complex has been microscopically observed as small pores in the cell surface [10]. The MAC proteins, C6 through C9 are structurally related with some differences in size and complexity. They share several common structural motifs including thrombospondin (TSP), low-density lipoprotein receptor class A (LDLRA), epidermal growth factor precursor (EGF), and MAC/perforin (MACPF) domain. The high degree of sequences and structural motifs similarities have suggested that C6 [11,12], C7 [13], C8a [14], C8b [15,16], and C9 [17,18] are ancestrally related. It is generally believed that MAC proteins arose by a series of gene duplications of an ancestral perforin-like gene [19,20] and therefore considered to be members of the gene family that includes the perforins although the evolution process is still under debate [20–22]. It's no doubt that more molecular evolutionary analyses are needed to elucidate the exact evolutionary process in fish terminal complement pathway.

The ninth component of complement (C9) is a single-chain glycoprotein that is involved in the formation of MAC on the surface of target cells [23]. Bacterial cell death can be caused solely by C9 [24]. The C9 component has been cloned and characterized in Japanese flounder (*Paralichthys olivaceus*) [25], pufferfish (*Fugu rubripes*) [26], carp (*Cyprinus carpio*) [27], rainbow trout (*Oncorhynchus mykiss*) [28,29], and grass carp (*Ctenopharyngodon idella*) [30]. They all presented the conserved TSP, LDLRA, EGF, and MACPF domains as their amphibian and mammalian counterparts did. Besides that, the fish C9 molecules also show their special characteristics such as the presence of a second TSP domain which is

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doi:10.1016/j.fsi.2012.02.010

absent in other vertebrates' C9 on the C terminal. And there is a unique glycine-rich region only present in grass carp and zebrafish C9 between the LDLRA domain and the MACPF domain [30]. These special characteristics are likely to have evolved into these present forms in fish for the sake of meeting the survival demands enforced by the aquatic environment in which is full of countless and numerous types of microbes [31]. So it is quite interesting to investigate the evolution of C9 between fish and mammals for the reason of the huge differences between the environments they lived in.

Miiuy croaker, *Miichthys miiuy*, belongs to the family Sciaenidae of order Perciformes and mainly distributes from the western Japan Sea to the East China Sea [32]. In China, it has been widely cultured since the late 1990s for its good taste and high nutritive and medicinal value [33]. However, many bacterial and viral diseases especially occurred in juvenile miiuy croaker have caused reduction of output and profits. Researches on its native and adaptive immune system are useful and helpful to understand the defense mechanisms of miiuy croaker against bacterial infection. Recently, a spleen cDNA library of miiuy croaker full of immune-related genes has been reported by our laboratory [34] and a partial C9 sequence has been screened.

In this study, a truncated C9 cDNA of miiuy croaker was cloned and sequenced. It only presented the three domains on the N terminal. Furthermore, the expression pattern of C9 after stimulated by pathogen was also analyzed to elucidate the possible role of C9 in the response to bacterial infection. Finally, to explore the possible evolutionary process of this truncated C9, an evolutionary analysis of C9 between fish and mammals was conducted for the first time.

2. Materials and method

2.1. Bacterial injection

Miiuy croakers were obtained from Zhoushan Fisheries Research Institute (Zhejiang, China). All fish were held in the laboratory for two weeks prior to use in experiments to allow for acclimatization. Only the fish with similar size and body weight were used. Miiuy croakers were randomly divided into two groups, control and injection groups. In the injection group, each fish was intraperitoneally challenged with 1 ml suspension of *Vibrio anguillarum* approximately 1.2×10^8 colony forming units while the control fish were challenged with the 0.9% saline. At 6 h, 12 h, 24 h, 36 h, 48 h and 72 h post-injection, the infected fish were sacrificed and the tissue samples were frozen in the liquid nitrogen immediately and stored at −80° centigrade until RNA isolation.

2.2. Isolation of RNA and 3′RACE

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The concentration of the total RNA was measured by spectrophotometer and 1 μg was reverse-transcribed to cDNA using 3′-Full RACE Core Set (Takara) according to the protocols provided by the manufacturer.

In a preliminary study, we have identified the miiuy croaker C9 EST sequences [34]. The obtained EST sequence contained 5′ terminal untranslated region (UTR) and partial coding region (with NCBI Accession No. of GW669752). To isolate the full length cDNA of C9 gene, one specific primer, C9-3′RACE, was designed for amplification the 3′ end (see Table 1).

RACE-PCR was performed according to the manufacturer's instructions (Takara). The reaction conditions of normal PCR were as follows: a pre-denaturalization at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. PCR was performed on a Bio-Rad S1000 (Bio-Rad). The amplified fragments were separated and purified with a QiaexII gel extraction kit (Qiagen). The products were then cloned into pMD19-T vector (Takara), propagated in *Escherichia coli* TOP10, and sequenced using Big Dye Terminator on ABI 3730 sequencer (Applied Biosystems).

Table 1
Primers used to amplify the miiuy croaker C9 gene.

Primer	Sequence (5′→3′)	Application
C9−3′RACE	CGAGGGTCCTGGAAAGCAGTGAGCAA	For 3′RACE of C9
C9-RT-F	ATGATTACTACGATGGGAGG	Expression of C9
C9-RT-R	TCTTGTTCACAGTGGAGGAG	
β-Actin-RT-F	GAGCCGCACGCTTCTTT	Expression of β-Actin
β-Actin-RT-R	CTGCTGTAGCCGAGGAC	
Splice-F	CATCAGTCAAAGGGGGTACTCT	The short C terminal domain
Splice-R	CATTGGGAATCCACAAATGTC	

To prove the accuracy of this short C9 sequence, primers (Splice-F and Splice-R, Table 1) were designed to amplify and sequence the C terminal part of miiuy croaker C9 molecule of another five different individuals.

2.3. RT-PCR analysis

A pair of primers, C9-RT-F and C9-RT-R, was used for amplifying gene fragment (in Table 1). Real-time quantitative PCR was conducted on a 7500 Real-time PCR system (Applied Biosystems). Amplifications were carried out at a final volume of 20 μl, containing 1 μl cDNA sample, 10 μl SYBR Green Real-time PCR master mixtures (Takara), 0.4 μl ROX II, 1 μl of each primer, and 6.6 μl ddH_2O. And the reaction carried out without the template was used as blank control. PCR amplification was performed in triplicate wells, using the following conditions: 10 s at 95 °C, followed by 40 cycles consisting of 5 s at 95 °C and 34 s at 60 °C, dissociation curve analysis was performed after each assay to determine target specificity. Expression of β-actin was used as internal control of gene expression analysis. The primers β-actin-RT-F and β-actin-RT-R were used for RT-PCR of β-actin expression (Table 1).

2.4. Sequence analysis and alignment

Sequences of C9 components were analyzed for similarity with other known sequences by BLAST program and the multiple sequence alignments were generated using Clustal X program [35] and adjusted based on amino acid sequences. The signal peptide was predicted on SignalP 3.0 server [36]. The protein domains and motifs were identified by Scanprosite [37] and Motifscan programs. The divergence and percent identity values of each sequence pair in the alignment were calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign [38]. Percent identity compared sequences directly, without accounting for phylogenetic relationships. Note that divergence is not usually the inverse of percent identity (i.e. the sum of the percent identity and divergence values for a given pair is not usually 100).

2.5. Evolutionary analysis

To investigate the evolution pressure on this truncated C9 gene, nine fish, two frog and fourteen mammalian C9 sequences were retrieved from the GenBank or Ensemble database. Fish phylogeny relationship was taken from the conventional viewpoint of Nelson [39]. And mammalian phylogeny tree was taken from the recent researches [40–42]. Nonsynonymous (d_N) and synonymous (d_S) nucleotide substitution rates were estimated using the likelihood