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Molecular cloning, characterization and expression of the complement component Bf/C2 gene in grass carp

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ABSTRACT

The complement system is an integral part of the host immune system and plays an immunoregulatory role at the interface between the innate and acquired immune responses. Factor B (Bf) serves as the catalytic subunit of complement C3 convertase in the alternative pathway (AP), while in the classical pathway (CP), this function is subjected to C2. In this study, we cloned and characterized the two Bf/C2 genes of grass carp, gcBf/C2A and gcBf/C2B. The gcBf/C2A and gcBf/C2B cDNA sequences are 2259 and 3004 bp in length, and the open reading frames (ORFs) of gcBf/C2A and gcBf/C2B were found to encode peptides of 752 and 837 amino acids, respectively. The genes share 30.7% amino acid identity with each other and 32.4–38.3% and 31.4–33% with the Bf and C2 genes in humans and mice. GcBf/C2A and gcBf/C2B were expressed in a wide range of grass carp tissues, with the highest level of expression of both genes detected in the liver. After a challenge with *Aeromonas hydrophila*, gcBf/C2A was significantly upregulated, especially at 4 h after infection, and the significantly higher expression of gcBf/C2B (27.3-fold) was found in the head kidney at 24 h post-challenge. The expression of gcBf/C2A was quickly upregulated at 1 day post-hatching and peaked at 5 days post-hatching. The maximum expression of gcBf/C2B was found at 1 day post-hatching. In conclusion, our data enables a better understanding of the physiological function of the Bf/C2 complement genes in vertebrates.

1. Introduction

The complement system is an integral part of the host immune system and plays an immunoregulatory role at the interface of innate and acquired immune responses [1]. Complement activation results in the opsonization of pathogens and their removal by phagocytes, as well as cell lysis [2]. Complement activation is known to occur through three different pathways: the alternative, classical and lectin pathways, which involve the formation of convertases to produce protein fragments by the cleavage of C3 and C5. Factor B (Bf) serves as the catalytic subunit of C3 convertase in the alternative pathway (AP) [3,4], while in the classical pathway (CP), this function is mediated by C2 [5,6]. In addition, Bf and C2 are single chain glycoproteins, which also serve as the catalytic moiety of the C5 convertases. Bf and C2 show a significant overall sequence similarity, sharing the same domain structure; this includes three complement control protein (CCP) domains at the N-terminal, one Von Willebrand factor A (VWFA) domain and a serine protease domain at the C-terminal [7]. These are considered to have arisen from a common ancestor, which was probably more 'B-like', although no direct evidence is available to identify when the Bf and C2 genes diverged [8].

The Bf and C2 genes are closely linked and reside in the class III region of the mammalian major histocompatibility complex (MHC) [9]. The same region also contains genes for the complement component, C4, the steroid 21-hydroxylase, the *Hsp70* heat shock protein, tumor necrosis factor α (TNFα), lymphotoxin, valyl-tRNA synthetase, and various other genes of unknown function [10]. Deficiencies of the Bf and C2 genes lead to increased susceptibility to bacterial infection [11]. In recent years, extensive studies have been focused on the biological role of the complement components Bf and C2 in mammals, including disease association studies [12–14] and gene knockout studies [15]. These have suggested that genetic polymorphisms in Bf and C2 are associated with a number of diseases [13,14,16–18]. In teleost fish, some investigations have focused on characterization and structural analysis of Bf/C2, but

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little was conducted on immune responsiveness about Bf/C2 function. In large yellow croaker and catfish, the Bf/C2 genes were upregulated after pathogen challenge, but they have different expression patterns in various organs [19,20].

Grass carp is one of the most important freshwater food fish species in China because of its delicious meat and economic importance. However, viral disease has caused high mortality in both wild and cultured fish, and had a severe impact on grass carp aquaculture. A better understanding of the innate immune response in grass carp can facilitate the development of effective methods for disease control. In this study, we cloned two grass carp complement Bf/C2 genes, which were designated as gcBf/C2A and gcBf/C2B; their amino acid sequence were analyzed, and their gene expression patterns were assessed in various tissue samples during the early development of grass carp. Expression levels were also assessed after grass carp were challenged with the *Aeromonas hydrophila* bacterium. Our data provide information on the constitutive and inductive expression of transcripts of fish Bf/C2 genes, as well as a better understanding of the crucial innate and acquired immune response of grass carp.

2. Materials and methods

2.1. Cloning and sequencing of Bf/C2 cDNA

Based on expressed sequence tags (ESTs) from the grass carp cDNA library [21], two pairs of gene-specific primers (F1 and R1, F2 and R2) were designed to amplify the internal region of gcBf/C2A and gcBf/C2B, respectively. The PCR cycling conditions were as follows: 31 cycles of 98 °C for 10 s, 61 °C for 15 s and 72 °C for 2 min. The resultant products were isolated using a Gel Extraction Kit (Tiangen Biotech Ltd., Beijing, China), cloned into pGEM-T vector (TaKaRa, Shiga, Japan) and transformed into *Escherichia coli* strain Top10 competent cells according to the manufacturer's instructions. Putative clones were screened by PCR using the M13F/M13R primers under the same PCR cycling conditions, and five randomly selected clones were sequenced on a 3730XL DNA sequencer (ABI, Foster City, CA, USA). To obtain the full-length cDNA sequence of gcBf/C2A and gcBf/C2B, 5′ and 3′ RACE was performed using gene-specific primers and adapter primers. The universal primer mix (UPM) contained a mixture of the long form (UPM long) and the short form (UPM short).

For 3′-RACE, PCR was performed with primers GcBf/C2A-3RACE1 or GcBf/C2B-3RACE1 and GcBf/C2B-3RACE2. The annealing temperature of the PCR was 55 °C. For 5′-RACE, first strand cDNA synthesis was primed using CDS and the SMART IIA primer (SMART RACE cDNA Amplification kit, Clontech, USA). The synthesized cDNA was then amplified by PCR using GcBf/C2A-5RACE1 and universal primers or GcBf/C2B-5RACE1 and universal primers. The annealing temperature of PCR was 68 °C. All primers are listed in Table 1.

Table 1
Primers used for sequence analysis.

Primer	Primer sequence (5′ - 3′)
UPM long	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT
UPM short	CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
GcBf/C2A-5RACE1	GAGGTTCCACCAGTTGAATAAGAGC
GcBf/C2B-5RACE1	CTTCAACCCTAACAGTCCTACCCAA
GcBf/C2A-3RACE1	TTGATGAGGGCACCAGT
GcBf/C2B-3RACE1	TCCTCAGGGAGACCAAGAGAAATC
GcBf/C2B-3RACE2	GAATAACAGACATAAAATGACTTGC
F1	GATGGGAGATGATGTGAA
R1	GAGGTTATAGCGAGGATG
F2	CAGCCGCTTGATTTACCA
R2	CTGCCGTCAGCACCATAC
GcBf/C2ArpF	AGGAACTAATATCGCCCAAGC
GcBf/C2ArpR	ATCATCTCCCATCCCAAACAC
GcBf/C2BrpF	TCCGTATTGGAGACAGAGTGC
GcBf/C2BrpR	TCCTCCTAAAGCCTGAGCAAC
Actin-F	CCTTCTTGGGTATGGAATCTTG
Action-R	CCTGAGCGTAAATACTC

2.2. Sequence analysis

The alignment search tool, BlastN, from the National Center for Biotechnology Information (NCBI) was used to search for homologous sequences in GenBank. The cDNA of gcBf/C2A and gcBf/C2B was translated into its potential open reading frame (ORF) by the ORF Finder algorithm (http://www.ncbi.nlm.nih.gov/gorf/). The putative amino acid sequences were analyzed for the presence of signal peptides with the SignalP software (http://www.cbs.dtu.dk/services/signalP/) [22]. Domain analyses were carried out with several resources, including Simple Modular Architecture Research Tools (SMART; http://smart.gembl-heidelberg.de/) [23], Pfam 20.0 (http://pfam.wustl.edu/) [24] and Scanprosite (http://www.expasy.org/tools/scanprosite/) [25] programs. Multiple sequence alignments were performed with the CLUSTALW 1.8 program [26]. The needle program (http://www.ebi.ac.uk/Tools/emboss/align/) was used to determine the identities between different complement sequences. A phylogenetic tree was constructed based on the deduced full-length amino acid sequences with the Maximum Likelihood (ML) algorithm in MEGA 5.0 [27], and the reliability of the analysis was assessed by 1000 bootstrap replicates.

2.3. Fish collection and immunity challenge

Grass carp that were 50 g in body weight were obtained from the Wujiang National Farm of Four Major Domesticated Chinese Carps, Jiangsu Province, China. All fish were healthy and acclimatized at 23 ± 1 °C for 14 days in a circulating water system. Various tissues from grass carp, including blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin, were used for RNA isolation in order to analyze the expression of gcBf/C2A and gcBf/C2B in healthy grass carp.

To examine the expression profiles in different tissues from grass carp challenged with *A. hydrophila*, 24 juvenile fish were evenly divided into two groups and cultured under the same conditions. Water temperature was maintained at 23 ± 1 °C, and dissolved oxygen concentration was maintained above 5 mg/mL by an oxygen tank. Fish from one group were intraperitoneally injected with formalin-killed *A. hydrophila* S2 (Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) at a dose of 2.0×10^6 cells (suspended in 100 μL PBS) per fish, whereas fish from the other group (control group) were injected with 100 μL sterile PBS per fish. Three fish were sampled at 4 h, 1 day, 3 days and 7 days post-injection from each group, respectively. Blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heard, gill, intestine and fin were collected from each fish for total RNA isolation.

To examine the expression profiles of embryos and fries at different developmental stages, samples for the unfertilized egg, 0-h post-hatching, at the 16-cell, morula or gastrula stages, eye sac appearance, caudal fin appearance, muscular effect, heartbeat, and 1-, 2-, 3-, 4-, 5-, 6-, 7-, 10- and 15-days post-hatching were collected from the Wujiang National Farm of Four Major Domesticated Chinese Carps, Jiangsu Province, China. Embryos and fries were reared in a hatching trough with constant water flow. The water temperature was maintained at 21 ± 1 °C. On the fifth day post-hatching, the fries which could steadily swim were fed with