



Molecular cloning of Gibel carp (*Carassius auratus gibelio*) complement component C3 and its expression profile after Cyprinid herpesvirus 2 infection

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ABSTRACT. The complement systems play an important role in innate and adaptive immunity. In this study, the complement C3 gene, designated *CagC3*, was cloned and sequenced from Gibel carp (*Carassius auratus gibelio*). The expression pattern of *CagC3* in different tissues of healthy Gibel carp and after challenge with Cyprinid herpesvirus 2 (CyHV-2) were evaluated using quantitative real-time PCR. The full-length *CagC3* cDNA was 5131 bp with an ORF of 4950 bp, encoding a predicted protein of 1649 amino acids. The deduced amino acid sequence showed that *CagC3* has conserved domains and residues known to be critical for C3 function. Phylogenetic analysis demonstrated that *CagC3* clustered with homologs from common carp and grass carp (*Ctenopharyngodon idella*). *CagC3* is expressed in all examined tissues of healthy Gibel carp, with the highest expression in liver. *In vivo*, after CyHV-2 challenge, *CagC3* transcription was significantly upregulated in liver, spleen and kidney with the peaks at 24 hr, 2 d, and 2 d, respectively. *In vitro*, *CagC3* expression in the Gibel carp brain cell line showed the same pattern as that *in vivo* after stimulation with CyHV-2 or poly(I:C). However, *CagC3* expression was downregulated at 24 hr after induction with lipopolysaccharide (LPS), and then reached the peak at 2 d. These results suggest that *CagC3* is involved in the innate immune response of Gibel carp to viral infection.

KEY WORDS: cloning, complement component C3, Cyprinid herpesvirus 2 (CyHV-2), Gibel carp (*Carassius auratus gibelio*), immune response

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The complement system participates in host protection, acting as the first line of defense against microbial intruders, promoting inflammatory reactions, and coordinating the adaptive immune response [3, 29]. It contains more than 35 soluble plasma proteins that play key roles in innate and adaptive immunity after activation [7, 35]. The third complement component, C3, is a key protein that plays a major role in host defense against infections [26]. Cleavage of inactive C3 generates a large fragment (C3b) and a smaller fragment (C3a), a crucial step in the complement activation cascade, which can be initiated by one or more of the three distinct pathways, called the alternative, classical and lectin complement pathways [24]. After C3 activation, the three distinct pathways converge into a final common pathway, known as the cytolytic pathway, in which the membrane attack complex forms, mediating lysis of target cells and playing a role in anti-infection immunity. Therefore, C3 is the crossing point of the three pathways for complement activation and acts as one of the bridges linking innate and acquired immunity [4, 20, 23].

In fish, a series of studies have been performed on the gene structure, type, function and expression of C3. A single isotype of C3 has recently been isolated from the spotted wolffish (*Anarhichas minor Olafsen*) [1], Atlantic cod (*Gadus morhua*) [14], halibut (*Hippoglossus hippoglossus*) [14], miiuy croaker (*Miichthys miiuy*) [34], orange-spotted grouper (*Epinephelus coioides*) [28], rohu (*Labeo rohita*) [27] and large yellow croaker (*Larimichthys crocea*) [40], which, like mammalian C3, were encoded by a single gene. In contrast, multiple forms of C3 have been described in rainbow trout (*Salmo gairdneri*) [37], gilthead sea bream (*Sparus aurata*) [36] and common carp (*Cyprinus carpio*) [22]. Differences in the binding specificity of C3 to various targets and in the hemolytic activity have been reported for trout [37], common carp [36] and sea bream [18]. In cod and halibut [14], C3 is expressed in a wide range of organs, with expression detected in different stages of larval development. However, these results contrast with studies on C3 expression in the spotted wolffish [1], in which C3 could only be detected in the liver. In addition, there

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are considerable numbers of publications that describe the expression response of C3 after stimulation of teleosts with bacteria, LPS and poly(I:C), but few studies have focused on the expression of C3 after viral infection.

Gibel carp (*Carassius auratus gibelio*) is a subspecies of crucian carp (*Carassius auratus*), and is widely cultured in almost the whole of China [42]. However, outbreaks of disease of this species have become of great concern; for example, a severe disease caused by Cyprinid herpesvirus 2 (CyHV-2) has emerged and caused huge economic losses [41]. CyHV-2, also known as herpes-viral hematopoietic necrosis virus of goldfish, is the second member of the genus *Cyprinivirus* isolated from carp [8]. In this study, the full cDNA encoding the Gibel carp complement component C3 was cloned and its domain architecture, phylogenetic relationships and mRNA expression *in vitro* and *in vivo* analyzed. These results provide a foundation for further study of the immunological responses of Gibel carp to CyHV-2 infection and for the control of this disease.

MATERIALS AND METHODS

Animals, cell line and virus

Gibel carp (about 150 g/individual) were obtained from the experimental farm of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. The fish were kept in tanks with circulated and aerated water at 25°C (2 × 1.5 × 1.5 m) for 15 days and fed twice a day with a commercial diet before experiments. Gibel carp brain (GiCB) cell line was established and conserved by our laboratory; cells were grown at 25°C in M199 Medium (M199; Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) [17]. CyHV-2 was isolated by our laboratory [41] and propagated in GiCB cells.

Primer design, cloning and sequencing of CagC3

A pair of degenerate primers (CagC3F and CagC3R) was designed to obtain the internal region of *CagC3* according to the conserved sequence of complement C3 homologs from other fish [common carp C3-H1 (GenBank accession number BAA36619.1), common carp C3-H2 (BAA36620.1), common carp C3-S (BAA36621.1), and grass carp C3 (AAQ74974.1)]. To obtain the full-length cDNA of *CagC3*, rapid-amplification of cDNA ends (RACE)-PCR was performed using the Clontech SMART cDNA synthesis kit (following the manufacturer's instructions) to identify the 5'- and 3'- regions of *CagC3*. Four specific primers were based on the amplified partial Gibel carp C3 sequence described above. Using nested PCR, primers 3F1 and 3F2 were used for 3'-RACE and 5R1 and 5R2 for 5'-RACE. To confirm the resulting full-length cDNA sequence of *CagC3*, specific primers (C3F/C3R) were designed. All the primers used in this study are listed in Table 1. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Residual DNA was removed by DNase I (Invitrogen) treatment. First strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and stored at -20°C. cDNA was used as template in 50- μ l PCR reactions; the PCR cycling conditions were: 30 cycles of 94°C for 10 sec, 60°C for 30 sec, and 72°C for 45 sec. The PCR products were purified using the Wizard® SV Gel and PCR Cleanup System (Promega, Madison, WI, USA), ligated into vector pMD19-T (TaKaRa, Dalian, Liaoning, China) and then transformed into *Escherichia coli* DH5 α competent cells. Five randomly selected clones were sequenced by Beijing Tianyi Huiyuan Bioscience & Technology Inc. (Beijing, China).

Sequence analysis of CagC3

The cDNA of *CagC3* was translated into its potential open reading frame (ORF) using the ORF finder algorithm (<http://www.ncbi.nlm.nih.gov/gorf/>). Identities between *CagC3* and other known C3 amino acid sequences were determined by BLAST (NCBI). Domain analyses were carried out with Simple Modular Architecture Research Tools (SMART; <http://smart.gembl-heidelberg>).

Table 1. Primer sequences used in this study

Name	Primer sequence (5'-3')	Applications
CagC3F	ACTKGGCATCTGTGTGGCAGAACC	Homologous cloning
CagC3R	GGCCAGTATTCAAYCCAGKTCTKC	Homologous cloning
3F1	ACCATCATGGTGTTCAGGCTGTG	First 3' RACE
5R1	CGGTCCTTCACCTGCGTGCGATAC	First 5' RACE
3F2	CAGGTGCTGAAAGAAGGAAGACTGAT	Nest 3' RACE
5R2	CGCAACAGCGTAAGGATTGGTCAG	Nest 5' RACE
C3F	TGCGGCAGTGATTAGACTCGGTACG	full-length cDNA
C3R	TGTACCATTTTTATTTCAGACAGTAT	full-length cDNA
CagC3-qF1	AGTCAAATGGTGGGAAGCAGAAAG	qRT-PCR
CagC3-qR1	TACGTATACCGAGACATCGAAGG	qRT-PCR
CagActin-F	CTCCCCTCAATCCCAAAGCCAA	qRT-PCR
CagActin-R	ACACCATCACCAGAATCCATCA	qRT-PCR
Universal Primer Mix (UPM)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
Nest Universal Primer (NUP)	AAGCAGTGGTATCAACGCAGAGT	Nest RACE

F indicating forward primer and R reverse primer. RACE, rapid-amplification of cDNA ends; qRT-PCR, quantitative real-time-PCR.

de/) [31], Pfam 20.0 (<http://pfam.wustl.edu/>) [5] and Scanprosite (<http://prosite.expasy.org/scanprosite/>) [9] programs. Multiple sequence alignments were performed with the CLUSTALW 2.1 program [11]. The predicted mass and isoelectric point (pI) of the putative protein were calculated using the ProtParam tool at the ExPASy website (<http://www.expasy.org/tool/protparam/>). A phylogenetic tree was constructed with the neighbor-joining (NJ) method in MEGA 5.0 software [39] and the reliability of the analysis was assessed by 1,000 bootstrap replicates.

CagC3 expression in vivo after CyHV-2 infection

To assess the tissue distribution of *CagC3* expression in healthy gibel carp, nine different tissues including liver, spleen, kidney, brain, muscle, heart, gill, intestine and skin were collected from every sample fish. To examine *CagC3* expression *in vivo* in different tissues after virus infection, carp in the virus-infected group were injected intraperitoneally with 0.5 ml CyHV-2 (10^6 TCID₅₀/ml); carp in the control group were injected intraperitoneally with an equal amount of Dulbecco's phosphate buffered saline (DPBS) (Sigma). Virus-injected carp (n=5 per time point; 40 total) and PBS-injected carp (n=5 per time point; 40 total) were sacrificed at 0, 6, 12 and 24 hr, and 2, 3, 6 and 9 d after challenge.

CagC3 expression in vitro after induction with virus, poly(I:C) or LPS

In vitro, GiCB cells, as a sensitive cell line to CyHV-2 [17], were maintained at 28°C in 6-well culture plates at 1×10^6 cells/well in M199 supplemented with 10% FBS. For viral infection, GiCB cells were infected with CyHV-2 at a multiplicity of infection of 1, 10 µg/ml LPS (final concentration) or 10 µg/ml poly(I:C) (final concentration), respectively. Controls were treated with DPBS. All the above infections were performed in parallel. The cells were collected 6, 12, 24, 48 and 72 hr post-infection. All samples were stored at -80°C until use.

Quantitative real-time PCR expression analysis

In quantitative real-time PCR (qRT-PCR), synthesized cDNA was used as the template; along with two pairs of gene specific primers (CagC3-qF1/CagC3-qR1 or CagActinF/CagActinR) designed using Primer Premier 5.0 software. The qRT-PCR amplifications were carried out in total reaction volumes of 20 µl, containing 10 µl SYBR real-time PCR premixture (BioTeKe, Beijing, China), 1 µl diluted cDNA sample, 1 µl of each primer (0.5 µM), and 7 µl ddH₂O, in a Real-Time PCR Detection System (Rotor-Gene 6000, Qiagen, Dusseldorf, Germany). The PCR program was: one cycle of 95°C for 5 min, then 40 cycles of 94°C for 15 sec, 60°C for 15 sec and 72°C for 20 sec. For each cDNA sample, reactions were performed in triplicate and measured by the crossing-point (*Ct*) values. Expression of β-actin was used as the internal control for analysis of C3 gene expression. The relative *CagC3* expression ratio was determined by the $2^{-\Delta\Delta CT}$ method [16]. The data are expressed as mean ± SD. Expression differences between control and treatment groups were assessed for statistical significance ($P < 0.05$) using Student's *t*-test and the SPSS statistical software package.

RESULTS

Sequence of CagC3 and homology alignment

The full-length cDNA of *CagC3* is 5,131 bp (GenBank accession number: KF110786) and contains an ORF of 4,950 bp, encoding a predicted protein of 1649 amino acids (aa) with an estimated molecular mass of 184.81 kDa. The pI of *CagC3* is predicted to be 6.28. A pair of specific primers C3F/C3R (Table 1) was used to confirm the full-length cDNA of *CagC3* in the PCR reaction. As shown in Fig. 1, a specific fragment, approximately 5,100 bp, consistent with the expected size was obtained in the PCR reaction with reverse transcriptase and was subsequently confirmed by sequencing analysis, while no band was found in the reaction without reverse transcriptase (Fig. 1).

Based on multiple alignment of the predicted amino acid sequence of *CagC3* with homologs from other species, the *CagC3* sequence includes a putative cleavage site and eight well-conserved domains (Fig. 2). The putative cleavage site is 'RXXR' which occurs from aa 662 to 665. The first (N-terminal) domain is alpha-2-macroglobulin (A2M_N), from aa 136 to 230. Moving through the primary structure toward the C-terminus, the second domain is alpha-2-macroglobulin_2 (A2M_N_2, aa 459–600). The next domain, occurring after the cleavage site, is anaphylatoxin (ANATO, aa 687–722). Next is another alpha-2-macroglobulin domain (A2M, aa 765–858), followed from aa 988 to 1018 by an alpha-macroglobulin thioester bond-forming region. Sixth, the complement component region of the alpha-2-macroglobulin family is found (A2M_comp, aa 1040–1272). The next domain is a receptor-binding domain of alpha-2-macroglobulin proteins (A2M_recep, aa 1385–1480). Finally, there is a basic domain called the Netrin C-terminal domain (C345C, aa 1519–1631).

Deduced amino acid sequence comparisons indicate that *CagC3* shows 81%, 77%, 52% and 51% identities with the C3 proteins of grass carp, zebrafish, grouper (*Epinephelus coioides*) and medaka (*Oryzias latipes*), respectively. The highest amino

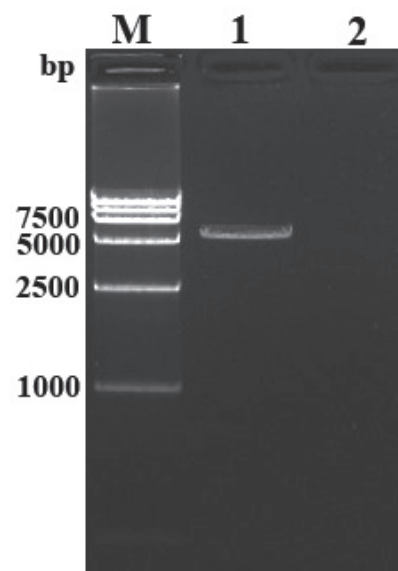


Fig. 1. Amplification results of the full-length cDNA of *CagC3* by RT-PCR assay. Lane M: DL1.5 kb DNA ladder marker; Lane 1: Amplification of *CagC3* in the PCR reaction with reverse transcriptase; Lane 2: Amplification of *CagC3* in the PCR reaction without reverse transcriptase.

gibel carp	METEDVCSFASKKGYRTIVSVEKSSISVSYVIFPMTLGNHMIKASAYDAIYSDGVRKPLKVVSEGLVLPVHRKNVE	[938]
carp	METEDVCSFASKKGYRTIVSVEKSSISVSYVIFPMTLGNHMIKASAYDAIYSDGVRKPLKVVSEGLVLPVHRKNVE	[931]
zebrafish	MESADVCSLASKKGYRTIVTIPKSSLSVPPYVIFPMTLGNHMIKAAAYDAVYTDGVRKPLKVVSEGLVLPVLRPNVE	[932]
mouse	LHNPAFCSLATAKRYRQIVTIPPKSSLSVPPYVIFVPLKIQQVEVKAAVVNHFI SDGVRKPLKVVPEGMIRNKTVAIHT	[946]
human	LHNPAFCSLATAKRRHQIVTIPPKSSLSVPPYVIFVPLKIQQVEVKAAVVYHFI SDGVRKPLKVVPEGIRNKTVAVRT	[946]
[Thiol-ester		
gibel carp	LNPVKNGEKP IIVKSEIF---VDRVPDTPANTYISITGEEIACITVEQALISGDFMGRLLIQPHGCGEQNMIIVMTLFPVIAT	[1014]
carp	LNPVKNGEKP IIVKSDIP---VDRVPDTPANTYISITGEEIACITVEQALISGDFMGRLLIQPHGCGEQNMIIVMTLFPVIAT	[1007]
zebrafish	LNPVKNGDSP IIVRADIP---ADRVPGTIPAHITISITGEEIACITVEQALISGDFMGRLLIQPHGCGEQNMIIVMTLFPVIAT	[1008]
mouse	LDPEKLCQG-GVQKVDVPAADLSDQVDPDTESETRILLGQSPVVMQAFDAVDGERLKHLLVTPAGCGEQNMIIVMTPTVIAV	[1025]
human	LDPERLCRE-GVQKEDI PADLSDQVDPDTESETRILLGQTPVAQMTDADVAERLKHLLVTPAGCGEQNMIIVMTPTVIAV	[1025]

[A2M comp		
gibel carp	HYLDSTSQWEIVGMERRNEALNIHTG YRQLGYRKT DGSYAAWTRPSS TWTLYAVAKIFAMANDFAQTEENVLGSALK	[1094]
carp	HYLDSTSQWEIVGMERRNEALNIHTG YRQLGYRKS DGSYAAWKDRPSS TWTLYAVAVFAMANDFATIEKNVLSALK	[1087]
zebrafish	HYLDSTNQWEIAGMKRRDEALGHITKTYNRQYARKPDGSYAAWIDRPSS TWTLYAVAKIFAMANDLITIEEVLGSALK	[1088]
mouse	HYLDDETEQWEKFGLEKRQGALEL I KKYTQQLAFKQPS SAAFNRRPSS TWTLYAVVRFVSLAANLIALDSVLCGAVK	[1105]
human	HYLDDETEQWEKFGLEKRQGALEL I KKYTQQLAFKQPS SAAFVRRPSS TWTLYAVVRFVSLAVNLIALDSVLCGAVK	[1105]

A2M comp		
gibel carp	WLVIHLKLPDGSFRBESAVIHGEMVGDVR-GKDAEASLTAFAVVIAMQEAIREICAGSVGSLPESIRKAVVFLDGRLPQITN	[1173]
carp	WLVIHLKLPDGSFRBESAVIHGEMVGDVR-GKDADA SLTAFAVVIAMQEAIREICGSEVGSLSHESIRKAVVFLGRVFPQITN	[1166]
zebrafish	WLVIHLKLPDGSFRBKAAVIHGEMVGDVQ-GRDAEASLTAFAVVIAMQEGRELCGATVGSLPESIRKAVSFLGKLNKLTN	[1167]
mouse	WLIILEKPKDPGVFQEDPVIHQEMIGGFRNAKEADVSLTAFAVLIALQEAIRDCEGQNSLPGSITKAGDFLEAYNMQR	[1185]
human	WLIILEKPKDPGVFQEDAPVIHQEMIGGLRNNNEKDMALTAFAVLI SLQEAKDICEEQNSLPGSITKAGDFLEAYNMQR	[1185]

A2M comp		
gibel carp	PYAVAMTSYAMNDNKLKDKILMKHSSQKEAGRSITVFGQHHSLEATAYAVLALVKAKDFDKAGEAVHWLNRRQSHYGG	[1253]
carp	PYAVAMTSYAMANENKLNKDLMKYSSQHEAGRSITVFGQHYHSLLEATAYAVLALVKEKDFDKAGEAVHWLNRRQSHYGG	[1246]
zebrafish	PYAVALTSYAMAGADRLNKAVLMKFSSTKGEAGTYVNVFGQYHTREATAYAVLALVKARDFNAAGEAVHWLGSQHYSYGG	[1247]
mouse	PYVVAIAGYALALMNLKEEYPLGKFLNTAKDRNVEEEDQQLYNVEATSYALLLALLLQKDFSVPPVVRWLNBRQRYGGG	[1265]
human	SITVVAIAGYALAQMGRLKGPLLNKFLTTAKDKNVEEEDGKQLYNVEATSYALLLALLLQKDFDFVPPVVRWLNBRQRYGGG	[1265]

A2M comp		
gibel carp	SCITQATIMVFQAWAEBYRTQVKDRHNFMEVELSVAGRSKPVRYTIKRNNAHLTRSDRVDIKQEFNVTAKGCTATLFSVL	[1333]
carp	SCITQATIMVFQAWAEBYRTQVKDRQNFNDVELSVAGRSKPVRYTIKKNNAHLTRSDRVDINKEFNVTAKGCTATLFSVL	[1326]
zebrafish	SCITQATIMVFQAWAEBYRTQVKDSQNFNLVEAVEGRSKPSRYTIKRNDRHVSRSOKVDINKDFNVTAKGCTATLFSVL	[1327]
mouse	YGSITQATIMVFQALAQYQTDVPHKDLMDVSEHLPSSRSATTFRLWENGNLRSSEETKQNEAFSLTAKGKRGTLFSV	[1345]
human	YGSITQATIMVFQALAQYQKDPADHQELNDVSLQLPSSRSKI THRIHWESASLRSSEETKENEGFTVTAEGKRGTLFSV	[1345]

[A2M recep		
gibel carp	TLVYARPVVEKSDITFDFLTVKLEKENITKQG-AIETYKMTDFYFKS-DKTDATMILLDIGIPTGFIVENRDLLELSRG	[1411]
carp	TLVYARPVVEKSDITFDFLTVKMEKDNANQG-AIETYKMTDFYFKS-DKTDATMILLDIGIPTGFIVENRDLLELSRG	[1404]
zebrafish	TLVYARPVVEKSDITFDLSLTKDKDPEIKHADAIETFKLTMDFYFKN-PKSPATMILLDIGIPTGFIVENRDLLELTRG	[1406]
mouse	AVYHAKLKSQVT-CKKFDLRSIRPAPETAKKPEEAKNTMFLICTKYLGDVDATMSILDSMMTGFAPDTPDKLELLASG	[1424]
human	TMVYHAKADQLT-CKKFDLKYTIKPAPETEKRPQDAKNTMFLICTRYRGDQDATMSILDSMMTGFAPDTPDKLQLANG	[1424]

A2M recep		
gibel carp	KERYIQRFEMDKVLSERGSLLYLDRVLRKQRQLAERMHKMLNVGLQPAAVTIYBYSPNARCTKFFHPERTDGAISI	[1491]
carp	KERYIQRFEMDKVLSERGSLLYLDRVLRKQRQLAERMHKMLNVGLQPAAVTIYBYSPNARCTKFFHPERADGAISR	[1484]
zebrafish	KEKYLERFEMDKALSDRGSLLYLKMSNRESERIAERMHKMLNVGLQPAAVTIYBYSPNARCTKYFHPER-EGVITYR	[1485]
mouse	VDRIYSRYEMNKAFSNKNLTIYLEKISHTEDCLTAKVHYFNGLIQGSKVYVSYNLEESCTRFYHPKDDGMLSK	[1504]
human	VDRIYSRYELDKAFSDRNTLIYLDKVSHEDDCLAKVHYFNWELIQGAVKVVAYNLEESCTRFYHPKDEKGLNKLK	[1504]

[C345C		
gibel carp	LKGDLCQCAEENGSLQK-KNHVGDVRLDKACEAGMDYVYRVTVVGMDLKQSDIYDLRVDQVLRKGTVDVDEKVRPFP	[1570]
carp	LKGDLCQCAEENGSLQK-KNHI GDEERFNACEAGMDYVYRVTVVGMDLKQADIDVLEVEQVLRKGTVDVDEKRRRPF	[1563]
zebrafish	LKGDMLCQCAEENGSLQK-KGGFKDEBRNIAKACESGMDYVYRVKVKMDLAAADITVQMEVEQVLRKGTQVIEGQVSKF	[1564]
mouse	LCHSEMRCAEENGSLQKQSEKINLNVRLDKACEPCVDYVYKTELNTI BLDDDFBYTMTIQQVTKSDEVEQAGQKRF	[1584]
human	LQRDELRCQCAEENGSLQKSDDKVTLLEERLDKACEPCVDYVYKTRLVKQVLSNDFYIMAIETITKSGSDEVEQAGQKRF	[1584]

[C345C]		
gibel carp	LARPSQRKPLELVEGSSYLIMGRSVDLPGLGSSLOYIFGEQTWIIPYPTREBSQIPEHKQRYLGISDLQSLTDCGAT	[1649]
carp	LARPTRNHLGLVEGSSYLIMGRSVDLPDLGSSLOYIFGEQTWIIPYPTREBSQIPEHRDRIYIGISLQNSLLKEGAT	[1642]
zebrafish	LGRPSKESLGFVQGSYLIMGRSVDLPKLGSSLOYILGEQTWIIPYPTREBSQSKSAEHRDRIYIGITELNMLTKEGAT	[1643]
mouse	ISHIKRNALKLQKGRKYLWGLSSDLWGEKPNPSTYITGKDTWVWHVPEABECQDQKYKQCEELGAFTESMVYVCGPN	[1663]
human	ISPDKREALKLEEKHYLWGLSSDFWGEKPNLSYITGKDTWVWHVPEEDECQDEENKQKQDGLGAFTESMVYVCGPN	[1663]

Fig. 2. Continued.

acid identity is 91% with common carp C3-H1. However, the identities with the proteins from tammar wallaby (*Macropus eugenii*) and human (*Homo sapiens*) are only 44%.

Phylogenetic analysis of CagC3

The relationships among vertebrate C3 sequences were revealed in a phylogenetic tree (Fig. 3). The unrooted phylogenetic tree divided into two groups: fish and mammals. The fish group has two branches, and the CagC3 sequence belongs to the branch containing cyprinid fish. According to the phylogenetic tree, CagC3 is most closely related to that from common carp, and has high similarity with homologs from zebrafish and grass carp. The tree was consistent with traditional taxonomy and phylogenetic transition.

Expression of *CagC3* in vivo after infection with *CyHV-2*

The relative expression levels of *CagC3* in various tissues were determined by qRT-PCR. A fragment consisting of 123 bp was amplified from each of the tissues. Retrieval and sequencing results verified the detection of the *CagC3* fragment. The expression of *CagC3* was observed in all nine normal (healthy) tissues examined (Fig. 4A). However, the expression profiles of *CagC3* in different tissues showed significant differences. *CagC3* mainly expressed in the liver, was moderately expressed in the gill, spleen, heart and brain, and was expressed at a low level in muscle, intestine, skin and kidney.

In vivo, after challenge with *CyHV-2*, the highest expression of *CagC3* was observed in liver, and high expression levels were also observed in spleen and kidney (Fig. 4). In the liver, the expression level increased significantly from 0 to 24 hr post-infection, reached the peak at 24 hr, then decreased sharply from 24 hr to 3 d and maintained a stable level from 3 to 9 d (Fig. 4B). In the spleen (Fig. 4C), expression of *CagC3* increased sharply from 6 to 12 hr, reached the peak at 2 d and then decreased from 2 to 9 d. In the kidney (Fig. 4D), the expression of *CagC3* increased from 0 to 24 hr, reached the peak level at 2 d, and remained relatively high at 3 d. Subsequently, the expression of *CagC3* decreased sharply and the level was low from 6 to 9 d.

Expression of *CagC3* in vitro after induction with virus, poly(I:C) or LPS

In vitro, the mRNA expression of *CagC3* in GiCB cells infected with *CyHV-2* increased significantly at 48 hr and declined at 72 hr. Similarly, mRNA expression levels of *CagC3* in GiCB cells induced by poly(I:C) were obviously elevated at 24 hr compared with the control group, reached the peak at 48 hr, and recovered to the normal level at 72 hr. Interestingly, LPS induced different *CagC3* mRNA expression trends: the mRNA expression fell from 6 to 24 hr, reached the minimum at 24 hr, then rose sharply from 24 to 72 hr (Fig. 5).

DISCUSSION

As the central component of the complement system, C3 may play an important role in innate and acquired immunity. C3 has been investigated in many teleost species [2]. In contrast to mammals, almost all teleost fish studied thus far have been found to possess multiple forms of C3: two isoforms have been found in *Oryzias latipes* [12], *Dicentrarchus labrax* [18] and *Trematomus bernacchii* [19], five in *Sparus aurata* [36], eight in *Oncorhynchus mykiss* [37], and four in *Cyprinus carpio* [22] and *Danio rerio* [6]. In the present study, the complement component C3 gene from Gibel carp was cloned and characterized and the expression profiles were analyzed for the first time. However, it should be noted that this study examined only one isoform of C3, and whether there are other isoforms of C3 in Gibel carp is worthy of further investigation.

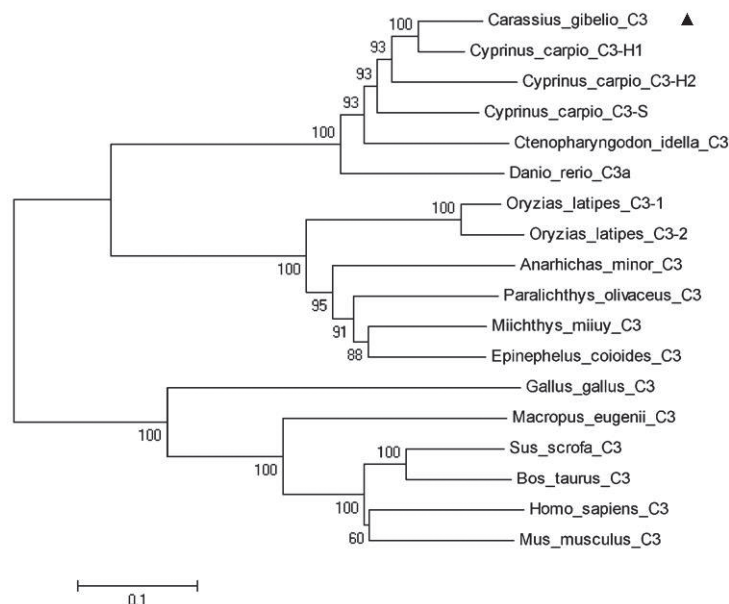


Fig. 3. Phylogenetic tree of C3 from Gibel carp and other species constructed with the neighbor-joining (NJ) method in MEGA 5. The deduced amino acid sequences used and their GenBank accession numbers are: *Carassius auratus gibelio* C3 (KF110786), marked with ▲; *Cyprinus carpio* C3-H1 (BAA36619.1); *Cyprinus carpio* C3-H2 (BAA36620.1); *Cyprinus carpio* C3-S (BAA36621.1); *Ctenopharyngodon idella* C3 (AAQ74974.1); *Danio rerio* C3.1 (NP_571317.1); *Oryzias latipes* C3-1 (NP_001098552.1); *Oryzias latipes* C3-2 (NP_001098553.1); *Anarhichas minor* C3 (CAC29154.1); *Paralichthys olivaceus* C3 (BAA88901.1); *Miichthys miiuy* C3 (AFC89899.1); *Epinephelus coioides* C3 (BAA88901.1); *Gallus gallus* C3 (NP_990736.1); *Macropus eugenii* C3 (C3 AAW69835.1); *Sus scrofa* C3 (NP_999174.1); *Bos taurus* C3 (CAJ31249.1); *Homo sapiens* C3 (AAA85332.1); and *Mus musculus* C3 (NP_033908.2). The reliability of the analysis was assessed by 1,000 bootstrap replicates; bootstrap support percentages are shown at nodes.

Analysis of the deduced amino acid sequence showed the presence of eight well-conserved domains in CagC3, as observed in other species. The structures and functions of the eight domains were similar to the complement component C3 of gilthead sea bream [18], which showed the typical domain architecture of alpha-2-macroglobulin family proteins. It is worth noting that the

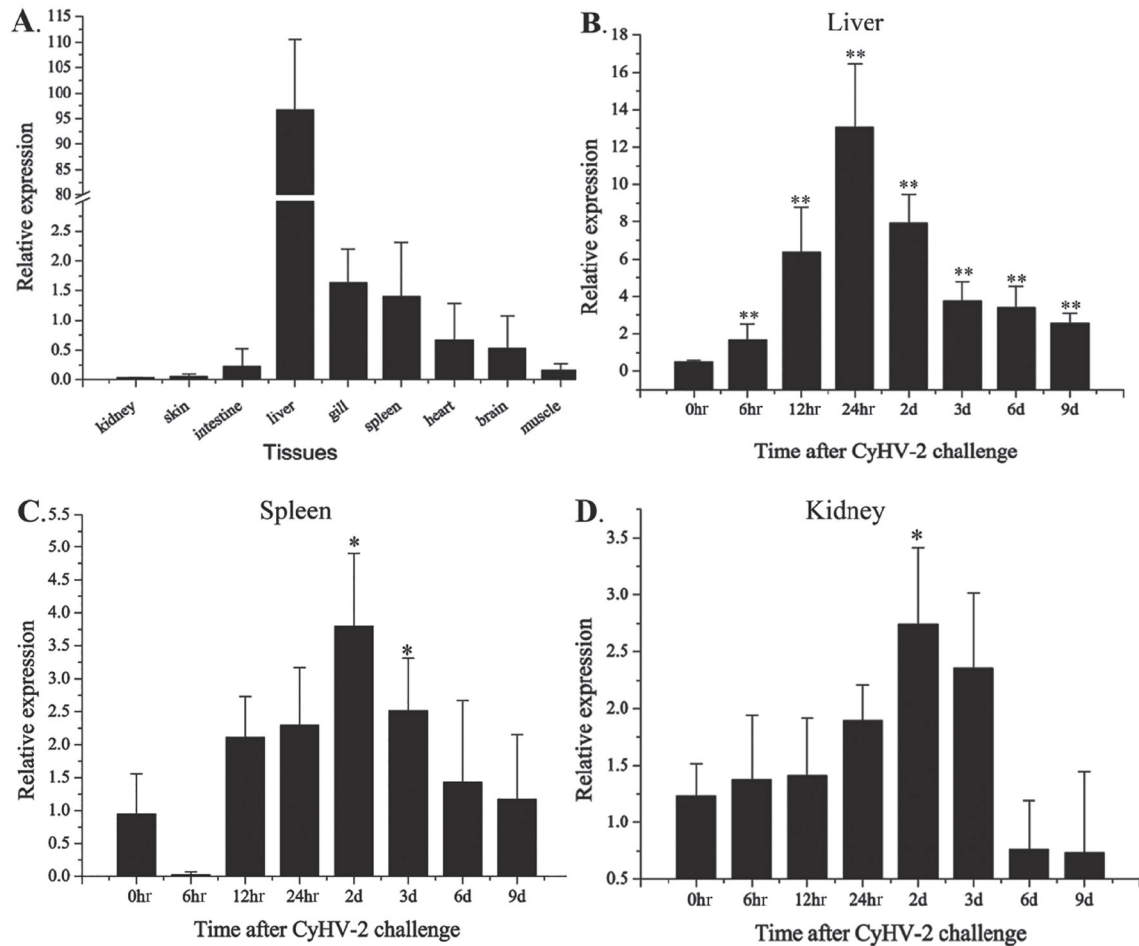


Fig. 4. Expression of *CagC3* in Gibel carp determined by qRT-PCR. A, Expression in nine tissues of healthy fish (kidney, intestine, liver, gill, spleen, brain, heart, muscle and skin). Expression after challenge with CyHV-2 in (B) liver, (C) spleen, and (D) kidney. All data were normalized to β -actin. The data are expressed as means \pm SD (n=5) and were analyzed by calculating $2^{-\Delta\Delta CT}$. Significant differences (* $P < 0.05$; ** $P < 0.01$) in *CagC3* expression in the challenge groups are relative to the expression in that tissue at 0 hr.

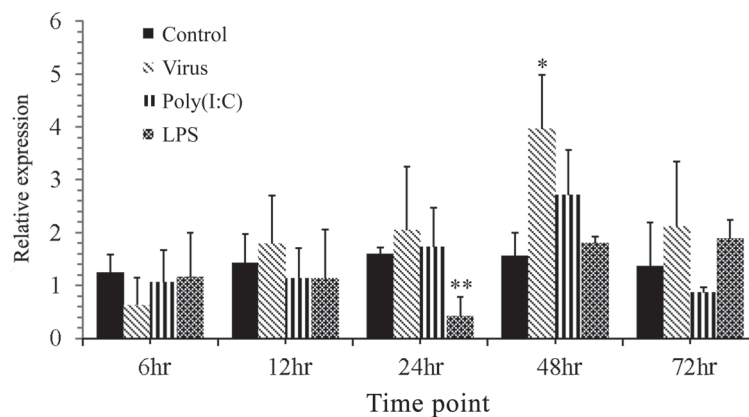


Fig. 5. Expression of *CagC3* mRNA in Gibel carp brain (GiCB) cells induced by CyHV-2, LPS or poly(I:C) challenge, determined by qRT-PCR at various time points. All data were normalized to β -actin. The data are expressed as means \pm SD (n=5) and were analyzed by calculating $2^{-\Delta\Delta CT}$. Significant differences (* $P < 0.05$; ** $P < 0.01$) in *CagC3* expression are relative to the expression in control group.

five-residue thioester domain (GCGEQ), which is thought to be critical in the formation, stability and function of this region [15], is highly conserved in CagC3. Moreover, previous study revealed that Pro¹⁰⁰⁷ and Pro¹⁰²⁰ in human C3 are necessary for stable thioester formation [10]. Our analysis indicated that Pro¹⁰⁰⁷ is conserved in CagC3, whereas Pro¹⁰²⁰ is replaced by Leu (Fig. 2). Similar results were also found in trout C3-1, C3-3 and C3-4 isoforms [43]. In addition, in human C3, residues His¹¹²⁶ and Glu¹¹²⁸ have been shown to be critical in determining the binding specificity of C3 to its target surface [15, 21]. These two residues are also conserved in Gibel carp, as His¹¹¹⁵ and Glu¹¹¹⁷, respectively (Fig. 2). In trout, the His residue is conserved in the C3-1 and C3-3 isoforms, whereas in C3-4 this residue is substituted by Thr. It has been suggested that differences in these residues account, at least in part, for the observed variations in the binding efficiencies of the various trout C3 isoforms [43].

Analysis of the CagC3 ANATO and C345C domains revealed that these domains both contain six canonical cysteine residues which are known to be significant for their functions [38]. The potential α/β processing signal RKRR is present in CagC3 (Fig. 2). Previous studies have suggested that 'RXXR' is the cleavage site where C3 proteins are processed into the two-subunit chain structure [25]. The C3-convertase cleavage site and the residues that align to the two cofactor I cleavage sites (Arg-Ser¹³⁰⁴, Arg-Ser¹³²¹ in the human C3 numbering) are also conserved in CagC3 (Arg-Ser¹²⁹² and Arg-Ser¹³⁰⁹). Similar residue conservation was also found in *Labeo rohita* C3 (LRC3) [27]. These sequence data suggest that CagC3 functions in a similar way to the C3 molecules of other animals. Analysis of the deduced amino acid sequence of CagC3, multiple sequence alignment and phylogenetic analysis showed that the structure of CagC3 is similar to counterparts in other vertebrates, with 91% identity with common carp C3-H1.

In vivo, C3 can constitutively express in tissues including liver, spleen, kidney, brain, muscle, heart, gill, intestine and skin. Generally, a great deal of C3 is present in liver, which has been suggested to be the main site of C3 production in mammals and fish [1, 2]. These results were consistent with the previous reports on other teleost fish, such as common carp [22], Gilthead seabream [18], miuiy croaker [34], orange-spotted grouper [28], rohu [27] and large yellow croaker [40]. Spleen and kidney are very important lymphoid organs in teleost fish, and serve similar roles to bone marrow and lymph nodes in mammals [33]. After infection of Gibel carp with CyHV-2, the expression of CagC3 in liver, spleen and kidney varied significantly, consistent with previous studies in miuiy croaker [34], rainbow trout [32] and common carp [30]. Interestingly, the expression in liver reached its peak at 24 hr post-challenge, which was earlier than in spleen and kidney. This may imply that response to virus stimulation occurs firstly in liver; the same result has been found in Atlantic halibut [13] and miuiy croaker [34]. Although the increase was lower than in liver, the expression levels of C3 in the kidney and spleen were significantly higher than the levels in the control group and reached their peak 2 d after infection of Gibel carp with CyHV-2. These results indicate that organs other than the liver also play an important role in the complement immune system.

In vitro, GiCB cells were challenged with CyHV-2, poly(I:C) and LPS, showing different CagC3 mRNA expression patterns. The RNA expression of CagC3 from GiCB stimulated with LPS fell to the minimum in the first 24 hr, then increased sharply; on the contrary, the RNA expression of CagC3 post CyHV-2 or poly(I:C) challenge increased for the first 2 d. These differences in the gene induction patterns indicate that the complement system was activated by distinct pathways. CyHV-2 is a DNA virus and poly(I:C) is a synthetic analog of double-stranded RNA, so we suggest that the Gibel carp complement system could be activated similarly by DNA virus infection and RNA, at least *in vitro*.

In all, the full-length cDNA of the complement component C3 of Gibel carp was cloned and sequenced for the first time. The deduced amino acid sequence of CagC3 showed typical structural properties similar to other teleosts. Although only one isoform of complement component C3 is identified in the present study, presence of more isoforms cannot be ruled out. CagC3 genes were expressed in various tissues with the highest level in liver. Significant upregulation of CagC3 expression was observed in liver, spleen, kidney and the GiCB cell line after infection with CyHV-2, suggesting that CagC3 might play an important role in the immune response to viral infections in Gibel carp. The results reported herein provide a basis for further studies of CagC3 in the innate response and disease defense, which may potentially be useful for the control of viral disease caused by CyHV-2.

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