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Short Sequence Report

Molecular characterization of complement factor I reveals constitutive expression in channel catfish

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ABSTRACT

The complement system in vertebrates plays a crucial role in immune defense via recognition and removal of pathogens. Complement is tightly regulated by a group of both soluble and cell-associated proteins. Complement factor I is a soluble serine protease that regulates multiple pathways in complement activation. In this work, a complement factor I transcript was isolated and sequenced from channel catfish (*Ictalurus punctatus*) liver after screening expressed sequence tags. The full-length cDNA is comprised of 2284 bp in length, encoding a polypeptide of 668 amino acids. The complement factor I protein was found to be well conserved, with similar domain structures and architecture from fish to mammals. The catfish complement factor I exists as a single-copied gene in the catfish genome. Expression analysis revealed that the catfish complement factor I is constitutively expressed in all tissues and leukocyte cell lines tested, indicating its importance as a regulatory enzyme throughout channel catfish. While expression of complement factor I is often found to be in the liver in mammals, it is constitutively expressed in channel catfish and carp throughout in various tissues and organs.

1. Introduction

The complement system in vertebrates plays a crucial role in innate immunity and serves as a bridge between the innate and adaptive immune systems [1,2]. The complement system comprises a group of soluble and membrane-bound proteins responsible not only for alerting the immune system of invading pathogens and altered host cells, but also for their removal [3]. Depending on the initial stimulus, complement can be initiated by one or more distinct yet overlapping pathways: the classical, lectin, and alternative. The classical pathway is antibody-mediated, while the lectin pathway is activated after recognition and binding of microbial carbohydrate motifs by host lectins. In contrast, the alternate pathway is continuously active and provides a first line of host defense. The alternative pathway can be stimulated by a wide array of foreign molecules [3,4].

While each complement pathway is distinguishable based on the nature of recognition and enzymatic cascade, all three pathways converge in order to target cells for destruction. Convergence occurs at the step of central component (C3) activation. Component C3, and also its homologous component C4 (classical and lectin pathways), are systematically processed to form activated complement fragments C3b and C4b, respectively. These fragments, through a series of enzymatic reactions, lead to the formation of C3 and C5 convertases. The covertases either create an amplification loop to increase C3b production used in part for opsonization of target surfaces (C3 convertase), or initiate the formation of membrane–attack complexes that are involved in cell lysis (C5 convertase). Thus, the various complement fragments are responsible for major molecular and cellular effector functions of the complement system including inflammatory responses, phagocytosis, lymphocyte stimulation, and cell lysis [3,5].

The complement system is highly effective in response to foreign pathogens, but can be self-destructive if exaggerated activation occurs. The host is equipped with specific inhibitors that tightly regulate complement cascades. One such inhibitor, complement factor I (*CFI*), regulates complement by proteolytic cleavage of components C3b and C4b in the presence of specific cofactors. Degradation of these components regulates C3b and prevents the formation of C3 and C5 convertases, along with further downstream effector functions [3,5]. Accordingly, the host immune response is tightly controlled in multiple complement pathways by the activity of *CFI*.

Complement factor I is a soluble serine protease comprising of 583 amino acids in length with a relative molecular weight of 88 kDa in humans [6]. *CFI* protein is a heterodimer with a heavy chain and a light chain linked by disulfide bonds. Both heavy and light chains are encoded by the same gene, and the protein is cleaved after translation to form the functional heterodimers. The heavy chain

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contains the non-catalytic domains of factor I membrane attack complex (FIMAC), Cys-rich scavenger receptor (SR), and two of low-density lipoprotein receptor class A (LDLa). The FIMAC domain has similar properties to extracellular matrix proteins, and may function in protein–protein interactions [7]. Based on gene ontology, SR domains are normally membrane-bound with pattern recognition/lipoprotein receptor activity, while LDLa domains are involved in lipoprotein binding. The light chain contains the catalytic trypsin-like serine protease (Tryp_SP) domain. The *CFI* heavy chain appears to be involved in substrate recognition specificity, while the light chain contains the proteolytic activity [7].

Channel catfish (*Ictalurus punctatus*) is not only the top aquaculture species in the United States (USDA-NASS, 2008), but also a research model for comparative immunology. Much has been learned about the catfish immune system, and it is one of the best characterized among teleosts. Multiple genes involved in catfish innate immunity have been characterized; recently for instance, the NOD-like receptors [8] and members of the lectin family [9,10]. In addition, catfish remains the only fish species where clonal functionally distinct lymphocyte cell lines have been established [11–13]. However, research on characterization of complement components has been limited (for a review see [3]) in catfish, and in teleosts in general.

CFI genes have been reported from human [6], mouse [14], rat [15], *Xenopus* [16], the cartilaginous houndshark [7] and recently the nurse shark [17], and finfish common carp [18], but it has not been identified from catfish. In the present study, a channel catfish *CFI* transcript (Ip*CFI*) was fully sequenced and characterized, and expression in various tissues and leukocyte cell lines was determined. Results indicate that *CFI* is constitutively expressed in a wide array of healthy catfish tissues and leukocyte cell lines. Current evidence supports that *CFI* genes are highly conserved in structures throughout vertebrate evolution [7], but their expression patterns are divergent in teleost fish.

2. Materials and methods

2.1. Isolation of complement factor I cDNA from channel catfish

All channel catfish (*I. punctatus*) expressed sequence tags (ESTs) available from the GenBank dbEST were clustered using the CAP3 program [19]. A contiguous sequence (contig) was identified by BLASTX at the NCBI to be homologous to a partial sequence of complement factor I. A forward and reverse oligonucleotide primer was designed to amplify this transcript for cloning and sequencing.

Total RNA was isolated from a channel catfish liver using the TRIzol reagent (Invitrogen, Carlsbad, CA). The total RNA (500 ng) was used as template to create cDNA by the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was directly amplified using a reaction mixture containing 1 μl of a 1:100 dilution template cDNA with 1.5 μl (25 mM) $MgCl_2$, 0.8 μl of (10 mM) dNTP mix, 1 μl each of (1 μM) forward and reverse primer, 1 μl of 10x PCR buffer, 0.25 μl of *Taq* (JumpStart *Taq* DNA polymerase, Sigma–Aldrich, St. Louis, MO), and nanopure water up to a total volume of 10 μl. The thermal cycling profile for cDNA amplification was as follows: an initial denaturation step of 2 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, with a final extension of 72 °C for 5 min. The PCR product was analyzed by electrophoresis on a 1.3% agarose gel. A single band was obtained; the amplicon was gel purified, and then cloned using the pGEM-T Easy Vector System (Promega, Madison, WI). Plasmid DNA from three positive clones was sequenced on an ABI 3130*XL* Genetic Analyzer (Applied Biosystems, Foster City, CA) from both ends using T7 and SP6 primers. Using these sequences, the complete *CFI* cDNA was then obtained using both a primer-walking strategy and Rapid Amplification of cDNA Ends (RACE; SMART RACE cDNA Amplification Kit, Clontech, Mountain View, CA) with the same liver total RNA as template. The completed *CFI* cDNA sequence was used for Basic Local Alignment Search Tool (BLAST) searches and other *in silico* analyses.

2.2. Southern blot analysis

To ascertain genomic copy number of the *CFI* gene in channel catfish, Southern blot analysis was performed as previously described [10,20]. Genomic DNA was isolated from whole blood of three individual channel catfish using standard protocols [21,22]. Briefly, DNA (10 μg) was digested with 10 U of the restriction endonucleases *Eco* RI, *Hin*d III or *Pst* I (New England Biolabs, Beverly, MA). The digested DNA was electrophoresed on a 1% agarose gel along with a lambda DNA-*Hin*d III digested molecular weight ladder (New England Biolabs). The DNA was transferred to a positively-charged nylon membrane (Millipore, Bedford, MA) overnight by downward capillary transfer with 20x SSC buffer. The DNA was fixed to the membrane by the auto-crosslink feature on a UV crosslinker (Stratagene, La Jolla, CA). The membrane was pre-hybridized for 2 h with pre-hybridization solution containing Atlantic salmon sperm DNA [22], and then hybridized with an α-^{32}P dCTP labeled probe specific for *CFI* at 65 °C overnight. The membrane was washed twice (2x SSC, 0.1% SDS) and exposed to X-ray film overnight at −80 °C. The *CFI* gene-specific probe fragment was generated by PCR using forward (5′-TATCAGTGTCCACGGATCAG-3′) and reverse (5′-ATCTAGGGCGAGTCTCACTG-3′) primers. The probe was labeled by using the Random Primed DNA Labeling Kit (Roche Applied Science, Indianapolis, IN).

2.3. In silico characterization of channel catfish CFI cDNA

To fully characterize the Ip*CFI*, several molecular models were built including architecture of conserved domains, a phylogenetic tree, and a multiple sequence alignment. *CFI* sequences from various species were gathered from GenBank and used in the analyses (Table 1). The putative channel catfish *CFI* protein sequence was determined using the ExPASy web server and the Translate tool (http://www.expasy.ch/tools/dna.html). Conserved domain structures from channel catfish and other species were determined using the SMART domain program [23] or the Conserved Domain Database (CDD) at the NCBI. Next, a phylogenetic tree was constructed using the MEGA 4.0 software [24]. The protein sequences were aligned by ClustalW and then an initial neighbour-joining tree was created. Using this tree as a guide, a consensus minimum evolution tree based on 10,000 bootstrap replicates was built. Settings included total deletion of gaps and/or missing data, with the Poisson correction method enforced. Finally, a multiple sequence alignment was created by ClustalW using the Ip*CFI* putative protein translation and *CFI* protein sequences from various species (Table 1). Conserved features from this alignment were labeled accordingly.

2.4. Gene expression analysis of channel catfish CFI

To assess the level of gene expression of Ip*CFI*, reverse-transcription polymerase chain reactions (RT-PCR) were performed using total RNA from various channel catfish tissues and leukocyte cell lines. Each cDNA template (1 μl) was used directly in RT-PCR, along with gene-specific oligonucleotides (1 μM) designed from the Ip*CFI* sequence. Catfish *CFI*-specific forward (5′-GCACCTATCAGAACGACA TCGC-3′) and reverse (5′-CCCATCCAGAAATGGTGCAGGT-3′) primers were designed using the FastPCR software [25]. Thermal cycling conditions for each RT-PCR reaction included an initial denaturing step of 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, with a final extension of 72 °C for 5 min. Catfish β-actin forward (5′-AGAGAGAAATTGTCCGTGACATC-3′) and

Table 1
List of genes used for comparative genetic analysis. Sequences and accession numbers were obtained from GenBank. The domains were identified and annotated by Simple Modular Architecture Research Tool (SMART) or the Conserved Domain Database (CDD) at the NCBI. Complete domain descriptions are discussed in the text. *E*-values were determined using BLASTX with channel catfish *CFI* as query. *CFI*: complement factor I.

Species	Protein	Accession	E-value	Domains (N to C terminus)
Bos taurus	*CFI*	NP_001033185.1	7e-127	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Canis lupus familiaris	*CFI* isoform 2	XP_535694.1	3e-126	FIMAC-SR-LDLa-LDLa-Tryp_SPc
	CFI isoform 3	XP_863506.1	7e-126	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Cyprinus carpio	*CFI* isoform A	BAB88920.1	7e-177	FIMAC-SR-LDLa-LDLa-Tryp_SPc
	CFI isoform B	BAB88921.1	0	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Gallus gallus	*CFI*	XP_426329.2	9e-121	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Homo sapiens	*CFI* preprotein	NP_000195.2	1e-126	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Monodelphis domestica	*CFI*	XP_001362753.1	7e-124	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Mus musculus	*CFI*	NP_031712.2	9e-129	FIMAC-SR-LDLa-LDLa-Tryp_SPc
	Epithin	AAD02230.3		
Pan troglodytes	*CFI*	XP_526653.2	8e-127	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Pongo pygmaeus	*CFI*	Q5R5A4	9e-126	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Rattus norvegicus	*CFI*	NP_077071.1	2e-126	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Triakis scyllium	*CFI*	BAC01864.1	8e-115	FIMAC-SR-LDLa-LDLa-Tryp_SPc
Xenopus laevis	*CFI*	NP_001095259.1	4e-129	FIMAC-SR-LDLa-LDLa-Tryp_SPc

reverse (5′-CTCCGATCCAGACAGAGTATTTG-3′) primers were also included in each reaction as an internal control. Amplification products were analyzed by electrophoresis on a 1.3% agarose gel. Tested tissues included liver, trunk kidney, heart, stomach, gill, brain, intestine, muscle, skin, ovary, head kidney, and spleen, and tested cell lines included cytotoxic T-cell line TS32.15, cytotoxic T-cell line TS32.17, T-cell line 28.3, macrophage cell line 42 TA, B-cell line 3B11, and B-cell line 1G8.

In order to provide an assessment of the relative expression of *CFI* in various channel catfish tissues, real-time quantitative RT-PCR (qRT-PCR) was performed on the *CFI* transcript. Total RNA isolated from channel catfish ($N = 4$) tissues was pooled, adjusted to 100 ng, and used as template. One-step qRT-PCR was carried out using a LightCycler 1.0 (Roche Applied Science) and components from the Fast Start RNA Master SYBR Green I kit (Roche Applied Science), with modifications as we previously described [8]. Two separate qRT-PCR reactions were performed on 12 channel catfish tissues: (i) *CFI*, using gene-specific primers listed above for RT-PCR and (ii) 18S rRNA, using primers described elsewhere [10]. Specificity of amplification products was assessed by melting curve analysis. Standard curves were generated for each reaction by using serial dilutions (ng) of macrophage cell line 42 TA total RNA. Relative concentrations of each sample were determined. *CFI* expression was normalized with 18S rRNA expression. The spleen RNA sample was arbitrarily chosen as the calibrator (1X). Relative abundance of *CFI* was calculated for the remaining tissues by ratio with the calibrator.

3. Results and discussion

3.1. Sequence analysis of channel catfish CFI

The complete sequence of channel catfish *CFI* cDNA was obtained. The sequence was deposited into GenBank with the accession number GQ149234. The 5′ untranslated region (UTR) is 42 bp long. The Ip*CFI* open reading frame encodes a polypeptide of 668 amino acids. The 3′ UTR consists of 215 bp, with a poly-A signal (ATTAAA) 20 bp upstream of the poly-A tail. The *CFI* genes are highly conserved among all the species from which it has been characterized (Table 1). Although *CFI* has yet to be fully characterized in teleosts other than carp, analysis using BLAST suggests orthologues in other fish species likely exist [18]. BLASTX searches of Ip*CFI* to the *nr* database at the NCBI identified the protein LOC557557 in *Danio rerio* (GenBank ID: AAI29472) and an unnamed protein product in *Tetraodon nigroviridis*

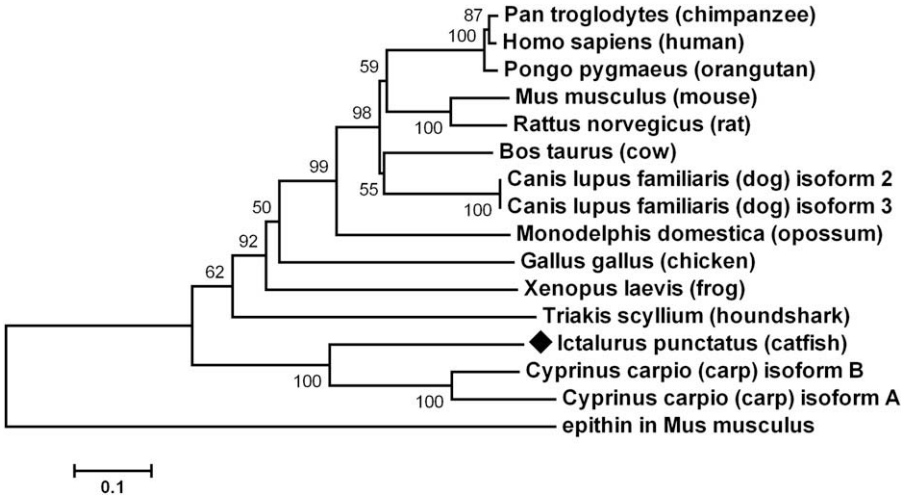

Fig. 1. Phylogenetic tree constructed using full-length polypeptides of complement factor I. The details of the sequences used for the construction of the phylogenetic tree are provided in Table 1. The position of the channel catfish complement factor I is indicated by a diamond. The *Mus musculus* epithin protein was used as an out-group [7,18]. The scale represents amino acid substitutions.

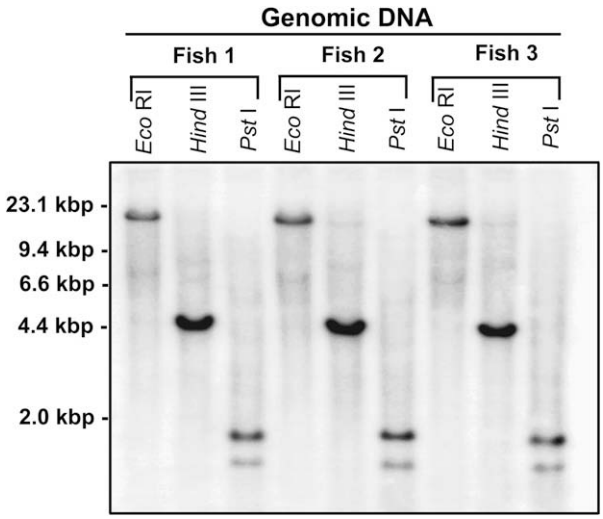

Fig. 2. Southern blot analysis of channel catfish complement factor I. The DNA was from three individual fish, fish 1, fish 2, and fish 3, and the DNA was digested with *Eco* RI, *Hin*d III, and *Pst* I. The molecular weight standards (in kbp) are indicated on the left margin.

(GenBank ID: CAF89951) as highly related. Similarly, an unnamed protein product (scaffold_2313, v.4) was identified by BLASTX search to the *Takifugu rubripes* genome as related to Ip*CFI*. Further investigation as to the degree in which *CFI* is found in various other teleosts would be warranted based on these findings.

As shown in Table 1, Ip*CFI* contained conserved domain structures and architecture, with a signal peptide at the N-terminus, followed by FIMAC, SR, two LDLa, and Tryp_SPc. This provides further evidence of the high structural conservation of this gene throughout vertebrate evolution [7], as all *CFI*s examined from selected species exhibit the same structural organization and domains based on amino acid sequence homology (Table 1). High conservation of *CFI* domains and architecture is also indicated by multiple sequence alignment (Supplemental Fig. 1). In the Tryp_SPc domain, all three amino acids that comprise the catalytic triad, His^{466}-Asp^{513}-Ser^{610} residues (Ip*CFI* numbering), as well as the Asp^{604} residue in the bottom of the specificity pocket were identical by composition and position in all species tested. A large insert of amino acids between the signal peptide and the FIMAC domain from the carp *CFI* isoform B [18] is clearly observed, while channel catfish and other species tested have much less of an insert in this region. The FIMAC domain is Cys-rich; all 10 Cys residues described elsewhere [7] were identified in all species. The exception is the position of Cys residue 10 (Cys^{162}) from Supplemental Fig. 1. The position of this Cys is shifted two positions toward the C-terminus in channel catfish and *Xenopus CFI*. While all 10 Cys resides in the FIMAC domain were found to be present, the position of the C-terminal Cys (Cys^{162}) residue may be flexible, as also shown elsewhere [18]. *CFI* translates both a heavy and light chain and, during post-translational modification, the polypeptide is cleaved at a tetrapeptide processing site in order to form the native protein. The tetrapeptide site is conserved in most species, with a single amino acid difference observed in channel catfish, houndshark, and *Xenopus CFI* (Supplemental Fig. 1). Based on pairwise alignment scores, Ip*CFI* exhibits the highest identity scores of 53% and 50% with carp isoform A and isoform B, respectively, and lowest identity with chicken (36%) and houndshark (35%) (data not shown). Interestingly, the channel catfish *CFI* shows lowest identity score with the houndshark. For further analysis, ClustalW pairwise identity scores were computed using putative proteins from the very recently characterized nurse shark *CFI* isoforms [17]. The catfish *CFI* exhibits identity scores of 34%, 34%, 33%, and 34% with nurse shark *CFI* isoforms 1-4, respectively. Taken together, these data could suggest recent evolution of *CFI* in shark [17] and further, recent divergence of *CFI* between cartilaginous and teleost fish species.

Phylogenetic analysis placed Ip*CFI* in the same cluster with the other teleost *CFI*s, with high bootstrap support (Fig. 1). The mammalian *CFI* proteins also form a distinct clade, with high support, with the lone exception of the opossum *CFI*. Interestingly, the opossum *CFI* protein branches from the mammalian clade, with high support, even though *CFI* proteins share such a high degree of homology. This result could be expected, as the recently sequenced opossum genome [26] is placed at a midpoint between eutherian mammal and non-mammalian vertebrate evolution [27]. While the evolutionary relationships observed with *CFI* proteins appear to be consistent [7,18], further studies inclusive of other *CFI* proteins would assist in the creation of a more comprehensive phylogeny.

3.2. *Copy number of channel catfish* CFI

Genomic copy number of the channel catfish *CFI* gene was determined by Southern blot analysis. Catfish possess a single copy of the *CFI* gene in the genome. As shown in Fig. 2, a single band was identified through hybridization of a *CFI* probe to genomic DNA digested with *Eco* RI and *Hin*d III restriction endonucleases. Two bands were observed with *Pst* I, but an internal *Pst* I site existed within the *CFI* probe. Single copy *CFI* genes were also found in houndshark [7] and humans [6,28]. In common carp [18], two transcripts of *CFI* (isoforms A and B) were identified with evidence that they were expressed from distinct genes. However, common carp is regarded to harbor a tetraploid genome, while channel catfish and humans are diploid organisms.

3.3. *Expression of channel catfish CFI*

CFI transcripts were detected in all cell lines and tissues tested (Fig. 3), suggesting that *CFI* transcripts are constitutively expressed in channel catfish. Further, *CFI* expression was quantified to determine relative amounts present in each tissue (Fig. 4). The highest source of *CFI* expression was found to be in the liver. High levels of relative expression were also observed in spleen, gill, heart, and skin tissues. Lower levels of expression were observed in trunk kidney, ovary, and head kidney tissues (Fig. 4). Results from both RT-PCR (Fig. 3) and qRT-PCR (Fig. 4) indicate that *CFI* expression in channel catfish is ubiquitous. *CFI* plays a critical role as an immune protein, as *CFI* can regulate multiple complement pathways by specific cleavage of C3b and C4b. *CFI*, in turn, protects the host from

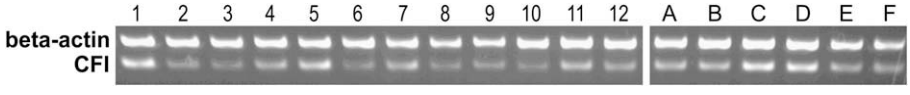

Fig. 3. Analysis of channel catfish complement factor I mRNA in various tissues and leukocyte cell lines with semi-quantitative RT-PCR. Tissues include: 1, Liver; 2, Trunk kidney; 3, Heart; 4, Stomach; 5, Gill; 6, Brain; 7, Intestine; 8, Muscle; 9, Skin; 10, Ovary; 11, Head kidney; and 12, Spleen. Cell lines include: A, cytotoxic T-cell line TS32.15; B, cytotoxic T-cell line TS32.17; C, T-cell line 28.3; D, macrophage cell line 42 TA; E, B-cell line 3B11; and F, B-cell line 1G8. The position of the complement factor I amplicon is marked as CFI. Beta-actin mRNA was used as an internal control.

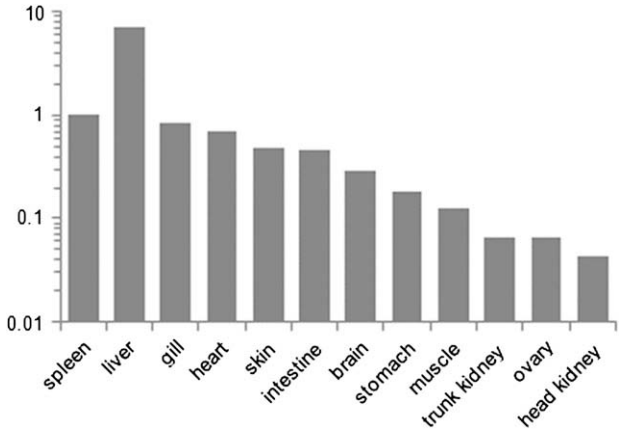

Fig. 4. Relative expression of complement factor I in channel catfish determined using qRT-PCR. The *Y*-axis represents normalized relative expression values ($\log_{10}$) of complement factor I. Tissue RNA samples are labeled along the *X*-axis. Expression levels in all tissues are presented relative to that in the spleen tissue (1X).

hyper-activation of the complement system that could lead to damage of self-tissues [29]. The degree in which *CFI* expression was identified here reveals the importance of *CFI* to be immediately available at the active site in catfish. Therefore, local (tissues) and peripheral (circulating cells) *CFI* synthesis may be crucial to the teleost immune system. This reasoning is especially valid given that bony fish complement could recognize a much wider range of foreign substances than its mammalian counterpart [3,29–35], whereby expanded activation would require closer regulation.

Previous reports have indicated variation as to the extent of *CFI* expression in peripheral blood cells and different tissues. In mammals, *CFI* expression in human dendritic cells was positive [36], while *CFI* expression in other peripheral blood cells from human and rat have varied [15,37–40]. Results from RT-PCR analysis of channel catfish B-cell, T-cell, cytotoxic T-cell, and macrophage cell lines clearly indicate *CFI* expression in these leukocytes (Fig. 3). Differences in *CFI* tissue preference have also been observed. In mammals, the predominant source of *CFI* biosynthesis and secretion appears to be by the liver [15,41], and *CFI* was found to be only transcribed by liver in the houndshark [7]. However, in the other teleost species where *CFI* expression was investigated, carp displays a wide array of tissue distribution [18], similar to channel catfish (Figs. 3 and 4). Carp *CFI* isoform B was tested for expression in the renal kidney, head kidney, hepatopancreas, heart, and spleen, and these tissues were all positive for *CFI* expression. Further, carp *CFI* isoform A was shown to be expressed in the hepatopancreas and ovary, indicating tissue preferences among the different carp *CFI* isoforms. Gonzalez et al. [42] also indicated expression of carp *CFI* in skin, blood, and liver. The results from carp corroborate a pattern of constitutive expression of *CFI* in multiple tissues as observed with channel catfish (Figs. 3 and 4). Evidence from the current and previous studies [18,42] indicates that, while structural conservation of *CFI* is marked throughout vertebrate evolution [7], its expression patterns appear to have evolved differently between cartilaginous and ray-finned fish. Clearly, there remains great interest in investigation of additional *CFI* genes to observe whether constitutive expression in multiple tissues holds true for other bony fish.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsi.2009.06.007.

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