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Molecular cloning, characterization and expression analysis of TLR9, MyD88 and TRAF6 genes in common carp (*Cyprinus carpio*)

Pawapol Kongchum [a,b], Eric M. Hallerman [a], Gideon Hulata [c], Lior David [d], Yniv Palti [b,*]

[a] Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
[b] National Center for Cool and Cold Water Aquaculture, USDA-ARS, 11861 Leetown Road, Kearneysville, WV 25430, USA
[c] Institute of Animal Science, Agricultural Research Organization, Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel
[d] Department of Animal Sciences, R.H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel

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ABSTRACT

Induction of innate immune pathways is critical for early host defense, but there is limited understanding of how teleost fishes recognize pathogen molecules and activate these pathways. In mammals, cells of the innate immune system detect pathogenic molecular structures using pattern recognition receptors (PRRs). TLR9 functions as a PRR that recognizes CpG motifs in bacterial and viral DNA and requires adaptor molecules MyD88 and TRAF6 for signal transduction. Here we report full-length cDNA isolation, structural characterization and tissue mRNA expression analysis of the common carp (cc) TLR9, MyD88 and TRAF6 gene orthologs. The ccTLR9 open-reading frame (ORF) is predicted to encode a 1064-amino acid (aa) protein. We found that MyD88 and TRAF6 genes are duplicated in common carp. This is the first report of TRAF6 duplication in a vertebrate genome and stronger evidence in support of MyD88 duplication is provided. The ccMyD88a and b ORFs are predicted to encode 288-aa and 284-aa peptides, respectively. They share 91% aa sequence identity between paralogs. The ccTRAF6a and b ORFs are both predicted to encode 543-aa peptides sharing 95% aa sequence identity between paralogs. The ccTLR9 gene is contained in a single large exon. The ccMyD88a and ccMyD88b coding sequences span five exons. The TRAF6b gene spans six exons. PCR amplification to obtain the entire coding sequence of ccTRAF6a gene was not successful. The 2104-bp fragment amplified covers the 3′ end of the gene and it contains a partial sequence of one exon and three complete exons. The predicated protein domains of the ccTLR9, ccMyD88 and ccTRAF6 are conserved and resemble orthologs from other vertebrates. Real-time quantitative PCR assays of the ccTLR9, MyD88a and b, and TRAF6a and b gene transcripts in healthy common carp indicated that mRNA expression varied between tissues. Differential expression of duplicate copies were found for ccMyD88 and ccTRAF6 in white and red muscle tissues, suggesting that paralogs may have evolved and attained a new function. The genomic information we describe in this paper provides evidence of sequence and structural conservation of immune response genes in common carp.

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1. Introduction

Cells of innate immune systems rely on a set of pattern recognition receptors (PRRs) which have a general ability to detect certain molecular structures present in pathogens [1]. Toll-like receptors (TLRs) function as PRRs that recognize conserved molecular structures broadly shared by microbes known as pathogen-associated molecular patterns (PAMPs) and trigger the signaling pathways that activate immune cells in response to pathogen infection. Among several TLRs that have been identified, TLR9 was characterized as the receptor that recognized unmethylated CpG dinucleotides in DNA, which are commonly found in bacterial and viral genomes, and subsequently activated cellular immune responses [2–5] Synthetic CpG oligodeoxynucleotides, a potent ligand for TLR9, have been proven to have an immunostimulatory effect on immune cells. Studies in fishes including common carp [6,7], two flounders [8,9], rainbow trout [10] and Atlantic salmon [11,12] demonstrated that CpG stimulation activated antibacterial and antiviral immune responses. Häcker et al. [13] showed that in mammals, recognition of CpG-DNA activated the toll-like receptor/interleukin-1 receptor (TLR/IL-1R) signaling pathways via myeloid differentiation marker 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6).

MyD88 is a cytoplasmic toll/interleukin-1 receptor (TIR) domain-containing adaptor molecule involved in the signaling through the

* Corresponding author. Tel.: +1 304 724 8340x2134; fax: +1 304 725 0351.
E-mail address: yniv.palti@ars.usda.gov (Y. Palti).

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IL-1R and TLR families [2]. This molecule is used by all TLRs, except TLR3, and activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines [14,15]. MyD88 interacts with TLRs through TIR domain-TIR domain interactions and uses its death domain to interact with the death domain-containing protein IL-1R-associated protein kinase (IRAK). Besides its function as an adaptor molecule in TLR/IL-1R signaling, MyD88 also has an important role in the IFN-γ signaling pathway in mammals, where in macrophages it bridges the cytoplasmic domain of IFN-γ receptor 1 and mixed-lineage kinase 3 (MLK3) in a pathway that through the activation of p38 regulates mRNA stability of cytokines such as tumor necrosis factors and IFN-γ inducible protein 10 (IP-10) [16].

TRAF6 is a cytoplasmic adaptor protein of the TRAF family that mediates signals induced by the tumor necrosis factor receptor (TNFR) superfamily and the interleukin-1 receptor (IL-1R) [17,18]. It is as an important intermediate of CpG-DNA-induced signal transduction through the TLR/IL-1R – MyD88 pathway which leads to the activation of the IKK complex and JNK kinases [13]. A study of TRAF6 function in mice [19] suggested that TRAF6 is a critical molecule that regulates the processes required for maturation, activation, and development of dendritic cells. In addition, the human TRAF6 was shown to be involved in the stimulation of apoptosis [20].

The ability of TLR9 to detect viral DNA and the important roles that MyD88 and TRAF6 play in the TLR9 signal transduction make them likely candidates for involvement in the host antiviral response. In teleosts, full-length cDNA sequences encoding TLR9, MyD88 and TRAF6 molecules have been isolated [21–28]; however, these gene transcripts have not been reported in common carp. In our previous work [29], we cloned and sequenced genomic fragments of several immune response genes from common carp including TLR9, TRAF6 and two paralogous MyD88 genes. In this paper, we describe the isolation of full-length cDNA, genomic organization, phylogenetic relationship and tissue-specific mRNA expression distributions of the common carp TLR9, MyD88 and TRAF6. This information will expand our knowledge of the structure and evolution of antiviral immune response genes in teleosts and vertebrates. The gene sequence data will also facilitate our on-going research project to develop molecular tools for selective breeding of common carp lines that are resistant to cyprinid herpesvirus-3 (CyHV-3), a causative agent of a lethal disease in common carp and koi.

2. Materials and methods

2.1. Experimental fish, tissue collection and RNA extraction

Koi carp (approximately 100 g body weight) purchased from a local pet store were used for gene isolation. Adult common carp (400g–500 g body weight) obtained from the E. W. Shell Fisheries Center, Auburn University, AL were used for tissue expression studies. Different tissues/organs were dissected from anaesthetized fish and immediately frozen in liquid nitrogen and stored at −80 °C until use. Tissue sampling was conducted in concordance with the National Center for Cool and Cold Water Aquaculture Institutional Animal Care and Use Committee protocol number 043. Total RNA was extracted using TRI Reagent (Sigma–Aldrich) and treated with RQ1 RNase-Free DNase (Promega) following the manufacturer's instructions. The concentration of the total RNA was quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and its integrity was visualized on an agarose gel.

2.2. Full-length cDNA isolation

The full-length cDNA sequences of the common carp TLR9, MyD88a, Myd88b, TRAF6a and TRAF6b were obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturers' instructions. First strand cDNA was generated from 1 μg total RNA extracted from kidney and spleen. The 3′-RACE cDNA was synthesized using 3′-RACE CDS Primer A (5′-AAGCAGTGGTATCAACGCA GAGTAC$(T)_{30}$VN-3′). For 5′-RACE, cDNA was synthesized using 5′-RACE CDS Primer A (5′-$(T)_{25}$VN-3′) and SMART II A Oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′). The 3′- and 5′-RACE-ready cDNAs were then used as template for cDNA fragment amplification by PCR using gene-specific primers and Universal Primer A Mix (0.4 μM long oligonucleotide, 5′-CTA ATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and 2 μM short oligonucleotide, 5′-CTAATACGACTCACTATAGGGC-3′). Gene-specific primers used for the amplification of RACE cDNA fragments (Table S1) were designed based on common carp genomic DNA sequences previously deposited in GenBank (accession numbers GU321985, GU321986, GU321987 and GU064560) using FastPCR software (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). PCR amplifications were performed using a hot-lid thermocycler with the following thermal cycling profiles: 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min for 30 cycles, followed by a final extension of 72 °C for 10 min. Nested PCR was performed with a gene-specific primer and a nested universal primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′) to obtain specific PCR product. PCR amplicons were then cloned into a plasmid vector for nucleotide sequencing as previously described [29]. Briefly, PCR amplicons were cloned into the pCR2.1-TOPO vector and transformed into competent *E. coli* (TOPO TA Cloning Kit, Invitrogen). Transformants were spread on a Luria–Bertani (LB) agar plate containing X-gal and 100 μg/ml ampicillin. Insert-positive colonies were grown in LB broth with 100 μg/ml of ampicillin followed by Magnificent Broth (MB) with 50 μg/ml of kanamycin, and the plasmid DNA was isolated and sequenced.

2.3. Characterization of intronic regions

Primer pairs covering entire open-reading frames of the common carp TLR9, MyD88 and TRAF6 genes were used to amplify gene fragments from genomic DNA. The primers used are listed in Table S2. PCR was carried out in an 11-μl reaction volume consisting of 10 μl reaction mixture (1× PCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM each of forward and reverse primers, 0.5 U AmpliTaq Gold (Applied Biosystems) DNA polymerase), and 1 μl (~20 ng) gDNA template. PCR amplifications were performed as follows: 1 cycle of 94 °C for 10 min, then 35 cycles of 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min, followed by 1 cycle of 72 °C for 7 min. PCR products were cloned and sequenced as described above. The genomic nucleotide sequences were compared to cDNA sequences to determine the exon–intron boundaries of the genes.

2.4. Sequence analyses

Sequence assemblies were carried out using Sequencher software (Gene Codes Corporation). Nucleotide sequences were translated to amino acid sequences by the ExPASy Translate tool (http://www.expasy.ch/tools/dna.html). The predicted amino acid sequences were blasted against the NCBI database using BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sequence identities and similarities. The identification and annotation of protein domains were performed using the web-based SMART program (http://smart.embl-heidelberg.de/). The presence of a signal peptide was analyzed using the web-based SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Phylogenetic analyses were conducted on amino acid and DNA sequences using MEGA 3.1 software [30]. Multiple alignments were generated with ClustalW implemented in