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Identification, characterization and the interaction of Tollip and IRAK-1 in grass carp (*Ctenopharyngodon idellus*)

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ABSTRACT

Tollip and IRAK-1 are key components of the TLR/IL-1R signaling pathway in mammals, which play crucial roles as mediators of the TLR/IL-1R signal transduction pathways. Although several TLRs have been found in fish, molecular associations, protein–protein interactions or the role of the TLR signaling pathway in infection-induced immunity in fish has received little attention. In this study, Tollip and IRAK-1 sequences of grass carp were isolated from a head kidney cDNA library. Full length transcripts and sequences of promoter regions were obtained by 3′ and 5′ RACE and genome walking, respectively. Reporter gene-promoter constructs and real-time RT-PCR analysis was used to determine grass carp Tollip and IRAK-1 transcription pattern in tissues. Recombinant proteins were used for antibodies production. Phylogenetically, the grass carp loci clustered with previously reported Tollip and IRAK-1genes, respectively, and their sequences shared the highest identity with the genes of zebrafish (*Danio rerio*). The promoter region of grass carp Tollip and IRAK-1 proved to be active. After viral infection transcript levels of both loci were upregulated in most immune-related tissues in a time-dependent manner. Using antibodies produced in this study, immunofluorescence analysis indicated that Tollip and IRAK-1 were uniformly distributed and co-localized in the cytoplasm of CIK cells. After viral infection, however, Tollip and IRAK-1 both trended toward the cell membrane. Our results demonstrate the existence of Tollip and IRAK-1 proteins in teleost species, and suggest that Tollip-IRAK-1 complexes are being recruited to receptor complexes after stimulation with virus. These results provide novel insights into the role of the TLR signaling pathway in teleosts, especially the action of teleost Tollip and IRAK-1 and the interaction of these molecules as part of this pathway.

1. Introduction

Innate immune responses from *Drosophila* to *Man* utilize remarkably conserved molecular components, many of which contain homologous protein–protein interaction domains [1,2] such as the Toll–IL-1 receptor (TIR) domain and the death domain (DD). Toll-like receptors (TLRs), interleukin-1 receptor (IL-1R), and interleukin-18 receptor (IL-18R) are single-transmembrane proteins with conserved cytoplasmic TIR domains, which facilitate the activation of a similar high molecular mass complex when stimulated with their cognate ligands [3]. Toll-interacting protein (Tollip) and IL-1 receptor-associated kinase 1 (IRAK-1) may be incorporated into this high molecular mass complex [4]. It has been shown that in untreated cells, Tollip forms a complex with IRAK-1. Recruitment of Tollip-IRAK-1 complexes to activated receptor complex occurs through association of Tollip with cytoplasmic TIR domain. Co-recruited MyD88 then triggers IRAK-1 autophosphorylation, which in turn leads to the dissociation of IRAK-1 from Tollip (and TLRs, IL-1Rs, IL-18Rs) [4], and its association with TNF receptor-associated factor 6 (TRAF-6) to signal activation of either NF-κB or mitogen-activated protein kinases [5].

The Tollip gene was first described in the mouse, human and *Caenorhabditis elegans* [5], while the IRAK-1 gene was first found in humans [6]. To date, Tollip and IRAK-1 protein sequences have been isolated from mammals, birds, nematodes and fish. In mammals, Tollip has been shown to be involved in two main functions. The first is that Tollip was found to play a role in the activated IL-1 receptor complex, suggesting that it was an integral part of the IL-1RI signaling cascade [5]. Later, Tollip was shown to associate with TLR2 and TLR4 receptors as well, which triggers suppression of TLR-mediated cellular responses [4]. In 2004, the role of Tollip in negative regulation of the IL-1β and TNF-α signaling pathway was

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proposed [7]. The second function concerns the role of Tollip in protein sorting through interaction with target of Myb1 (Tom1), ubiquitin and clathrin [8,9]. An endosomal function of the protein was first suggested by Katoh et al. [10,11]. In addition, Ciarrocchi and D'Angelo [12] have shown the involvement of Tollip in the sumoylation process, and in the control of both nuclear and cytoplasmic protein trafficking.

IRAKs play crucial roles as mediators in the TLR/IL-1R signal transduction pathways [13]. IRAKs constitute a protein family with four members, IRAK-1, IRAK-2, IRAK-3/M, and IRAK-4. Lin et al. (2010) reported the crystal structure of the MyD88/IRAK-4/IRAK-2 death domain (DD) complex, which surprisingly reveals a left-handed helical oligomer that consists of 6 MyD88, 4 IRAK-4 and 4 IRAK-2 DDs. Assembly of this helical signalling tower is hierarchical [14]. IRAK-1 has been identified as a key component of the IL-1R signaling pathway in mammals [6]. Upon ligand binding to TLRs or IL-1R, IRAK-1 is recruited to the receptor complex to activate the downstream signaling pathway leading to proinflammatory responses [15]. Furthermore, IRAK-1 is involved in activation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3), STAT1 and interferon regulation factor 7 (IRF7), as well as in downstream gene expression [13,16,17]. Phosphorylated IRAK-1 also undergoes ubiquitin-mediated degradation or sumoylation resulting in nuclear translocation and transcriptional activation of inflammatory target genes [18]. In 2000, Burns et al. [5] provided evidence that Tollip forms a complex with IRAK-1 in resting cells using yeast two–hybrid interaction assays and by co-immunoprecipitation from 293T cells.

Based on molecular structure and phylogenetic analysis, Rebl et al. identified two closely related Tollip-encoding genes in Atlantic salmon (*Salmo salar*) and the respective orthologous mRNA molecules in rainbow trout (*Oncorhynchus mykiss*) [19]. Subsequently, an IRAK-1 cDNA sequence named ScIRAK-1 was also identified in mandarin fish [20,21]. Although several TLRs [22–28] have been found in fish, the role of the TLR signaling pathway, the molecular associations and protein–protein interactions in infection-induced immunity in fish has received little attention.

In the study reported here, we therefore characterized Tollip and IRAK-1 genes of grass carp and compared their amino sequence with other known Tollip and IRAK-1 proteins. We analyzed their expression at the transcript and protein level in virus infected grass carp and in control and viral infected CIK (*Ctenopharyngodon idellus* kidney) cells. The aim of our study was to provide further insight into the genetic basis and protein–protein interactions participating in the TLR signaling pathway in response to viral infection in teleost fish. The investigation of molecular associations and interactions in this study provide novel insights into the role of the TLR signaling pathway, especially with regards to teleost host responses to infection.

2. Materials and methods

2.1. Cell culture and animals

CIK cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (MEM-10) at 28 °C as described previously [29]. Grass carp were supplied by the Guanqiao farm in Wuhan, China.

2.2. Virus and viral infection of grass carp

The Grass carp reovirus (GCRV) 873 strain was generously provided by Prof. Q. Fang of the Wuhan Institute of Virology, Chinese Academy of Science [30]. The virus titers, given as tissue culture infective dose ($TCID_{50}$/0.1 ml), were calculated by the method of Reed and Muench [31]. Nine-month-old grass carp with an average weight of 126 g were raised in clean tanks at 28 °C. Sixty grass carp were randomly divided into two groups, one control group and a GCRV-infected group. In the GCRV-infection group, each fish was intraperitoneally injected with 150–200 μl GCRV for a dose of approximately 10^7 $TCID_{50}$ kg^{-1} body weight. Fish in the control group were injected with the same amount of saline. The various tissue samples of control (1 d after injection, control grass carp, $n = 3$) and virus-infected grass carp (at 2 d, 3 d, 4 d, 5 d, 7 d post-infection, infected grass carp, each time point $n = 3$) were immediately removed and frozen in liquid nitrogen, or stored at −80 °C until RNA isolation.

2.3. Screening of cDNA libraries, 3′ and 5′ RACE

The full cDNA sequence of Tollip was isolated from a head kidney cDNA library of grass carp. Through PCR amplification using primers shown in Table 1 (IRAF-F1/IRAK-R1) designed from conserve regions of known IRAK-1 genes, one cDNA fragment of 1051 bp was found to be homologous to known IRAK-1 genes. The

Table 1
Primers used for cloning and expression studies.

Primer	Sequence (5′-3′)	Application
IRAF-F1	ATCAGATCAGACTGAACTACGCCTT	
IRAK-R1	CTCTAGCATGACCACTCCAAAACT	
IRAF	ATCGCCCTCTCTTGGTTACAGCG	3′ RACE
IRAR	TGAGGATGTCTGTGCGTCTGGG	5′ RACE
GT-R1	CTTACCTGTCCCCGTTGCGTGCT	Genomic walking (first round PCR)
GT-R2	GTTGCGTGCTAATTGTTGTTGCC	Genomic walking (second round PCR)
GI-R1	TAATGGCGAAGAATGCCTGGCTC	Genomic walking (first round PCR)
GI-R2	TCATAAGCCACTGCTACACCGC	Genomic walking (second round PCR)
ETo-F1	GGTCGCCTCAGCATCACTGTA	RT-PCR primer used in expression study
ETo-R1	CATAGACGGCATACCCAAGTCG	RT-PCR primer used in expression study
EIR-F1	ATACAGGCATCCCAACATAATGG	RT-PCR primer used in expression study
EIR-R1	ACAGCGTAGTCGGTCTTCTAAAG	RT-PCR primer used in expression study
β-actin-F	GGATGATGAAATTGCCGCACTGG	RT-PCR control used in expression study
β-actin-R	ACCGACCATGACGCCCTGATGT	RT-PCR control used in expression study
ETo-F2	AGGATCCGATTAGCACGCA ACGAGGAC	Expression in *E. coli*
ETo-R2	TAAGCTTCTCTGCCATCTG TAGCAAGG	Expression in *E. coli*
EIR-F2	CATGAATTCCAAGCAGATTGGAG AAGGAGGT	Expression in *E. coli*
EIR-R2	CATCTCGAGGCCAGAGGTCTT CAACCCTG	Expression in *E. coli*
5′ CDS	$(T)_{25}$ VN-3′ N = A, C, G, or T; V = A, G, or C	5′ RACE PCR
BD SMART oligo	AAGCAGTGGTATCAACGCAGAGT ACGCGGG	5′ RACE PCR
5′ UPM	CTAATACGACTCACTATAGGGCA AGCAGTG-GTATCAACGCAGAGT CTAATACGACTCACTATAGGGC	5′ RACE PCR
3′ Adapter	GGCCACGCGTCGACTAGTAC	3′ RACE PCR adaptor
Oligo dT adapter	$GGCCACGCGTCGACTAGTACT_{17}$	First strand cDNA synthesis
AP1	GTAATACGACTCACTATAGGGC	Genomic walking adaptor primer 1
AP2	ACTATAGGGCACGCGTGGT	Genomic walking adaptor primer 2