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Identification and molecular characterization of the interleukin-10 receptor 1 of the zebrafish (*Danio rerio*) and the goldfish (*Carassius auratus* L.)

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ABSTRACT

This is the first report of the identification and molecular characterization of an interleukin-10 receptor 1 in bony fish. By gene synteny analysis, we identified the zebrafish interleukin-10 receptor 1 (IL10R1) and using this IL10R1 sequence, we cloned the goldfish IL10R1 cDNA transcript. The identified fish IL10R1 protein sequences had a putative JAK1 binding site, only one of the two STAT3 binding sites, that are present in all other vertebrates IL10R1 proteins as well as C-terminal serine rich areas, believed to be responsible for the anti-inflammatory properties of IL10R1. Phylogenetically, the fish IL10R1 proteins grouped independently of the amphibian, avian and mammalian IL10R1s. Quantitative gene expression analysis of the IL10R1 of zebrafish and goldfish revealed highest mRNA levels in the spleen tissues. High mRNA levels were also observed in the zebrafish muscle in contrast to low mRNA levels in the muscle of the goldfish. Moderate IL10R1 mRNA levels were seen in most other tissues examined and lowest gene expression was in the liver of both fish species. Goldfish monocytes stimulated with a recombinant goldfish interleukin-10 (rgIL-10) or with heat killed fish pathogens, *Aeromonas salmonicida* or *Trypanosoma carassii*, exhibited significantly reduced mRNA levels of the IL10R1. Furthermore, we produced a recombinant form of the goldfish IL10R1 (rgIL10R1) and using *in vitro* binding studies, demonstrated that the dimerized rgIL-10 specifically interacted with rgIL10R1. Our results suggest that interleukin-10 system has been highly conserved throughout evolution.

1. Introduction

Interleukin-10 (IL-10) was initially identified in the supernatants of Con-A-stimulated T cells based on its ability to inhibit the synthesis of pro-inflammatory cytokines (Fiorentino et al., 1989). Since then it has been established that the interleukin-10 cytokine is indispensable for the regulation of the inflammatory and other immune processes. In fact the importance of this soluble mediator in homeostatic and immune regulation was evident from observations in IL-10 knockout mice (Kuhn et al., 1993; Rennick and Fort, 2000; Rennick et al., 1997), where IL-10$^{-/-}$ mice, despite being raised in a pathogen free environment, developed severe enterocolitis in response to their natural enteric flora. These IL-10$^{-/-}$ mice also exhibited highly polarized Th1 bias, with excessive production of pro-inflammatory mediators. It is now well accepted that the primary targets of the immunosuppressive effects of IL-10 are cells of the monocyte/macrophage lineage, whereas other leukocytes are influenced by this cytokine indirectly through bystander effects (Bogdan et al., 1991; Ding and Shevach, 1992; Fiorentino et al., 1991a,b). It is also becoming clear that the central mechanism behind the IL-10-induced down-regulation of inflammatory processes occurs through IL-10-mediated inhibition of inflammatory cytokine synthesis (Oswald et al., 1992).

The biological roles of interleukin-10 are attributed to a homodimerized form of this cytokine that is structurally similar to IFNγ (Syto et al., 1998; Walter and Nagabhushan, 1995; Walter et al., 1995; Zdanov et al., 1995). The IL-10 receptor complex is composed of ligand binding (Liu et al., 1994; Tan et al., 1995, 1993), and accessory (Kotenko et al., 1997; Spencer et al., 1998) receptor subunits (IL10R1 and IL10R2, respectively). Upon ligation, the IL-10 receptor complex signals through the JAK-STAT signalling pathway, where the signal transducer of activation 3 (STAT3), transcription factor is recruited and activated through phosphorylation by JAK1 and Tyk2 (Weber-Nordt et al., 1996). This STAT3 activation is believed to be the primary downstream cellular event that manifests in the anti-inflammatory effects mediated by IL-10 (Riley et al., 1999; Takeda et al., 1999).

Homologs of IL-10 have been identified in several bony fish species including puffer fish (Zou et al., 2003), carp (Savan et al., 2003), trout (Inoue et al., 2005) zebrafish (Zhang et al., 2005), sea bass (Pinto et al., 2007), cod (Seppola et al., 2008) and goldfish (Grayfer

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et al., 2011). A single copy of the puffer fish IL-10 was initially identified and was found to exhibit low tissues expression (Zou et al., 2003). The mRNA levels of the carp, trout and zebrafish IL-10 were highest in the head kidney, spleen and gill tissues of the respective fish species and increased after LPS stimulations (Inoue et al., 2005; Savan et al., 2003; Zhang et al., 2005). Cod injected with formaline-killed *Vibrio anguillarum* and polyI:C were observed to up-regulate spleen IL-10 gene expression (Seppola et al., 2008) while sea bass IL-10 mRNA levels increased in spleen and kidney upon immune stimulation with UV-killed *Photobacterium damselae ssp. Piscicida* (Pinto et al., 2007). Interestingly, a second isoform of the rainbow trout IL-10 has recently been identified, where the two cytokines shared 92% identity, had distinct post-transcriptional regulatory motifs in their untranslated regions, and exhibited increased gene expression in response to a number of different immune stimuli (Harun et al., 2011). We recently reported that the stimulation of goldfish monocytes with a recombinant goldfish IL-10 (rgIL-10) resulted in decreases in the expression of pro-inflammatory genes, concomitant increase in expression of suppressor of cytokine signalling-3 (SOCS-3) and activation (by phosphorylation) and nuclear translocation of the goldfish STAT3 (Grayfer et al., 2011).

Despite the increasing number of reports describing fish IL-10 ligands, very little is known about the IL-10 receptors of bony fish. In an initial attempt to evaluate the gene synteny organization of the IL-10R2 locus in lower vertebrates, the chicken cluster composed of GART, IFNAR1, IFNAR2 and IL-10R2 was identified, while the puffer fish counterparts were not found (Reboul et al., 1999). Subsequently, the same research group succeeded in identifying the puffer fish IL-10R2 chain based on protein sequence and gene structure homology (Lutfalla et al., 2003). To our knowledge, with the exception to the above work, there are no other reports describing IL-10 receptors of teleosts. The salmon and zebrafish IL-10R2 sequences are available on GenBank. To date, the teleost IL-10R1 has not been identified. This report represents the first identification and characterization of a bony fish interleukin-10 receptor 1.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20 °C in a flow-through water system on a simulated natural photoperiod, and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments. All of the fish ranged from 10 to 15 cm in length and whenever possible an equal number of both sexes were used. Zebrafish (*Danio rerio*) were a kind gift from Dr. Declan W. Ali (Department of Biological Sciences, University of Alberta).

2.2. Macrophage cultures

The procedures for the isolation and cultivation of primary kidney macrophages (PKM) and the medium (NMGFL-15) used for their cultivation have been described previously (Neumann et al., 2000a). The PKM cultures consisted of heterogeneous populations of cells including early progenitors, monocytes and mature macrophages as determined by flow cytometry, morphology, cytochemistry and function (Neumann et al., 2000a). The 3-day cultures contain primarily monocytes while older cultures (6–8 days) are predominated by mature macrophages (Neumann et al., 2000a,b).

2.3. Isolation of goldfish splenocytes, peripheral blood leukocytes, kidney granulocytes

The isolation of goldfish splenocytes, PBLs and granulocytes has been described previously (Grayfer and Belosevic, 2009b).

2.4. Synteny analysis of the zebrafish IL10R1

Synteny analysis was performed using NCBI server, map viewer option. The IL10R1 loci of the human (*Homo sapiens*), chicken (*Gallus gallus*) and frog (*Xenopus* (*Silurana*) *tropicalis*) were compared and were observed to share a high degree of gene synteny surrounding the IL10R1 gene of the respective species. These neighboring genes were located in the zebrafish (*D. rerio*) genome and were found to flank a putative zebrafish IL10R1 gene.

2.5. Cloning of the goldfish IL10R1

Primers designed against the zebrafish IL10R1 sequence (forward 1: TGT GGG ATC CCC CCA ATA AAG CCC; forward 2: TCA GGG CCA AAT ACG GTG AAG; reverse 1: GGC TTT CTG TGA ATC TTA; reverse 2: GGT TTC AGC CTC AGC TGA AGG) were used to obtain partial sequences of 307 and 869 bp in length. The thermocycling parameters used were as follows: 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, a gradient of 55 ± 5 °C for 45 s, 72 °C for 2 min 30s and a final extension of 72 °C for 12 min. The obtained products were inserted into the TOPO TA pCR2.1 cloning vector (Invitrogen), transformed into TOP10 competent cells (Invitrogen), and gown on LB-Kanamycin agar plates. The resulting colonies were screened by colony PCR using the M13 forward (GTA AAA CGA CGG CCA G) and M13 reverse (ACA GCT ATG ACC ATG ATT AC) primers using the following thermocyling conditions: 95 °C for 10 min, followed by 25 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 2 min 30s and a final extension of 72 °C for 12 min. Corresponding colonies of interest were grown up over night in 1 mL LB-Kanamycin at 37 °C, the plasmids were isolated using QIAprep miniprep columns (Qaigen) and sequenced using the M13 forward and M13 reverse primers described above. The sequencing results were then blasted against the NCBI database and identified as the candidate goldfish IL10R1. RACE PCR was performed using the SMARTer RACE cDNA amplification kit (Clonetech) in accordance with the manufacturers instructions to identify the remaining open reading frame and untranslated regions of the goldfish IL10R1 transcript. The RACE PCR primers employed were forward: AGC AGG AAG ATA GCG GCT GTG GCA GT and reverse: GGC ACA ATG TCC CAC TCA GCA TCA GGA CC. The full-length sequence of the goldfish IL10R1 cDNA transcript (No. JN203498) has been submitted to GenBank.

2.6. In silico analyses

Protein sequence alignments of fish, amphibian, avian and mammalian IL10R1 were done performed using the Clustal W software (http://www.ebi.ac.uk/clustalw/). Signal peptide regions of respective IL10R1 proteins were identified using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and the transmembrane regions were predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The fibronectin type III domain and the immunoglobulin-like fold of the goldfish IL10R1 were predicted using the InterPro Scan online server (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The putative *N*-glycosylation sites of the goldfish IL10R1 were determined using the NetNGlyc Server v. 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The percent identities between the IL10R1 proteins of different species were calculated using the LALIGN server (http://www.ch.embnet.org/software/LALIGN_form.html). Phylogenetic analysis was conducted using Clustal X and NJ-plot