Fish & Shellfish Immunology 28 (2010) 918–926

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Identification, characterization and genetic mapping of *TLR1* loci in rainbow trout (*Oncorhynchus mykiss*)

Yniv Palti [a,*], M. Fernanda Rodriguez [a,1], Scott A. Gahr [a,2], Maureen K. Purcell [b], Caird E. Rexroad III [a], Gregory D. Wiens [a]

[a] United States Department of Agriculture, Agriculture Research Service, National Center for Cool and Cold Water Aquaculture, 11861 Leetown Road, Kearneysville, WV 25430, USA
[b] Western Fisheries Research Center, U.S. Geological Survey, 6505 NE 65th Street, Seattle, WA 98115, USA

ARTICLE INFO

Article history:
Received 11 December 2009
Received in revised form
26 January 2010
Accepted 3 February 2010
Available online 11 February 2010

Keywords:
Toll-like receptor
TLR1
Rainbow trout
Innate immunity
Genetic map
Gene expression
Gene duplication

ABSTRACT

Induction of innate immune pathways is critical for early anti-microbial defense but there is limited understanding of how teleosts recognize microbial molecules and activate these pathways. In mammals, Toll-like receptors (TLR) 1 and 2 form a heterodimer involved in recognizing peptidoglycans and lipoproteins of microbial origin. Herein, we identify and describe the rainbow trout (*Oncorhynchus mykiss*) *TLR1* gene ortholog and its mRNA expression. Two *TLR1* loci were identified from a rainbow trout bacterial artificial chromosome (BAC) library using DNA sequencing and genetic linkage analyses. Full length cDNA clone and direct sequencing of four BACs revealed an intact *omTLR1* open reading frame (ORF) located on chromosome 14 and a second locus on chromosome 25 that contains a *TLR1* pseudogene. The duplicated trout loci exhibit conserved synteny with other fish genomes that extends beyond the *TLR1* gene sequences. The *omTLR1* gene includes a single large coding exon similar to all other described *TLR1* genes, but unlike other teleosts it also has a 5′ UTR exon and intron preceding the large coding exon. The *omTLR1* ORF is predicted to encode an 808 amino-acid protein with 69% similarity to the *Fugu TLR1* and a conserved pattern of predicted leucine-rich repeats (LRR). Phylogenetic analysis grouped *omTLR1* with other fish *TLR1* genes on a separate branch from the avian *TLR1* and mammalian *TLR1*, *6* and *10*. *omTLR1* expression levels in rainbow trout anterior kidney leukocytes were not affected by the human *TLR2*/*6* and *TLR2*/*1* agonists diacylated lipoprotein (Pam_2CSK_4) and triacylated lipoprotein (Pam_3CSK_4). However, due to the lack of *TLR6* and *10* genes in teleost genomes and up-regulation of *TLR1* mRNA in response to LPS and bacterial infection in other fish species we hypothesize an important role for *omTLR1* in anti-microbial immunity. Therefore, the identification of a *TLR2* ortholog in rainbow trout and the development of assays to measure ligand binding and downstream signaling are critical for future elucidation of *omTLR1* functions.

Published by Elsevier Ltd.

1. Introduction

Receptors that recognize conserved pathogen molecules are part of the ancient innate arm of the immune system and are conserved in both invertebrate and vertebrate lineages. Toll-like receptors (TLRs) are a family of transmembrane proteins that recognize conserved pathogen structures to induce immune effector molecules. In vertebrates, TLRs can distinguish among classes of pathogens and serve an important role in orchestrating the appropriate adaptive immune responses [1]. TLRs are type I membrane proteins that contain an extracellular N-terminus with leucine-rich repeat region (LRR) and an intracellular C-terminus with a Toll/IL-1 receptor domain (TIR). The cytoplasmatic TIR domain harbors conserved amino acids that have been shown to be involved in the signaling as well as in the localization of the TLR [2,3], while the LRR region is involved in pathogen recognition [4]. A limit of approximately 10 TLRs per vertebrate species has been described, and in mammals most of the TLRs have been shown to identify distinct pathogen associated molecular patterns (PAMPs) [5–8]. Overall, 19 distinct TLR genes have been identified to date from various animal species [7,8].

Two major TLR subfamilies were identified in human. *TLR1*, *2*, *4*, *5*, *6* and *10* are the members of the first sub-family recognizing microbial lipids, sugars and proteomes [9–15]. *TLR3*, *7*, *8* and *9* are the members of the nucleic acid subgroup recognizing nucleotide

* Corresponding author. Tel.: +1 304 724 8340x2134; fax: +1 304 725 0351.
E-mail address: yniv.palti@ars.usda.gov (Y. Palti).
[1] Current address: Monsanto Company, Res Bldg N3SA, 800 N. Lindbergh Blvd, Creve Coeur, MO 63167, USA.
[2] Current address: Biology Department, St. Vincent College, 300 Fraser Purchase Rd., Latrobe, PA 15650, USA.

1050-4648/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.fsi.2010.02.002

derivatives of viral or bacterial origin [16–20]. The *TLR1*, *2*, *6* and *10* genes form a phylogenetically related cluster based on sequence similarities and genomic structures [7,8], and in their dimeric combinations they cover broad variations of bacterial peptidoglycans and lipoproteins [21]. They are primarily located on the cell surface and upon activation they induce NF-κB expression through the recruitment of IL-1R signaling molecules including, myeloid differentiation primary response protein 88 (*MyD88*), IL-1R-activated kinase, TNFR-associated factor 6 and NF-κB-inducing kinase [22–24]. In mammals, the synthetic diacylated (Pam_2CSK_4) and triacylated (Pam_3CSK_4) lipoproteins are known experimental agonists of *TLR2*/*6* and *2*/*1* heterodimers [13,23,24].

TLR orthologs have been described in several fish species. A complete repertoire of ten or more TLRs was identified in the *Fugu* and the zebrafish genomes [25–27]. *TLR6* and *10* have not been identified in teleosts to date, and of the five teleost genomes sequenced (zebrafish, *Fugu*, *Tetraodon*, medaka and stickleback) *TLR4* was only identified in zebrafish [7,8]. A number of TLR genes were identified, characterized and mapped in rainbow trout including *TLR3*, *5*, *5S*, *7*, *8*, *9*, *20* and *22* [28–34]. Although found in all the teleost genomes sequenced, rainbow trout (*Oncorhynchus mykiss*) *TLR1* and *TLR2* orthologs have not been reported. Due to their important role in anti-microbial immunity we hypothesize that the rainbow trout genome contains *TLR1* and *TLR2* orthologs.

In this paper, we report the *TLR1* ortholog in rainbow trout and describe its genomic location, gene organization, expression pattern, and regulation. Rainbow trout are widely used in basic research [35] and they are economically important for aquaculture and sport fishing. A better understanding of anti-bacterial immunity is necessary to reduce disease loss in aquaculture and for comparative study of immune system evolution in teleost fish.

2. Materials and methods

2.1. Genes identification and sequencing

The rainbow trout gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=r_trout) [36] was screened by BLAST for ESTs with high homology to the human *TLR1*, *6* and *10* (accession numbers NP_003254, NP_006059 and AAI09112). One EST was identified (accession CA341973) and the complete sequence of the cDNA clone of origin (1RT157C11) was obtained by primer walking as previously described [33,37].

The 5′ RACE protocol was performed in spleen and kidney rainbow trout RNA samples (1 μg/μL) using the GeneRacer kit (Invitrogen, Carlsbad, CA) according to product instructions. Two nested primers (Table 1) were designed for this experiment. PCR reactions were set-up as follows: 1 μL template cDNA (25 ng/μL), 2 μL Reaction Buffer (20 mM $MgCl_2$ included), 1 μL $MgCl_2$ (10 mM), 2 μL dNTP (200 mM), 2 μL each primers (10 μM), 0.4 μL Pfu Taq polymerase (Invitrogen, Carlsbad, CA). The PCR products were isolated from agarose gel using the QIAquick kit (Qiagen, Valencia, CA). The sequencing reactions were set-up as follows: 1 μL template cDNA, 1 μL Ready Reaction mix (ABI, Foster City, CA), 1.75 μL BD buffer, 0.5 μL sequencing primer, 5.75 μL dH_2O.

Table 1
PCR primers and amplicon sizes.

Primer	Sequence	Purpose	Amplicon size (bp)
157C11_1	CGGTGCATGGAGGTAGGTTTCTGG	5′ RACE	N/A
157C11_2	AGAGGTCCAGGGCTTCGGTGCA	5′ RACE	N/A
157C11_T7_3	AAACCAACGAGTTGAGGCTG	BAC Lib screen	435
157C11_SP6_2	ACCTGAGCACTTCAGACGTG	BAC Lib screen	435
*TLR1*_For	CAGACGCCCTGTTGATGTTC	RNA expression	90
*TLR1*_Rev	CCTTCACAAGTTCCACCACG	RNA expression	90
*EF-1α*_For	GGGCAAGGGCTCTTTCAAGT	RNA expression	169
*EF-1α*_Rev	CGCAATCAGCCTGAGAGGT	RNA expression	169

PCR primers were designed from conserved gene segments (Table 1) for screening of the NCCCWA Swanson 10× bacterial artificial chromosome (BAC) library PCR super-pools as previously described [37]. BACs identified by PCR as positive for *TLR1* were fingerprinted using *HindIII* as previously described [38] to identify sets of overlapping clones. BAC DNA was isolated following a miniprep protocol (Qiagen, Valencia, CA). Following *HindIII* digestion and gel electrophoresis, gel images were captured using a Molecular Dynamics Typhoon 9210 Variable Mode Imager and exported as TIF files. Banding patterns were analyzed using Image 3.10 and FPC (Fingerprinted Contigs) V6 software [39] to assemble overlapping BACs into contigs [40]. DNA samples from four BAC clones (294O23, 156A18, 318E05 and 150A12) were isolated using a Large Construct Kit (Qiagen, Valencia, CA), according to the manufacturer protocol. The primer walking method [33,37] was used to obtain genomic sequence directly from the BACs. The samples were sequenced on an ABI 3100 Automated Sequencer (ABI, Foster City, CA).

2.2. Microsatellite markers isolation, genotyping and genetic linkage analysis

A shotgun library was prepared from each of the positive BAC clones and 96–192 sub-clones were sequenced to isolate microsatellite genetic markers as previously described [30,41]. The subclone sequences were used in BLAST sequence similarity searches for identifying neighboring genes and conducting comparative genomics analyses.

The NCCCWA mapping panel of 5 families was genotyped with microsatellite markers as previously described [42]. Four microsatellites (GenBank Accessions GF101807, GF101808, GF101809 and GF101810) were genotyped using the tailed protocol as previously described [30,31,43]. Output files were analyzed using GeneMapper version 3.7 (ABI, Foster City, CA), formatted using Microsoft Excel and stored in a Microsoft Access database.

The four microsatellite markers were placed on the rainbow trout genetic map by two-point linkage analysis as previously described [28,30,31,42]. Genotype data were added to the current NCCCWA genetic map [42] and MULTIMAP [44] was used to conduct two-point linkage analyses to identify the closest markers from the published map having the highest LOD scores.

2.3. Peptide sequence prediction and alignment

The *omTLR1* ORF and its translated amino-acids sequences were predicted using the ExPASy Translate tool (http://us.expasy.org/). Homologous genes from other species were identified using BLASTX global alignment (Table 2). Amino-acid sequences were aligned using ClustalW [45] (http://www.ebi.ac.uk/clustalw/) and this alignment was used for phylogenetic analysis in the program *MEGA3* [46] (http://www.megasoftware.net/). A consensus phylogenetic tree was generated using the neighbor-joining algorithm (Poisson correction/exclusion of gaps) and support for the tree was determined using 10,000 repetitions of bootstrap analysis. Conservation of syntenic relationships was deduced by identifying the genome locations of putative homologs in the genome sequences of zebrafish, stickleback, medaka and Tetraodon using the Ensembl Genome Browser (http://www.ensembl.org/). The simple modular architecture