Developmental and Comparative Immunology 44 (2014) 173–179

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Toll-like receptor 22 of gilthead seabream, *Sparus aurata*: Molecular cloning, expression profiles and post-transcriptional regulation

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ARTICLE INFO

Article history:
Received 8 November 2013
Revised 3 December 2013
Accepted 4 December 2013
Available online 12 December 2013

Keywords:
TLRs
Macrophages
Granulocytes
Bacterial infection
AU-rich elements
Teleosts

ABSTRACT

TLR22 is a fish-specific TLR that recognizes dsRNAs. In the present study, a TLR22 homologue gene from gilthead seabream (sbTLR22) was identified and characterized. The full coding sequence contained a single open-reading frame of 2895 nucleotides encoding a predicted protein of 964 amino acids in length. Its 3′-UTR was relatively long, 1380 nucleotides, and contained three AU-rich sequences frequently associated with mRNA instability. Functional studies showed that the sbTLR22 transcript had a short half-life, although the three AU-rich sequences in its 3′-UTR did not seem to be related with this fact. The sbTLR22 was highly expressed in the spleen, thymus and gills of healthy fish. After *Vibrio anguillarum* infection, the mRNA levels of sbTLR22 increased greatly in head kidney, blood and peritoneal exudate, but were only moderately induced in spleen and liver, suggesting the involvement of sbTLR22 in the immune response against bacterial infections. In addition, acidophilic granulocytes and macrophages, both considered professional phagocytes in seabream, displayed cell-type-specific sbTLR22 expression profiles when stimulated with different pathogen-associated molecular patterns (PAMPs). Although acidophilic granulocytes expressed sbTLR22, polyinosinic:polycytidylic acid (poly I:C) was unable to up-regulate the expression of this receptor. In contrast, poly I:C induced the expression of sbTLR22 in macrophages, in a process that was partially endosome-dependent. Taken together, our results suggest that sbTLR22 is involved in bacterial infection and might sense bacterial PAMPs.

1. Introduction

Toll-like receptors (TLRs) are key receptors of the innate immune system where they recognize and respond to components of invading microorganisms, which are termed pathogen-associated molecular patterns (PAMPs). Upon TLR activation, the expression of a number of inflammatory cytokines, interferons and chemokines are induced, and oxygen and nitrogen radicals increase. The signaling pathways induced by TLRs culminate in the activation of nuclear factor-κB (NF-κB) transcription factors, mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38, and C-Jun N-terminal kinase (JNK) (Brikos and O'Neill, 2008).

To date, 17 different types of TLR have been identified in fish. Although similar to their mammalian counterparts in structure and key features, functional characterization of several fish receptors has revealed distinct features of TLR signaling pathways and ligands (Phelan et al., 2005; Rebl et al., 2010; Sepulcre et al., 2009). Unlike in the human TLR repertoire, TLR6 and TLR10 are absent in the fish lineage, while some TLRs, such as soluble TLR5, TLR22 and TLR23, are only found in fish (Rebl et al., 2010).

In recent years, TLR22 has been identified in several fish species. The involvement of a TLR in the host response to a pathogen is suggested from the regulation of this receptor after experimental challenge with such pathogen. As regards fish TLR22, the results obtained in previous reports are diverse and TLR22 expression seems to be modulated either by bacterial or viral components. In goldfish, TLR22 was induced in leukocytes by lipopolysaccharide (LPS), *Aeromonas salmonicida* and *Mycobacterium cheloni* (Stafford et al., 2003). Inactivated *A. salmonicida* pathogens induced TLR22 expression in the spleen, head kidney and peripheral blood lymphocytes of rainbow trout (Rebl et al., 2007). In orange-spotted grouper, there was significant up-regulation of TLR22 expression in the spleen after *Vibrio alginolyticus* challenge, and in isolated spleen and head kidney leukocytes after LPS and polyinosinic:polycytidylic acid (poly I:C) stimulation (Ding et al., 2012). Expression of TLR22 was induced in peripheral blood leukocytes by both peptidoglycan and poly I:C (Hirono et al., 2004). TLR22 expression was induced in zebrafish larvae (*Danio rerio*) exposed to immunostimulants such as LPS, peptidoglycan or poly I:C (Sundaram et al., 2012). TLR22 expression was up-regulated in anterior kidney and

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0145-305X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2013.12.003

spleen tissues, and also in primary anterior kidney cells of large yellow croaker upon stimulation with poly I:C (Xiao et al., 2011). Finally, the infection of grass carp with grass carp reovirus up-regulated TLR22 gene expression in spleen (Su et al., 2012).

TLR22 also seems to be involved in thermal stress resistance, since TLR22 was down-regulated by heat-shock in Atlantic cod (*Gadus morhua*) (Hori et al., 2010). By contrast, in zebrafish, TLR22 expression was not modified by temperature stress (Sundaram et al., 2012). In summary, TLR22 is mostly up-regulated after a pathogen challenge but also it can be down-regulated. For example, in cod, two paralogues of TLR22 were down-regulated in gills after *Vibrio anguillarum* bath challenge (Hori et al., 2010).

Seya et al. (Matsuo et al., 2008; Seya et al., 2009) demonstrated that TLR22 receptor from pufferfish recognizes dsRNA, and hypothesized that an endoplasmic TLR3 and a cell surface TLR22 would recognize dsRNA and participate in teleost interferon production. TLR22 would serve as a surveillance molecule for detecting dsRNA virus infection and for alerting the immune system for antiviral protection in fish. Although TLR22 seems to be involved in viral recognition, evidence from the literature also points to an important role for TLR22 in the initial innate response to bacteria. Since the modulation of TLR22 differs greatly among the fish species studied and its ligand(s) need further investigation, we decided to identify and characterize TLR22 from gilthead seabream (sbTLR22). In the present work, therefore, we have performed a detailed expression analysis after infection of fish with *V. anguillarum* as well as in purified professional phagocytes after *in vitro* activation with different PAMPs. In addition, the post-transcriptional regulation of sbTLR22 is studied for the first time. Finally, the functional studies carried out suggest a role for sbTLR22 in bacterial infections.

2. Materials and methods

2.1. Cloning of full-length cDNA of sbTLR22 and expression constructs

A full cDNA sequence of sbTLR22 was obtained from a gilthead seabream cDNA library made in λ ZAP Express (Stratagene) enriched with sequences up-regulated following immune stimulation. The full coding sequence of sbTLR22 was deposited in the European Nucleotide Archive (ENA) under accession number HG794237).

The 3′-untranslated region (UTR) sequence from sbTLR22 was obtained by digestion of full-length sbTLR22 with *Nae*I–*Sma*I and then subcloned into the PGL3-Promoter vector (Promega), a firefly luciferase reporter construct driven by the SV40 promoter. PGL3 was previously digested with *Xba*I and blunted with Klenow. The resulting construct was sequenced using an ABI PRISM 377 (Applied Biosystems). The luciferase reporter construct containing the 3′-UTR from sbTNFα was previously described (Roca et al., 2007).

2.2. Luciferase assays

Plasmid DNA was prepared using the Mini-Prep procedure (Qiagen). Transfections were performed with a cationic lipid-based transfection reagent (LyoVec, Invivogen) according to the manufacturer's instructions. Briefly, HEK293 cells were plated in 96-well plate (20,000 cells/well) together with 10 μl of transfection reagent containing a total of 100 ng of plasmid DNA (90 ng of either the reporter construct or the parental vector and 10 ng of the vector used for transfection efficiency). Transfection efficiency was monitored by co-transfecting the *Renilla* luciferase construct pRL-CMV (Promega). Luciferase activities (firefly and *Renilla*) were determined 48 h after transfection using the Dual-Glo Luciferase Assay System and the accompanying protocol (Promega).

Table 1
Amino acid sequence identity (%) between sbTLR22 and other teleost TLR22 genes.

	TLR22 *Paralichthys olivaceus*	TLR22 *Takifugu rubripes*	TLR22 *Oncorhynchus mykiss*	TLR22 *Danio rerio*
Full-length	56	55	42	37
TIR domain	82	83	76	68
Extracellular domain	51	49	36	31

Table 2
ENA accession numbers of TLRs used in this study.

Species	Protein	Genbank/Ensembl accession number
Sparus aurata	TLR22	HG794237
Takifugu rubripes	TLR22	BAF91187
	TLR21	BAC66138
	TLR3	AAW69373
Salmo salar	TLR9	ABV59002
Oncorhynchus mykiss	TLR22	CAF31506
	TLR9	ACC93939
Ictalurus punctatus	TLR21	ABF74623
Danio rerio	TLR22	XP_697657
	TLR3	AAI07956
	TLR9	AAI63628
Gallus gallus	TLR21	CAG32259
	TLR3	ABL74502

2.3. Amino acid sequence analysis

Sequence homology analysis was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the blast tool at Ensembl (Ensembl Genome Browser, http://www.ensembl.org). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (EXPASY) (http://www.expasy.org/). The protein domain features were predicted by Simple Modular Architecture Reach Tool (SMART) (http://smart.embl-heidelberg.de/) (Schultz et al., 1998). Multiple alignments were performed with entire amino acid sequences using Clustal W and a phylogenetic tree of TLR22 deduced amino acids was constructed by MEGA 5 (http://www.megasoftware.net). The gene accession number of all sequence analyzed are indicated in Table 2.

2.4. Animals

Healthy specimens (300 g mean weight) of the hermaphroditic protrandrous marine fish gilthead seabream were kept at the Spanish Oceanographic Institute (Mazarrón, Murcia) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting). Fish were fasted for 24 h before sampling.

The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the Bioethical Committee of the University of Murcia (approval number #537/2011) for the use of laboratory animals.

2.5. Experimental infections

Ten fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing 10^8 live *V. anguillarum* R82 cells (serogroup 01) (Toranzo and Barja, 1990). Twenty-four