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Molecular and functional characterization of Toll-like receptor (Tlr)1 and Tlr2 in common carp (*Cyprinus carpio*)

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ABSTRACT

Toll-like receptors (TLRs) are fundamental components of innate immunity that play significant roles in the defence against pathogen invasion. In this study, we present the molecular characterization of the full-length coding sequence of *tlr1*, *tlr2a* and *tlr2b* from common carp (*Cyprinus carpio*). Each is encoded within a single exon and contains a conserved number of leucine-rich repeats, a transmembrane region and an intracellular TIR domain for signalling. Indeed, sequence, phylogenetic and synteny analysis of carp *tlr1*, *tlr2a* and *tlr2b* support that these genes are orthologues of mammalian TLR1 and TLR2. The *tlr* genes are expressed in various immune organs and cell types. Furthermore, the carp sequences exhibited a good three-dimensional fit with the heterodimer structure of human TLR1-TLR2, including the potential to bind to the ligand Pam_3CSK_4. This supports the possible formation of carp Tlr1-Tlr2 heterodimers. However, we were unable to demonstrate Tlr1/Tlr2-mediated ligand binding in transfected cell lines through NF-κB activation, despite showing the expression and co-localization of Tlr1 and Tlr2. We discuss possible limitations when studying ligand-specific activation of NF-κB after expression of Tlr1 and/or Tlr2 in human but also fish cell lines and we propose alternative future strategies for studying ligand-binding properties of fish Tlrs.

1. Introduction

Pattern recognition receptors recognize widely-conserved motifs of pathogens and are crucial for initiating immune responses against invading microorganisms. Toll-like receptors (TLRs) are a family of germline-encoded pattern recognition receptors and known to activate rapid inflammatory responses upon detection of their cognate ligands [1]. TLRs are type-I transmembrane proteins with numerous extracellular leucine-rich repeat (LRR) motifs which collectively form a horseshoe-shaped solenoid, which is responsible for ligand binding. The cytoplasmic part of each TLR contains a Toll/interleukin-1 receptor homology (TIR) domain which, upon dimerization of two TLRs, initiates a signalling cascade leading to activation of transcription factors such as NF-κB or AP-1, and subsequently to production of pro-inflammatory cytokines [2].

Most vertebrate genomes are recognized to have at least one gene representing each of the six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11) [3]. Also within the modern bony fish (Teleostei) the number of Tlr families generally is consistent with what is found for most (higher) vertebrates, although it is not unusual to find duplicated *tlr* genes due to several fish-specific whole-genome duplication events [4–8]. In addition to these duplications, some novel Tlrs seem to be "fish-specific", such as a soluble form of Tlr5, and Tlrs 18–27 [9,10], indicating that an expansion of Tlrs has occurred during the evolution of teleosts. To date, little is known about the ligand specificities of individual Tlrs in fish, since the LRR

Abbreviations: BSA, bovine serum albumin; GFP, green fluorescent protein; IRAK1, interleukin-1 receptor-associated kinase 1; LRR, leucine-rich repeat; LTA, lipoteichoic acid; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; PAMP, pathogen-associated molecular pattern; Pam_3CSK_4, *N*-palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys_4; PBL, peripheral blood leukocytes; PBS, phosphate buffered saline; PE, phycoerythrin; PGN, peptidoglycan; RLU, relative light units; RT-qPCR, real-time quantitative polymerase chain reaction; TIR, toll/interleukin-1 receptor; TLR, Toll-like receptor; TRAF6, TNF receptor-associated factor 6; UTR, untranslated region; WGD, whole genome duplication.

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ectodomains of Tlrs are not always highly conserved and sequence information alone cannot infer functional properties [10]. In apparent contrast to the variation in the ectodomains, intracellular TIR domains of fish Tlrs appear highly conserved and downstream signalling via well-described molecules such as MyD88, Irak1 and Traf6 identified in several fish species, suggest a conserved mechanism of innate immune signalling could exist [11]. Yet, studies into ligand-binding properties of fish Tlrs are essential to characterize their exact function within the immune system of fish.

The mammalian TLR1 family consists of TLR1, 2, 6, and also includes TLR10. TLR2 recognizes a variety of microbial components including lipoproteins/lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, parasite glycosylphosphatidylinositol anchors, and fungal zymosan (reviewed by Takeda et al. [1]). TLR2 functions as a heterodimer with either TLR1 or TLR6; the TLR2/TLR1 heterodimer recognizes a variety of triacylated lipoproteins [12], whereas the TLR2/TLR6 recognizes mycoplasma-derived diacylated lipoproteins [13]. TLR6 and TLR10 seem to have arisen as paralogs of TLR1 in the mammalian lineage, with TLR10 found in humans. Neither TLR6 nor TLR10 have been identified in genomes of any lower vertebrate, including teleosts. In fish, Tlr1 and Tlr2 were first identified in fugu [14] and zebrafish [15,16]. Subsequently, Tlr1 and/or Tlr2 have been described in several fish species; Japanese flounder (*Paralichthys olivaceus*) [17], channel catfish (*Ictalurus punctatus*) [18,19], rainbow trout (*Oncorhynchus mykiss*) [20,21], Tetraodon (*Tetraodon nigroviridis*) [22], orange-spotted grouper (*Epinephelus coioides*) [23], large yellow croaker (*Larimichthys crocea*) [24–26], and rohu (*Labeo rohita*) [27]. However, studies into ligand-binding properties of fish Tlr1 and/or Tlr2 molecules have been scarce.

We previously identified and characterized common carp (*Cyprinus carpio*) Tlr2 [28,29]. Transfection of human HEK293 cells with carp *tlr2* suggested the ability to bind the prototypical TLR2 ligands LTA, PGN and Pam_3CSK_4. Stimulation of carp macrophages with PGN induced *tlr2* gene expression, MAPK-p38 phosphorylation and led to an increased production of nitrogen and oxygen radicals. Here, we present the identification of Tlr1 and molecular characterization of the mRNA and genomic structure of both *tlr1* and *tlr2* from common carp. We compare the gene expression of *tlr1* and *tlr2* in the same tissue samples and purified cell populations and describe our efforts to characterize the function of putative Tlr1/Tlr2 heterodimers by studying subcellular localization and ligand-binding properties. We discuss possible limitations when studying ligand-specific activation of NF-κB after overexpression of Tlr1 and/or Tlr2 in human but also fish cell lines and propose alternative future strategies for studying ligand-binding properties of fish Tlrs.

2. Materials and methods

2.1. Animals

European common carp (*Cyprinus carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, The Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3×R8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) [30]. Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval from the local animal welfare committee (DEC) of Wageningen University.

2.2. Organ isolation

Carp were euthanized with 0.3 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/L $NaHCO_3$. Carp were bled from the caudal vein using a needle and syringe containing cRPMI medium (RPMI 1640 with 25 mM HEPES (Lonza, Basel, Switzerland) adjusted to an osmolality of 280 mOsm/kg with sterile water) containing 50 U/mL heparin (Leo Pharma, Ballerup, Denmark), 50 U/mL penicillin G (Sigma-Aldrich, St. Louis, MO, USA), and 50 μg/mL streptomycin sulphate (Sigma-Aldrich). For isolation of peripheral blood leukocytes (PBL), the heparinized blood was centrifuged at 100*g* for 5 min at 4 °C and then another 5 min at 300*g*. The buffy coat was collected, carefully layered on Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and centrifuged at 800*g* for 25 min at 4 °C without brake. The leukocyte layer was collected and washed twice with cRPMI. The obtained PBL were stored at −80 °C until used for RNA isolation. After bleeding the fish, the organs of interest were aseptically removed and immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation.

2.3. Isolation of leukocyte subtypes

Carp leukocyte subtypes were isolated by density gradient separation and/or magnetic cell sorting using specific antibodies as described before for thrombocytes [31], granulocytes [32], B cells [59,60], and macrophages [33]. In short, PBL or single-cell suspensions derived from carp organs were incubated with primary mouse monoclonal antibody: WCL-6 for thrombocytes (from blood), TCL-BE8 for neutrophils (from mid kidney), WCI-12 for B cells (from blood), and WCL-15 for monocytes/macrophages (from spleen). After incubation and washing, cells were stained with phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody. After washing and counting of cells, magnetic beads (anti-PE MicroBeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and allowed to bind, before washing and magnetic separation on LS Midi Columns using a MidiMACS Separator (Miltenyi Biotec). Head kidney-derived macrophages were isolated and cultured as described by Joerink et al. [34].

2.4. RNA isolation

Total RNA from carp organs and leukocytes was extracted using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands) including on-column DNase treatment with the RNase-free DNase set (Qiagen). Final elution was performed with 30 μL nuclease-free water. The integrity of the RNA was determined by agarose gel electrophoresis and the RNA quality and concentrations were assessed spectrophotometrically by measuring the absorbance at 260 nm and 280 nm (Nanodrop, Thermo Scientific, Waltham, MA, USA). RNA was stored at −80 °C until use.

2.5. cDNA synthesis

Prior to cDNA synthesis, 500 ng-1 μg of total RNA was subjected to an additional DNase treatment by using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase, according to the manufacturer's instructions. As control for genomic contamination, for each sample a reaction without SuperScript III Reverse Transcriptase was performed. cDNA samples were diluted 25 times in nuclease-free water before use as templates in real-time quantitative PCR experiments.

2.6. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed in a Rotor-