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Short communication

Molecular characterization and expression of CD2 in Nile tilapia (*Oreochromis niloticus*) in response to *Streptococcus agalactiae* stimulus

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ABSTRACT

The cluster of differentiation 2 (CD2), functioning as a cell adhesion and costimulatory molecule, plays a crucial role in T-cell activation. In this paper, the CD2 gene of Nile tilapia, *Oreochromis niloticus* (designated as On-CD2) was cloned and its expression pattern under the stimulation of *Streptococcus agalactiae* was investigated. Sequence analysis showed On-CD2 protein consists of two extracellular Ig-like domains, a transmembrane region, and a long proline-rich cytoplasmic tail, which is a hallmark of CD2, and several important structural characteristics required for T-cell activation were detected in the deduced amino acid sequence of On-CD2. In healthy tilapia, the On-CD2 transcripts were mainly detected in the head kidney, spleen, blood and thymus. Moreover, there was a clear time-dependent expression pattern of On-CD2 after immunized by formalin-inactivated *S. agalactiae* and the expression reached the highest level at 12 h in the brain and head kidney, 48 h in the spleen, and 72 h in the thymus, respectively. This is the first report on the expression of CD2 induced by bacteria vaccination in teleosts. These findings indicated that On-CD2 may play an important role in the immune response to intracellular bacteria in Nile tilapia.

1. Introduction

T-cell activation is primarily initiated through complex cell-to-cell interactions. According to the classic theory of immunology, it is believed that T-cell activation requires two sets of signals, the first of which is provided by the binding of T-cell receptors (TCRs) on T-cells to peptide-major histocompatibility complex (p-MHC) complex on antigen-presenting cells (APCs), while the second signal (costimulatory signal) is provided by interactions of costimulatory receptors on T-cells with their ligands on APCs [1,2]. In the absence of costimulatory signal, engagement of TCRs by MHC-bound peptide results in a state of anergy [3]. A number of costimulatory receptors are known to be able to deliver costimulatory signal, of which CD2 is one of the best-characterized [4,5].

As the member of the immunoglobulin superfamily (IgSF), CD2 is a type I integral membrane protein consisting of an extracellular region, a transmembrane region and a cytoplasmic tail. The extracellular region of CD2 comprises two IgSF domains, a membrane distal non-disulfide bonded V-type Ig domain that is responsible for binding to the ligands on APCs and a membrane proximal C2 Ig domain that contains two interchain disulfide bonds [6]. The cytoplasmic tail of CD2 is distinctive both for its large size and abundance of proline. This region is interacting with several proteins, such as CD2BP1 [7], CD2BP2 [8], CD2BP3 [9], and CD2AP [10], and plays important roles in the mediation of the signal triggered by the extracellular stimulus.

CD2 is a cell surface glycoprotein expressed on most T-cells and natural killer (NK) cells, functioning as a cell adhesion and costimulatory molecule. Ligation of CD2 by CD58 in humans or CD48 in

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rodents helps T-cells adhere to APCs, and provides costimulatory signals for T-cell activation [11–15]. The GPI-linked molecule CD59 has also been described to serve as a costimulatory ligand for CD2 [16], but these findings have been disputed [17]. In addition, CD2 has been shown to localize to lipid rafts upon stimulation with anti-CD2 mAb or CD58 and its recruitment is dependent on Lck binding [18,19], and stimulation with CD2 can induce phosphorylation of the TCR-proximal signaling complex [20]. CD2 has been identified from various mammalian species and its functions have been well characterized [21–24], but little information is available to date regarding fish CD2. CD2 has only been cloned from a few species of teleosts including ginbuna crucian carp *Carassius auratus langsdorfii* [25] and channel catfish *Ictalurus punctatus* [26], and the study of fish CD2 expression profiles and functional properties is rather limited.

Nile tilapia (*Oreochromis niloticus*) is one of the most important commercial fishes and widely cultured throughout the world. In recent years, infectious disease caused by *Streptococcus agalactiae* has been severe, resulting in great economic loss and becoming a big obstacle to tilapia aquaculture. How to eliminate such severe disease has become an emergency, and this, to most extent, depends on the understanding of the immune responses induced by *S. agalactiae*. However, fewer studies focused on the mechanism of immune response in tilapia, especially in the area of T-cell immunity [27,28]. Our previous studies described several potential binding partners of CD2, including CD59 [29], CD2BP2 [30] and Lck [31] from tilapia, which appear to contain important structural characteristics required for interacting with CD2, suggesting main components of CD2 signaling system may be conserved in teleosts. In this study, a CD2 gene (On-CD2) was cloned from Nile tilapia, *O. niloticus*, and its tissue distribution and mRNA expression profile in response to *S. agalactiae* stimulus were investigated. The present results contribute to better understanding of the mechanism of T-cell activation in teleosts.

2. Materials and methods

2.1. Fish and immunization

Samples of Nile tilapia (average weight of 100 ± 10 g, 3 months old) were obtained from a commercial farm in Zhanjiang, Guangdong province, China. Prior to experimentation, fish were acclimated in fiber-reinforced plastic tanks (1000 L each) with a stocking rate of 4 g L^{-1} under 28 ± 2 °C for 4 weeks. All tanks were supplied with flow-through aerated sand-filtered water, and a light and dark period of 12 h: 12 h was maintained. *S. agalactiae* ZQ0910, a virulent strain isolated from tilapia was used for immunostimulus [32]. The immunostimulation experiment was performed by intraperitoneal injecting (i.p.) the tilapia with 0.1 mL of formalin-inactivated bacteria resuspended in sterilized phosphate buffered saline (PBS) with the concentration of 1×10^7 cells mL^{-1} into the abdominal cavity and the tilapia injected with 0.1 mL of sterilized PBS were used as the control group. Then all processed tilapia were returned to tanks and treated as before. At time points of 0 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h and 96 h post-immunization, ten kinds of tissues including blood, brain, gill, head kidney, intestine, liver, muscle, skin, spleen and thymus were collected from the control and vaccinated groups, and immediately frozen by liquid nitrogen, followed by storage at −80 °C until use. In addition, to detect the expression of On-CD2 in response to peptidoglycan (LPS) and Lipoteichoic acid (LTA) stimulus, tilapia was intraperitoneal injected individually with 0.1 mL of LPS from *Escherichia coli* 055:B5 (5 mg/mL; Sigma, USA) and LTA from *Staphylococcus aureus* (5 mg/mL; Sigma, USA), with a dose of 5 μg LPS or LTA per gram of fish body weight [33,34], and three kinds of tissues including head kidney, spleen and thymus were collected at time points of 0 h, 12 h, 24 h, 48 h and 96 h post-stimulation. Tissues from three individuals were collected and pooled together as a replicate sample, and three replicates were taken for each sampling time point.

2.2. Cloning of cDNA for On-CD2

The sequences of all PCR primers used in this study were summarized in Table 1. Total RNA from head kidney was extracted using Trizol Reagent (Invitrogen, USA) as described in the manufacturer's instructions. The first-strand cDNA was synthesized from the total RNA using Reverse Transcriptase M-MLV (TaKaRa, Japan) according to the manufacturer's protocol and served as a template to amplify On-CD2 partial cDNA sequences by PCR using specific primers designed from our previous study on Nile tilapia transcriptome data (unpublished). To amplify the full-length sequence of On-CD2, the first-strand cDNA for 5′/3′-RACE (rapid amplification of cDNA ends) was synthesized with a SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) using head kidney RNA as the template and following the manufacturer's protocol. The full-length cDNA of On-CD2 was obtained by using 5′/3′-RACE methods with some gene specific primers designed based on the obtained partial sequences of On-CD2 cDNA. All the PCR products were ligated into the pMD18-T vector (TaKaRa, Japan) and transformed into competent *E. coli* cells. Then the positive clones were sequenced by sequenced SANGON BIOTECH (Shanghai, China). Finally, the partial sequence, 3′-end and 5′-end were assembled using contigExpress application software.

2.3. Bioinformatics analysis of On-CD2

The potential open reading frame (ORF) was analyzed with the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.htmL). The protein analysis was conducted with ExPASy tools (http://expasy.org/tools/). Location of domains was predicted using the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Multiple alignments of On-CD2 amino acid sequences were performed with the Clustalw2 program (http://www.ebi.ac.uk/Tools/clustalw2/). The similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenic trees were constructed by the neighbor-joining method using MEGA 4 software with 1000 bootstrap replications. The three-dimensional (3D) structure prediction was performed by SWISS-MODEL online software at the Expert Protein Analysis System (http://www.expasy.org/).

2.4. Quantitative analysis of On-CD2 mRNA expression

The differential expression levels of On-CD2 in pre- and post-immunized tissues were measured by fluorescent quantitative real-time PCR using gene-specific primers (Table 1). The first-strand cDNA was synthesized from the DNase treated total RNA using the Reverse Transcriptase M-MLV (TaKaRa, Japan) according to the manufacturer's protocol. The β-actin gene was used as an internal control to normalize the potential variations in RNA loading. The relative expression levels of On-CD2 were calculated using Nile tilapia β-actin expression as a reference, and the results were further compared to respective control group expression levels to determine the fold induction. Before qRT-PCR, each primer pairs were tested via standard RT-PCR to check for size specificity of the amplicon by 2.0% agarose gel electrophoresis and ethidium bromide staining, and target amplicons were sequenced to confirm specificity of the PCR products. In addition, the specificity of primers was further confirmed with the melting-curve after