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A single immunoglobulin-domain IgSF protein from *Sciaenops ocellatus* regulates pathogen-induced immune response in a negative manner

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ABSTRACT

The immunoglobulin superfamily (IgSF) is a large group of cell surface proteins that include various immunoregulatory receptors such as novel immune type receptors (NITRs), which are a family of diversified proteins found exclusively in bony fish. In this study, we identified and analyzed an IgSF protein, SoIgSF1, from red drum (*Sciaenops ocellatus*). SoIgSF1 is composed of 225 amino acid residues and moderately related to teleost NITRs. In silico analysis indicated that SoIgSF1 is a type I transmembrane glycoprotein and contains an N-terminal signal peptide sequence, a single extracellular immunoglobulin V domain, a transmembrane region, and a cytoplasmic region. However, unlike most NITRs, the cytoplasmic region of SoIgSF1 exhibits no consensus inhibitory or stimulatory signaling sequences but has two tyrosine-containing motifs that conform to the right-half sequence of the immunoreceptor tyrosine-based inhibitory motif (ITIM). Quantitative real time RT-PCR analysis showed that SoIgSF1 expression occurred mainly in immune organs and was drastically induced by viral and bacterial infection. Immunofluorescence microscopy indicated that viral infection of head kidney (HK) leukocytes induced surface expression of SoIgSF1, which was able to interact with antibodies against recombinant SoIgSF1. Antibody cross-linking of SoIgSF1 on HK leukocytes inhibited the expression of immune relevant genes and promoted viral and bacterial infection. Taken together, these results indicate that SoIgSF1, though lacking canonical intracellular signaling motifs, is involved in regulation of host immune response during pathogen infection possibly by functioning as a negative signaling receptor through a novel mechanism.

1. Introduction

The immunoglobulin superfamily (IgSF) is a large group of cell surface proteins that share the conserved structure of an immunoglobulin (Ig)-like domain, a transmembrane region, and a cytoplasmic region. To this IgSF family belong various immunoregulatory receptors that are expressed mainly by immune cells, such as killer cell Ig-like receptors (KIRs), leukocyte Ig-like receptors (LILRs), novel immunoglobulin-like transcript (NILT), and novel immune type receptors (NITRs) (Montgomery et al., 2011). NITRs are a family of diversified proteins found exclusively in teleost fish (Yoder, 2009). They form a subset of the Ig superfamily that goes through no rearrangements (Litman et al., 2001; Strong et al., 1999). NITR was first identified in Southern pufferfish (*Spheroides nephelus*) and subsequently found in a number of teleosts including zebrafish (Yoder et al., 2004, 2008), channel catfish (Evenhuis et al., 2007; Hawke et al., 2001), rainbow trout (Kock and Fischer, 2008; Østergaard et al., 2009; Yoder et al., 2002), Japanese flounder (Piyaviriyakul et al., 2007), medaka (Desai et al., 2008), and European sea bass (Ferraresso et al., 2009). No NITRs have been identified in higher vertebrates so far. However, studies of zebrafish NITRs have indicated that they share genetic and functional properties with mammalian LILRs, which led to the hypothesis that NITRs may be intermediates in the evolution of the leukocyte receptor cluster (Yoder et al., 2001).

Structurally, NITRs contain one or two extracellular Ig-like domains; when a single Ig domain is present, it is usually a variable (V) type; when two Ig domains are present, the second one is usually a V/C2 type. In most NITRs, the Ig domain is followed by a transmembrane region and a cytoplasmic tail. Based on the sequence features of their cytoplasmic regions, NITRs are classified into two primary groups, i.e., inhibitory NITRs, which are the major type, and stimulatory/activating NITRs. Stimulatory NITRs possess a positively charged residue in the transmembrane region, while inhibitory NITRs contain in the cytoplasmic region one or two immunoreceptor tyrosine-based inhibitory motif (ITIM) or ITIM-like motif (ITIM) (Strong et al., 1999; Yoder et al., 2004). ITIM is a conserved sequence (S/I/V/L–X–Y–X–X–I/V/L) found in mammalian inhibitory immune receptors such as KIRs and NK-cell receptors (Hsu et al., 2002; Martinez-Borra and Khakoo, 2008). Upon

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ligand–receptor interaction, ITIMs on the receptor are phosphorylated by Src family kinases and serve to recruit SH2 domain-containing enzymes such as phosphatases SHP-1 and SHP-2, which inhibit activation of the signaling process (Barrow and Trowsdale, 2006).

Red drum (*Sciaenops ocellatus*) was introduced into China in 1991 and is now being farmed as an economic fish species in China. However, very little study has been carried out to investigate the immunological mechanism of this species. In this study, we identified from red drum an IgSF protein, SoIgSF1, and analyzed its expression and function. We found that SoIgSF1 likely functions as a negative signaling receptor and is involved in regulation of viral and bacterial infection.

2. Materials and methods

2.1. Fish

Red drum (*S. ocellatus*) were purchased from a commercial fish farm in Fujian Province, China and maintained at 22 °C in aerated seawater. Fish were acclimatized in the laboratory for 2 weeks before experimental manipulation. Before each experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen. Fish were used for experiment only when no bacteria could be detected from any of the examined fish. Fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving tissue collection.

2.2. Cloning of SoIgSF1

A cDNA library of red drum head kidney (HK), spleen, and liver was constructed as described previously (Dang et al., 2010). Plasmid was isolated from 1200 clones and subjected to DNA sequence analysis; one clone was found to contain the cDNA of SoIgSF1 with 5′- and 3′-untranslated regions (UTRs). The nucleotide sequence of SoIgSF1 has been deposited in GenBank database under the accession number JQ031817.

2.3. Sequence analysis

The cDNA and amino acid sequences of SoIgSF1 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. Signal peptide search was performed with SignalP 3.0. The calculated molecular mass and theoretical isoelectric point were predicated by EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was created with the ClustalX program. Phylogenetic analysis was performed as described previously with ClustalX and the Neighbor-joining algorithm of MEGA 4.0 (Li et al., 2011).

2.4. Quantitative real time reverse transcriptase-PCR (qRT-PCR) analysis of SoIgSF1 expression in fish tissues

Brain, heart, gill, HK, spleen, liver, muscle, and blood were taken aseptically from three fish (average 12.4 g) and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Zheng et al., 2010). PCR efficiency (99.9%) was determined as described previously (Zheng and Sun, 2011). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of SoIgSF1 was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with translation initiation factor 3 (TIF3) as a control. TIF3 was relatively stably expressed in the tissues examined in this study (Sun B. and Sun L, unpublished data). The primers used to PCR the TIF3 gene were TIF3F (5′-ACACCCGTGAGACAGA CTTGC-3′) and TIF3R (5′-TCCCGACGATGGAAACTGAT-3′). The primers used to PCR SoIgSF1 were SoIgSF-F1 (5′-GCAGAGTGCTGGGGAA AATC-3′) and SoIgSF-R1 (5′-CAAATAGGTCGCATTCCAAGAGT-3′). The PCR assay was performed three times with the cDNA from each fish. The data are given in terms of mRNA levels relative to that of F3 and expressed as means plus or minus standard errors of the means (SE).

2.5. Expression of SoIgSF1 in HK in response to microbial challenge

The fish bacterial pathogen *Edwardsiella tarda* TX1 (Zhang et al., 2008) was cultured in Luria–Bertani (LB) broth at 28 °C to mid-logarithmic phase and resuspended in phosphate-buffered saline (PBS) to 1×10^7 colony forming units (CFU)/ml. The fish viral pathogen megalocytivirus RBIV-C1 (Zhang et al., 2012) was suspended in PBS to 10^5 copies/ml. Lipopolysaccharides (LPS) were prepared according to the method of Apicella et al. (1994) and suspended in PBS to 500 μg/ml. Red drum (average 13.1 g) were divided randomly into five groups and injected intraperitoneally with or without (control) 100 μl *E. tarda*, megalocytivirus, LPS, or 100 μl PBS alone. Fish (five for each time point) were euthanized at 4 h, 12 h, 24 h, and 72 h post-infection. Tissues were taken under aseptic conditions and used for qRT-PCR as described above. β-tubulin was used as an internal control for the PCR assay, because, of the currently known housekeeping genes in red drum, β-tubulin is the least-affected in transcription in kidney by bacterial and viral infection (Sun B and Sun L, unpublished data). The primers used to PCR the β-tubulin gene were TUBF (5′-CCCCAACAATGTCAA-GACCG-3′) and TUBR (5′-GCTGTGCTGTTGCCGATG-3′).

2.6. Purification of recombinant proteins and preparation of antisera

To prepare recombinant SoIgSF1 (rSoIgSF1), the plasmid pEtSoIgSF1, which expresses the IgV domain of SoIgSF1 linked to a protein tag (Trx) derived from the backbone plasmid pET32a (Novagen, San Diego, CA, USA), was created as follows. The coding sequence of IgV was amplified by PCR with primers SoIgSF-F2 (5′-GATATCGCCA CCATGCTGGTTCCTGTGATCAC-3′; underlined sequence, EcoRV site) and SoIgSF-R2 (5′-GCGCGATATCTTCACAACATACTGTTCG-3′; underlined sequence, EcoRV site); the PCR products were ligated with the T–A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the SoIgSF1-containing fragment, which was inserted into pET32a at the EcoRV site. rSoIgSF1 and, as a control, the Trx-tag were purified as follows. *Escherichia coli* BL21(DE3) (Tiangen, Beijing, China) was transformed with pEtSoIgSF1 and pET32a, respectively; the transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and expression of exogenous proteins was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM. After growing at 30 °C for an additional 5 h, the cells were harvested by centrifugation, and His-tagged proteins were purified using nickel-nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified proteins were dialyzed in PBS and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The concentrated proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized after staining with Coomassie brilliant blue R-250 (Supplementary data Fig. 1). The concentration of the proteins was determined using the