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Short communication

Stomatin-like protein 2 of turbot *Scopthalmus maximus*: Gene cloning, expression profiling and immunoregulatory properties

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ABSTRACT

Stomatin-like protein 2 (SLP-2) is a novel and unusual member of the stomatin gene superfamily. In this study, we obtained a full-length SLP-2 (SmSLP-2) cDNA from turbot (*Scopthalmus maximus*) spleen cDNA library. The cDNA sequence of SmSLP-2 contains a 5′-UTR of 107 bp, an ORF of 1050 bp, and a 3′-UTR of 959 bp. The ORF encodes a putative protein of 349 residues, which has a calculated molecular mass of 38.7 kDa. The SmSLP-2 protein possesses a prohibitin-homology (PHB) domain (residues 40 to 198) and shares 72.4–87.6% overall sequence identity with that of the teleost species. The highest expression of SmSLP-2 mRNA was found in the skin, followed by the head kidney, gut, spleen, liver, heart, gill and muscle. Moreover, both viral and bacterial pathogen infection resulted in the up-regulation of SmSLP-2 mRNA in the turbot head kidney and spleen in vivo. Subcellular localization analysis indicated that the SmSLP-2 proteins are mainly located in the peripheral membrane of ZF4 cells. This study also demonstrated that SmSLP-2 modulates IL-2 expression via active NFκB signaling pathway, and is possibly involved in host immune defense against bacterial and viral pathogens.

1. Introduction

Stomatin is a plasma membrane protein that was first isolated from human red blood cells [1]. To date, five different forms of mammalian stomatin have been identified, including stomatin, stomatin-like protein 1 (SLP-1), SLP-2, SLP-3 and podocin [2]. The members of the stomatin family are defined by the presence of a structurally conserved core domain called the stomatin-domain belonging to the stomatin-prohibitin-flotillin-HflC/K (SPFH) super family that is widely expressed in several tissues [3]. Orthologs of these proteins have been found in different cell types and organisms such as fish, insects, plants and bacteria [4,5]. Numerous reports suggest that the proteins from the stomatin family may be involved in the organization of the peripheral cytoskeleton and in the assembly of multichain receptors and overexpressed in several types of cancers [6–8].

SLP-2 is a novel and unusual member of the stomatin gene superfamily (also known as STOML2 or EPB72), since it does not have a hydrophobic membrane anchor at its N-terminus. Accordingly, it was suggested that SLP-2 separated early in evolution from the other four eukaryotic stomatin family members [9,10]. The structure of SLP-2 is consistent with its disposition as a peripheral membrane protein [11]. SLP-2 links stomatin or other integral membrane proteins to the peripheral cytoskeleton, plays a role in regulating the ion channel conductance and the organization of sphingolipid and cholesterol-rich lipid rafts [10]. Recently, several studies have reported that SLP-2 is involved in T cell responses, and development and progression of cancer [8,12,13]. The cDNA sequences of SLP-2 of several teleost species have been identified and stored in the GenBank. However, the biological activity and function of teleost SLP-2 in immune response remains unknown.

Turbot (*Scopthalmus maximus*) is a teleost farmed in north China as an economical species. In this study, the full-length cDNA of turbot SLP-2 (named SmSLP-2) was identified, and the expression and regulatory properties of SmSLP-2 were examined. Our results indicate that SmSLP-2 modulates Interleukin 2 (IL-2) expression and is possibly involved in host immune defense against bacterial and viral pathogens.

2. Materials and methods

2.1. Fish

Turbot were purchased from a commercial fish farm in

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Shandong Province, China and cultured at 20 °C in aerated seawater. Fish were acclimatized in the laboratory for 2 weeks before experimental manipulation. Six fish were randomly sampled to detect the presence of bacteria and megalocytivirus in blood, liver, kidney and spleen. Briefly, for detecting bacterial pathogens, the tissues were homogenized in sterile PBS, and the homogenates and blood were plated in triplicate in Luria–Bertani (LB) agar plates and incubated at 28 °C for 48 h. For detecting megalocytivirus, genome DNA was isolated from tongue sole blood, liver, kidney, and spleen. Viral DNA in the tissues was determined by quantitative real time PCR with megalocytivirus-specific primers. No bacteria or virus were detected from the examined fish. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) as reported previously [14]. The study was approved by the ethics committee of Institute of Oceanology, Chinese Academy of Sciences.

2.2. Gene cloning and sequence analysis

A cDNA library of turbot head kidney and spleen was constructed. One of the clones was found to contain the full length cDNA of SmSLP-2 with 5'- and 3'-untranslated regions (UTRs). SmSLP-2 nucleotide sequences have been deposited in the GenBank database with accession number: KT820173. The cDNA and amino acid sequences of SmSLP-2 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. The molecular mass and theoretical isoelectric point (pI) were predicted using EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Signal peptide search was performed with SignalP 3.0. Multiple sequence alignment was created with the ClustalX program.

2.3. Phylogenetic analysis

Multiple sequence alignments were created using CLUSTALW, while MEGA version 4.1 [15] was used to assess the similarities amongst the aligned sequences (www.ebi.ac.uk/clustalw/). A phylogenetic tree based on the deduced amino acid sequences was constructed using the neighbor-joining (NJ) algorithm, and the reliability of the branching was tested using bootstrap re-samplings with 1000 pseudo-replicates.

2.4. Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of SmSLP-2 under normal physiological conditions

Spleen, heart, gill, skin, head kidney, liver, muscle and gut were isolated aseptically from six turbot (average 16.1 g), and used for total RNA extraction with the RNAprep Tissue Kit (Omega Bio-tek, Doraville, GA, USA). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase kit (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) and stoRTF/stoRTR primers (Table 1). Melting curve analysis of the amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression levels of SmSLP-2 were analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with EF1a as a control. All data are given in terms of mRNA levels relative to that of EF1a and expressed as mean ± SEM. The assay was performed three times.

Table 1
Primers used in this study.

Primer	Sequences (5'→ 3') [a]	Application
stoSalIF	ctgcagtcgacgatgttacggacgctgtttcgag (sall)	Plasmid construction
stoSalIR	gtaccgtcgactgatgagtcgatgccgattggt (sall)	Plasmid construction
stoEcoRVF	gatatcatgttacggacgctgtttcgagccg (EcoRV)	Plasmid construction
stoEcoRVR	gatatctgatgagtcgatgccgattggt (EcoRV)	Plasmid construction
IL2PF	gctcaagcttaaatttaaaacaacaacgac (HindIII)	Plasmid construction
IL2PR	ttcgaagcttgcaggacttgaggtcactgtg (HindIII)	Plasmid construction
stoRTF	ggtgaggctcaagctgtctt	qRT-PCR
stoRTR	actctttggcgaggttcgag	qRT-PCR
RPSDF	aacacaggaagcagcagaac	qRT-PCR
RPSDR	acggcagtgatggtctctc	qRT-PCR
EF1aF	cgtcggcttcaacatcaagaac	qRT-PCR
EF1aR	gtgagcggtgtggcagtc	qRT-PCR

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

2.5. qRT-PCR analysis of SmSLP-2 under normal physiological conditions during pathogen infection

The fish bacterial pathogen *Edwardsiella tarda* TX1 was cultured in Luria–Bertani broth (LB) medium at 28 °C to an OD600 of 0.8. The cells were washed and resuspended in PBS to 1×10^7 colony forming units (CFU)/ml. The fish viral pathogen megalocytivirus RBIV-C1 was suspended in PBS to 5×10^4 copies/ml. Turbot (a total of 75) were randomly divided into 3 groups and injected intraperitoneally (i.p.) with 50 μl *E. tarda*, megalocytivirus or PBS. Fish (5 at each time point) were euthanized at 4 h, 12 h, 24 h and 48 h post-bacterial infection, and at 1 d, 2 d, 3 d, and 4 d post-viral infection. Tissues were collected under aseptic conditions. Total RNA extraction, cDNA synthesis and qRT-PCR were performed as described above using EF1a as the housekeeping gene (Table 1). The assay was performed three times.

2.6. Plasmid construction

To construct pTagGFP-SmSLP-2 that expresses SmSLP-2-TagGFP fusion protein, the coding sequence of SmSLP-2 was amplified with primers stoSalIF/stoSalIR (Table 1), and the PCR products were inserted into pTagGFP-N (Evrogen, Moscow, Russia) at the Sall site. To construct pSmSLP-2, which expresses His-tagged SmSLP-2, the coding sequence of SmSLP-2 was amplified with primers stoEcoRVF/stoEcoRVR (Table 1), and the PCR products were inserted into pCN3 at the EcoRV site. To construct pmIL2-Luc, which carries a luciferase reporter gene driven by mouse IL-2 promoter, the mouse IL-2 promoter sequence was amplified by PCR with primers IL2PF1/IL2PR1 (Table 1), and the PCR products were inserted into pMetLuc-2 (Clontech, Mountain View, CA, USA) at the HindIII site. The plasmids were purified using Endo-Free plasmid maxi kit (Omega Bio-Tek, Norcross, GA USA). All plasmid constructs were verified by restriction map analysis and DNA sequencing.

2.7. Cell culture and transfection

Zebrafish ZF4 cells (American type culture collection, USA) were cultured at 22 °C in H12 DMEM medium (Gibco, Carlsbad, CA, USA) containing penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% fetal bovine serum (FBS). For transfection, ZF4 cells were distributed into 24-well culture plates (2×10^5 cells/well) in Opti-MEM medium (Gibco). Transfection of the cells with pTagGFP-SmSLP-2 or pTagGFP-N was performed with Lipofectamine™ LTX and PLUS™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After transfection, the medium was replaced with fresh medium containing 1 μg/ml lipopolysaccharides (LPS)