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Short communication

Conserved inhibitory role of teleost SOCS-1s in IFN signaling pathways

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ABSTRACT

The suppressor of cytokine signaling 1 (SOCS-1) protein is a critical regulator in the immune systems of humans and mammals, which functions classically as an inhibitor of the IFN signaling pathways. However, data on functional characterisation of SOCS-1 in ancient vertebrates are limited. In this study, we report the function of teleost SOCS-1s in IFN signaling in fish models (zebrafish and *Tetraodon*) and human cells. Structurally, teleost SOCS-1s share conserved functional domains with their mammalian counterparts. Functionally, teleost SOCS-1s could be significantly induced upon stimulation with IFN stimulants and zebrafish IFNφ1. Overexpression of teleost SOCS-1s could dramatically suppress IFNφ1-induced Mx, Viperin and PKZ activation in zebrafish, and IFN-induced ISG15 activation in HeLa cells. Furthermore, a SOCS-1 variant that lacks the KIR domain was also characterised. This study demonstrates the conserved negative regulatory role of teleost SOCS-1s in IFN signaling pathways, providing perspective into the functional conservation of SOCS-1 proteins during evolution.

1. Introduction

The suppressors of cytokine signaling (SOCS) family proteins play important roles in a variety of signaling pathways (Yoshimura et al., 2007). The SOCS family is composed of 8 members (SOCS-1 to SOCS-7 and CISH) in mammals (Croker et al., 2008; Shuai and Liu, 2003). However, the molecular occurrence and functional performance of the SOCS family in ancient vertebrates during evolution remains largely unknown. In a previous study, we performed global identification and comparative analysis of SOCS homologs in teleost fish. Results showed that fish possessed at least 12 SOCS family members, including 8 known mammalian counterparts (SOCS-1 to SOCS-7, CISH) and 4 fish-specific members (SOCS-3b, SOCS-5b, SOCS-8, and SOCS-9) (Jin et al., 2008). Afterward, some of these family members, such as SOCS-1, SOCS-3, and CISH were cloned from various fish species, including zebrafish (*Danio rerio*), *Tetraodon nigroviridis*, rainbow trout (*Oncorhynchus mykiss*), and grass carp (*Ctenopharyngodon idella*) (Jin et al., 2007a,b; Wang et al., 2010, 2011; Xiao et al., 2010). However, their biological functions in fish remain poorly understood.

Among mammalian SOCS proteins, SOCS-1 has been characterised as the most important regulator of innate and adaptive immunity (Dimitriou et al., 2008; Endo et al., 1997; Naka et al., 1997). SOCS-1 negatively regulates a broader range of immune-relevant signaling pathways, such as NF-κB, JNK, and p38 (He et al., 2006; Ryo et al., 2003). However, it functions most classically as the negative regulator of the IFN signaling pathway, which reduces the responsiveness of many cell types to IFN-α, -β, and -γ (Fenner et al., 2006; Qing et al., 2005). In fish, SOCS-1 was found to be induced by IFN-γ in fibroid RTG-2 and macrophage RTS-11 cells (Wang et al., 2010). Besides, zebrafish IFNφ1 can also induce the expression of SOCS-1 in zebrafish embryos (Lopez-Munoz et al., 2011). These findings preliminarily indicate that teleost SOCS-1 might also be involved in IFN signaling regulation. However, direct evidence is needed to clarify this suggestion.

Recently, research on teleost IFN systems has significantly progressed. At least 4 virus-induced IFNs (IFNφ-1 to IFNφ-4) have been identified in zebrafish, and several IFN receptors, IFN signaling adaptors, IFN regulatory factors (such as mitochondrial antiviral signaling protein, MAVS), as well as JAK-STAT members have been characterised in various fish species (Holland et al., 2010; Lauksund et al., 2009; Stein et al., 2007; Xiong et al., 2012).

Abbreviations: SOCS-1, suppressor of cytokine signaling 1; CISH, cytokine-inducible SH2-domain containing protein; IFN, interferon; MAVS, mitochondrial antiviral signaling protein; JAK-STAT, the Janus kinase signal transducer and activator of transcription; ISG, IFN-stimulated genes; RIG-I, retinoic acid-inducible gene I; poly(I:C), polyinosinic–polycytidylic acid; MDA5, melanoma differentiation-associated gene-5; PKZ, protein kinase containing Z-DNA binding domains; *Dr*SOCS-1, *Danio rerio* SOCS-1; *Tn*SOCS-1, *Tetraodon nigroviridis* SOCS-1; *Dr*MAVS, *Danio rerio* MAVS; *Dr*IFNφ1, *Danio rerio* IFNφ1; IFN-I, type I IFN; TLR-3, toll-like receptor 3; RLRs, RIG-I-like receptors; DMEM, Dulbecco's modified Eagle's medium.

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Moreover, typical IFN-stimulated genes (ISGs) such as Mx, DNA binding protein kinase (PKZ), viperin, and ISG15 have also been identified in fish (Liu et al., 2002; Rothenburg et al., 2005). These results indicate that a precise IFN signaling and regulatory network might have originated in teleost fishes. However, a complete understanding of the teleost IFN network depends on further clarification of more other signaling and regulatory components, particularly negative regulators such as SOCS proteins. In the present study, we provide functional evidence of the involvement of teleost SOCS-1 molecules (SOCS-1s) in the negative regulation of IFN signaling. This study would not only enrich the current knowledge of SOCS proteins in IFN signaling regulation in ancient vertebrates, but also provide valuable information on the evolutionary history of SOCS proteins and IFN systems from fish to mammals.

2. Materials and methods

2.1. Experimental fish

One-year-old male and female wild-type AB zebrafishes (*D. rerio*) (weighing 0.5–1 g and 1–2 cm long) and green-spotted pufferfishes (*T. nigroviridis*) (weighing 4–6 g and 4–5 cm long) were kept in tanks with recirculating water at 26 °C, and were fed daily with commercial pellets at 0.7% of their body weight. All fish samples were acclimatised and evaluated for overall fish health for at least 2 weeks before the experiments. Only healthy fishes, determined by their general appearance and level of activity, were used in the study.

2.2. Characterisation of fish SOCS-1s

Structural characterisation and conservation of SOCS-1 proteins were comparatively analysed between the fishes and other species. All sequences used were retrieved from NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Multiple alignments were generated using the ClustalW program (version 1.83). Gene organisations (intron/exon boundaries) were illustrated by comparing the SOCS-1 cDNA with genome sequences, and the accompanying illustrations were drawn using GeneMaper 2.5 (http://genemaper.googlepages.com). Functional motifs in SOCS-1 proteins were analysed using the PROSITE database (http://expasy.org/prosite/) and the tertiary structure of SOCS-1s were determined using PyMOL (http://www.pymol.org/) (Huang et al., 2013). The phylogenies of the protein sequences were estimated with MEGA5 software using parsimony and the neighbor-joining method (Kumar et al., 2008).

2.3. Plasmid constructions

The zebrafish (*D. rerio*) SOCS-1 (*Dr*SOCS-1), *T. nigroviridis* SOCS-1 (*Tn*SOCS-1), and *Tn*SOCS-1 variant (*Tn*SOCS-1V) cloning vectors were constructed as previously described (Jin et al., 2007b). The ORFs of the SOCS-1 genes were cloned and inserted into pcDNA6/myc-HisB (Invitrogen, USA) to construct eukaryotic expression vectors, designated as pcDNA6-*Dr*SOCS-1, pcDNA6-*Tn*SOCS-1, and pcDNA6-*Tn*SOCS-1V. The human ISG15 and zebrafish Mx promoters (hISG15-pro-luc and *Dr*Mx-pro-luc) were cloned according to previously described sequence information (Altmann et al., 2004; Fan et al., 2008; Ritchie and Zhang, 2004). The NF-κB luciferase construct was purchased from Clontech (Palo Alto, CA, USA), and the pRL-TK vector was obtained from Promega (Madison, USA). The zebrafish MAVS (*Dr*MAVS) eukaryotic expression plasmid (pcDNA6-*Dr*MAVS) was kindly donated by Stéphane Biacchesi from Jouy en Josas, France (Biacchesi et al., 2009). The zebrafish IFNφ1 (*Dr*IFNφ1) expression plasmid (pcDNA3.1-*Dr*IFNφ1, with His tag only) was provided by Victoriano Mulero from the Department of Cell Biology and Histology, University of Murcia, Spain (Lopez-Munoz et al., 2009). All primers used in plasmid construction are shown in Supplementary Table S1. All constructed sequences were confirmed by sequencing analysis and the plasmids for transfection and microinjection were prepared endotoxin free using an EZNA™ Plasmid Midi Kit (Omega Bio-Tek, USA).

2.4. Cell culture and transient transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) FCS (Gibco, Life Technologies, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO_2. Cells (1×10^5/mL) were seeded into multiwell plates (Corning, USA) to allow growth until 70–90% confluence on the day of transfection and then transiently transfected with DNA in Opti-MEM I medium using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

2.5. Western blot analysis

Zebrafish embryos and HeLa cells injected or transfected with various expression plasmids or mock control vectors were collected for western blot analysis according to the protocol described previously (Xiong et al., 2012). The blots were probed with mouse myc/His Tag mAb (Abcam, USA) and HRP-conjugated goat anti-mouse IgG (Abcam, USA), and then incubated with ECL reagents (GE Healthcare, UK) according to the manufacturer's protocols. The emitted light was detected using a cooled CCD-camera (LAS-1000, Fujifilm).

2.6. Expression analysis of teleost SOCS-1s

To investigate whether teleost SOCS-1 is an IFN-stimulated molecule that involves in IFN signaling regulation, an induced-expression assay was examined in adult zebrafish or zebrafish embryos. For the former, the adult zebrafish were i.p. injected with 10 μL of IFN stimulant (poly I:C, Sigma–Aldrich, USA, 10 μg/fish) or mock PBS (control). After 12 h, total RNA from selected tissues, including the heart, spleen, liver, intestines, kidneys, gills, brain, skin, and muscles were isolated using TRIzol reagent (Gibco BRL) treated with RNase-free DNase I (Qiagen). For the latter, the expression pattern of *Dr*SOCS1 during embryonic development was initially determined. Then, *Dr*IFNφ1- and *Dr*MAVS-expression plasmids (*Dr*MAVS was used as an IFN stimulant) were injected into one-cell-stage embryos, and total RNA was isolated at the indicated time points. All obtained RNAs were then reverse transcribed using an ExScript RT reagent kit (TaKaRa, Japan) according to the manufacturer's protocol. Real-time PCR was performed on a Mastercycler® ep realplex real-time PCR system using a SYBR® Premix Ex Taq™ kit (TaKaRa, Japan) following the manufacturer's instructions. The experiment protocol consisted of the following: (1) 40 cycles of amplification at 95 °C for 30 s and at 60 °C for 20 s; (2) melting curve analysis at 95 °C for 5 s, at 65 °C for 15 s, and then at 95 °C for 15 s; and (3) cooling at 40 °C for 30 s. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method with SOCS-1 initially normalised against β-actin or GAPDH. The primers used are shown in Supplementary Table S1. Each PCR trial was performed with triplicate samples and repeated at least three times.

2.7. Role of teleost SOCS-1s in the IFN signaling pathway

The role of teleost SOCS-1s in the IFN signaling pathway was examined in zebrafish embryos using luciferase assay and