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Infectious salmon anemia virus segment 7 ORF1 and segment 8 ORF2 proteins inhibit IRF mediated activation of the Atlantic salmon IFNa1 promoter

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ABSTRACT

Infectious salmon anemia virus (ISAV) is an orthomyxovirus, which may cause multisystemic disease and high mortality of Atlantic salmon (*Salmo salar* L). This suggests that ISAV encodes proteins that antagonize the type I interferon (IFN-I) system, which is of crucial importance in innate antiviral immunity. To find out how ISAV might inhibit IFN-I synthesis, we have here studied whether the two ISAV proteins s7ORF1 and s8ORF2 might interfere with activation of the IFNa1 promoter mediated by overexpression of interferon regulatory factors (IRFs) or by the IFN promoter activation protein IPS-1. The IRF tested were IRF1, IRF3, IRF7A and IRF7B. Promoter activation was measured using a luciferase reporter assay where Atlantic salmon TO cells were co-transfected with the IFNa1 promoter reporter plasmid together with an IRF plasmid and the s7ORF1 or the s8ORF2 construct or a control plasmid. The results showed that s7ORF1 significantly inhibited IRF3 and IRF7B induced IFN promoter activity, while s8ORF2 significantly inhibited IRF1 and IRF3 induced promoter activity. Neither s7ORF1 nor s8ORF2 inhibited IPS-1 mediated promoter activation. Immunoprecipitation data suggest that both s7ORF1 and s8ORF2 can bind to all four IRFs. Taken together, this study thus shows that the ISAV proteins s7ORF1 and s8ORF2 antagonizes IFN-I transcription activation mediated by the IRFs. As such this work provides further insight into the pathogenic properties of ISAV.

1. Introduction

Infectious salmon anemia virus (ISAV) is a piscine orthomyxovirus, which may cause multisystemic disease in farmed Atlantic salmon (*Salmo salar* L.) [1,2]. Like influenza A virus, ISAV is enveloped and has a genome composed of eight linear, single-stranded RNA segments of negative polarity [3]. The segments encode ten putative proteins including hemagglutinin-esterase, fusion protein, matrix protein, nucleoprotein, proteins of the virus polymerase complex and two interferon antagonistic proteins [4–6].

To establish an infection, viruses need to inhibit or evade triggering the type I interferon system, which plays a crucial role in the innate immune response. Host cell recognition of virus RNA activates synthesis of type I interferons (IFN-I), which subsequently induce antiviral proteins in other cells. Frequent strategies used by viruses are targeting initiation of transcription of IFN-I or IFN-I induced genes [7]. As an example, the non-structural NS1 protein of influenza A virus possess multiple IFN antagonistic activities including inhibition of IFN-I synthesis [8]. To understand the pathogenic properties of ISAV, it is important to elucidate how it interacts with IFN-I system of salmon. In mammals, IFN-I is induced upon binding of viral RNA by the RNA helicases RIG-I and MDA5, which reside in the cytoplasm of most cells [9,10]. Upon binding viral RNA, RIG-I and MDA5 interact with the adaptor protein IPS-1 (also named MAVS), which mediates activation of the transcription factors IRF3, IRF7 and NFκB, resulting in start of IFN-I transcription [7,11–15]. IFN-I is secreted and induces a range of antiviral proteins in non-infected cells preventing further virus infection [9,16]. Atlantic salmon possesses multiple IFN-I subtypes of which IFNa, IFNb and IFNc appear to be most important in defence against virus infection [17–19]. IFNa is induced in most cells while IFNb and IFNc are produced by more specialized cells [17,20]. These IFNs both

Abbreviations: IFN, interferon; IRF, interferon regulatory factor; IPS-1, interferon promoter stimulating protein; ISAV, infectious salmon anemia virus; ORF, open reading frame.

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inhibit IPNV and ISAV replication [17,20]. IPS-1, IRF1, IRF3 and IRF7 are strong inducers of the IFNa1 promoter in Atlantic salmon and are thus potential targets for viral IFN antagonizing proteins [21–23]. Recently, several IPNV proteins were found to antagonize IPS-1 and IRF mediated activation of the IFNa1 promoter [24]. Previous studies have revealed two ISAV proteins, which antagonize IFNa induction in salmon [25]. They are encoded by an open reading frame (ORF) in segment 7 (s7ORF1) and an ORF in segment 8 (s8ORF), respectively. The s7ORF1 protein is a non-structural cytoplasmic protein while the s8ORF2 protein is a structural protein, which has a predominant nuclear localization [25–27]. The IFN antagonistic mechanisms of the two ISAV proteins have not been revealed. In this work, we demonstrate that s7ORF1 and s8ORF2 inhibit IFNa transcription mediated by overexpression of Atlantic salmon IRF1, IRF3, IRF7A and IRF7B. Moreover, we demonstrate that s7ORF1 and s8ORF2 bind to these IRFs.

2. Materials and methods

2.1. Cells

Atlantic salmon TO cells were obtained from Dr. Heidrunn Wergeland, University of Bergen, Bergen, Norway [28]. Atlantic salmon TO cells were cultured at 20 °C in L-15 medium (Invitrogen, Paisley, UK) containing penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}), 1% non-essential amino acid (NEAA, Invitrogen, Paisley, UK) and 8% fetal calf serum (FCS, Invitrogen, Paisley, UK). HEK293T cells were cultured at 37 °C and 5% CO_2 in Eagle's minimum essential medium (EMEM, Invitrogen, Paisley, UK) supplemented with penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}), 4 mM L-glutamine and 10% FCS.

2.2. Plasmids

The IFNa promoter assays were performed using the luciferase reporter plasmid pA1(-202), which contains the minimal promoter region of Atlantic salmon IFNa1 [29]. The ORFs of Atlantic salmon IPS-1, IRF1, IRF3 IRF7A and IRF7B genes subcloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) were available from previous work [21,30], and the resulting plasmids were named pIPS-1, pIRF1, pIRF3, pIRF7A and pIRF7B, respectively. Plasmids containing ISAV s7ORF1 and s8ORF2 genes were obtained from Dr. Siri Mjaaland at Norwegian School of Veterinary Science, Oslo, Norway [25]. The s7ORF1 and s8ORF2 genes were sub-cloned into the pcDNA3.1 vector and the plasmids were named ps7ORF1 and ps8ORF2. For immunoprecipitation experiments, the coding sequences of s7ORF1 and s8ORF2 were sub-cloned into pCMV-FLAG®-MAT-Tag®-1 expression vector (Sigma, St. Louis, MO). The coding sequences of IRF1, IRF3, IRF7A and IRF7B with a 3′ end overhang sequence (TACCCATACGATGTTCCAGATTACGCTTGA, encoding HA tag) were directionally cloned into the pcDNA3.1 vector, respectively. The resulting plasmids were named pIRF1-HA, pIRF3-HA, pIRF7A-HA and pIRF7B-HA, respectively. All plasmid constructs were verified by DNA sequencing. The plasmid expressing EGFP (pDestEGFP-C1) was obtained from Dr. Trond Lamark, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway [31].

2.3. Cell culture, transfection and reporter activity assay

TO cells were seeded in 24-well plates (Nunc) at a density of 2×10^5 cells per well. The cells were transfected using 10 μl Neon® Transfection System (Invitrogen, Carlsbad, CA) with 10 μl buffer R and 500 n plasmid in each well, and using 2 pulses of 1100 V and 30 ms each. In each well, cells were transfected with 50 ng of Renilla luciferase vector (Promega, Madsion, WI), 150 ng of pA1(-202), 150 ng of either pIRF1, pIRF3 pIRF7A, pIRF7B or pIPS-1, and 150 ng of either ps7ORF1, ps8ORF2 or control vector pcDNA3.1. The transfected TO cells were harvested after 48 h. According to manufacturer's instruction, the cells were lysed by 70 μl 1 × passive lysis buffer from Dual-Luciferase® reporter assay system (Promega, Madsion, WI). Ten microliters of lysate was dispensed with 100 μl of luciferase assay buffer II to measure the Renilla luciferase activity for normalization of transfection efficiency. Then the firefly luciferase activity was recorded after adding 100 μl of Stop & Glo buffer in the same well. Both luciferase activity assays were documented using a plate Luminometer, Luminoskan Ascent (Thermo Scientific, Beverly, MA). All samples for promoter assay were set up in triplicates and the results were presented as relative luciferase activities. All luciferase reporter assays were repeated at least three times.

2.4. Co-immunoprecipitation

HEK293T cells were seeded in 6-well plates (Nunc) at a density of 8×10^5 cells per well. Each well was transfected with 600 ng of ps7ORF1 or ps8ORF2 either alone or with 600 ng of pIRFs together by using Neon® Transfection System (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, the cells were washed twice by ice-cold phosphate buffered saline (PBS) and then lysed by 1 × HA lysis buffer (1 mM EGTA, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) with a protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, UK). The cell lysates were centrifuged for 15 min at 15000 × g. The supernatant was incubated with EZview™ Red ANTI-FLAG M2 Affinity Gel (Sigma, St. Louis, MO) at 4 °C for 2 h. The beads were centrifuged for 30 s at 8200 × g. The pellets were washed four times with ice-cold PBS and then eluted with 80 μl of 2 × SDS sample buffer according to the manufacturer's instruction. To study if pS7ORF1 or pS8ORF2 interacted with EGFP, HEK293T cells in 1 ml EMEM supplied with 5% FCS without antibiotics were seeded into 6-well plates (5×10^5 cells/well). Cells in each well (2 replicates) were co-transfected with 1 μg ps7ORF1 or ps8ORF2 and 1 μg pDestEGFP or pIRF3 or pcDNA3.1, using Lipofectamine 2000 Transfection Reagent (Life technologies). Cells were lysed and harvested 48 h post transfection using HA lysis buffer and protease inhibitor cocktail as above. Cell lysates were cleared with centrifugation (15000 × g in 15 min) and incubated with anti-FLAG M2 affinity gel for 2 h at 4 °C). The bound proteins with the interacting partners were eluted with 50 μl 2 × SDS sample buffer and subjected to SDS–PAGE and visualized by immunoblotting (see below).

2.5. Immunoblotting

The eluates from the co-immunoprecipitation were electrophoresed in a precast 4–12% gradient NuPAGE® Novex Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred onto a polyvinylidene difluoride membranes (Millipore, Billeria, MA) using Invitrogen NuPAGE system, according to manufacturer's instruction. The blots were blocked with 5% dry milk in 1 × TBS buffer (1 M Tris-HCl, pH 7.5, 5 M NaCl) for 2 h at room temperature, and exposed overnight with primary antibody (1:500 dilution; anti-HA tag antibody, Sigma, St. Louis, MO) and 5% dry milk in 1 × TBST buffer (TBS containing 0.1% Tween 20) at 4 °C. The membranes were washed three times with 1 × TBST buffer followed by incubation with secondary HRP-conjugated antibodies (1:2000 dilution; goat anti-rabbit antibody, Santa Cruz, Dallas, TX) and 5% dry milk in 1 × TBST buffer for 1 h at room temperature. The membranes were then washed three times with 1 × TBST buffer and subsequently developed using enhanced chemiluminescence (Pierce, Rockford,