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Regulation and function of interferon regulatory factors of Atlantic salmon

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ABSTRACT

Transcription factors of the interferon regulatory factor (IRF) family are major regulators of the early immune responses against viral infections. In particular, IRF1, IRF2, IRF3 and IRF7 of mammals are known to regulate the expression of type I interferons (IFNs), which constitute the obligate cytokines for antiviral defense. We therefore cloned the coding sequence of Atlantic salmon (As) IRF1, IRF2, IRF3 and IRF7B. Expression profiles were studied in Atlantic salmon TO cells after poly I:C (dsRNA) transfection, treatment with recombinant salmon IFNa1 and infection with infectious salmon anemia virus (ISAV). The main findings were that AsIRF1 was earliest up-regulated by all stimuli, while AsIRF3 and AsIRF7 had a similar activation profile induced at a slightly later time point. The ability to induce the Atlantic salmon IFNa1 promoter was measured in a luciferase reporter assay. The results showed that AsIRF1, AsIRF3 and AsIRF7B were able to induce the promoter in a dose-dependant manner. AsIRF2 repressed the promoter, while AsIRF7A and a splicing variant (AsIRF3D) lacking the interaction domain had almost no effect. Combination of AsIRF1 and AsIRF3 had a synergistic stimulatory effect on the promoter compared to each of the two IRFs alone. Overall, our findings suggest that AsIRF3 is the main regulator of salmon IFNa1 production along with AsIRF1, which is less potent. This confirms a similar role for salmon IRF3 as mammalian IRF3 to be one of the main IRFs eliciting salmon IFNa1 production. Surprisingly, AsIRF7A and AsIRF7B seemed to have a lesser role in salmon IFNa1 induction, which may indicate that these factors have a larger role in activating other IFN genes or interferon stimulatory genes of Atlantic salmon.

1. Introduction

Interferon (IFN) regulatory factors (IRFs) constitute a family of transcription factors which have major roles in regulating the cellular immune response upon virus infections and other cell stressors (Tamura et al., 2008). IRFs are also important in cell cycle progression, differentiation and reproduction (Ozato et al., 2007). Ten members (IRF1–IRF10) have been identified in higher vertebrates, although some of these genes have either been lost or appear non-functional in some species (e.g. *irf10* is absent in humans and mice). Additional IRF-like genes can be found in the lower vertebrates such as the *irf11* of zebrafish (Stein et al., 2007). The origin of the IRF family can be traced back to the formation of multicellular animals as they appear in all five main metazoan groups (Nehyba et al., 2009). The evolutional history reveals that the vertebrate IRF family members can be subdivided into four groups: IRF1-G (IRF1, IRF2), IRF3-G (IRF3, IRF7), IRF4-G (IRF4, IRF8, IRF9, IRF10) and IRF5-G (IRF5, IRF6). In mammals, the IRF3-G members are the major players in type I IFN induction as they both seem to be important factors in the IFN-β enhanceosome (Panne, 2008), and central for the antiviral response (reviewed in Paun and Pitha, 2007). Knock out studies have revealed that IRF7 is essential for the systemic antiviral IFN response, while IRF3 knock outs seem to have a reduced type I IFN response that can be rescued by IRF7 (Honda et al., 2005; Sato et al., 2000). Since IRF3 is ubiquitously and constitutively expressed in all tissues, this allows very early onset of *ifnb* transcription at the site of infection.

IRF1-G members appear to be important in regulating the early phase of various kinds of stress responses (Takaoka et al., 2008; Tanaka et al., 1996). IRF1 was first discovered to bind and activate the IFN-β promoter in mammals (Fujita et al., 1988). Knock out studies have later shown that IRF1 is important in activation of adaptive immune responses against pathogens (reviewed in Paun and Pitha, 2007; Tamura et al.,

Abbreviations: As, Atlantic salmon; CHSE, Chinook salmon embryo cells; DBD, DNA-binding domain; IAD, interaction domain; IFN, interferon; IRF, interferon regulatory factor; IRF-E, IRF-binding element; ISAV, infectious salmon anemia virus; ISGs, interferon stimulated genes; ISRE, interferon stimulated response element; Poly I:C, polyinosinic polycytidylic acid; RLA, relative luciferase activity (firefly luciferase divided by β-gal measurements); rSasalFNa1, recombinantly produced Atlantic salmon IFNa1 from HEK293 cells; TO, Atlantic salmon cells originating from the head kidney; VAD, virus activated domain.

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2008). IRF2 has been considered an inhibitor of IFN-α/β transcription, as it normally is constitutively expressed to mask the IRF-E (IRF-binding element) binding site in the IFN promoters, preventing IFN production in normally growing cells (Harada et al., 1989).

The IRF family harbors a conserved N-terminal DNA-binding domain (DBD) of ~115 amino acids that possess four or five tryptophan repeats with resemblance to the DBD of Myb transcription factors (Veals et al., 1992). The IRF DBD forms a helix-turn-helix motif and recognizes the IRF-E, G(A)AAA$^{G}/_{C}{}^{T}/_{C}$GAAA$^{G}/_{C}{}^{T}/_{C}$, which is the consensus for the IRF1-G (Tanaka et al., 1993). This sequence overlaps with the IFN-stimulated response element (ISRE), $^{A}/_{G}$NGAAANNGAAACT, which binds IRF9 in IFN-stimulated genes (ISGs) (Darnell et al., 1994). The C-terminus of all the members of the IRF family contains an IRF association domain (IAD), except the IRF1 and IRF2, which have a different interaction domain (IAD2) (Meraro et al., 1999; Sharf et al., 1995). The IRF3-G members harbor several serine residues at the C-terminus that are phosphorylated upon virus infection and constitute the virus activated domain (VAD) (Au et al., 2001; Lin et al., 1998; Marie et al., 2000).

Virus infections are mainly detected by receptors for nucleic acids in the cytoplasm (RIG-I, MDA5 or DAI) or in endosomes (Toll-like receptor 3, 7, 8 or 9) (Kawai and Akira, 2008; Wang et al., 2008). This event triggers signaling cascades, which lead to activation of NFκB, IRF3 and IRF7 (Chau et al., 2008; Fitzgerald et al., 2003; Hiscott, 2007). In mammals, activated NFκB and IRF3 and/or IRF7 translocate to the nucleus where they bind to the *ifnb* promoter to initiate IFN-β production. Secretion of IFN-β protects neighboring cells from viral infection by inducing transcription of antiviral proteins such as Mx, ISG15 and Protein Kinase R (PKR).

Fish possess IFNs with significant sequence similarity to mammalian type I IFNs (Aggad et al., 2009; Altmann et al., 2003; Lopez-Munoz et al., 2009; Lutfalla et al., 2003; Purcell et al., 2009; Robertsen et al., 2003; Sun et al., 2009; Zou et al., 2007). Atlantic salmon possesses four subtypes of type I-like IFNs (IFNa, IFNb, IFNc and IFNd), where IFNa1 and IFNa2 are believed to be the most important in the early phase of infection, similar to human IFN-β (Berg et al., 2009; Sun et al., 2009). IFNa1 and IFNa2 are encoded by different genes, but show 95% identity in amino acid sequence. The promoters of Atlantic salmon IFNa1 and IFNa2 genes are identical possessing one NFκB-element flanked by two IRF-binding elements (Bergan et al., 2006). On the other hand, the IFNb and IFNc promoters possess several IRF-binding elements, but no NFκB-element (Sun et al., 2009). Until now, little has been known about which IRF members activate the promoters of fish IFNs although all IRF family members (IRF1 to IRF11) have been reported in fish species (Collet et al., 2003a; Holland et al., 2008; Jia and Guo, 2008; Kileng et al., 2009; Ordas et al., 2006; Shi et al., 2008, 2009a,b; Stein et al., 2007; Sun et al., 2006, 2007; Xu et al., 2009; Zhang et al., 2003). Most fish reports have been on IRF1 and IRF2, but recently IRF3, IRF5 and IRF7 have been cloned and characterized with respect to expression profiles in tissues and cells. In general, functional studies on fish IRFs have been very sparse.

In order to expand the knowledge about IFN induction in Atlantic salmon, we here have cloned the coding sequence of Atlantic salmon (As) IRF1, IRF2, IRF3 and IRF7B (IRF7A has been reported by (Kileng et al., 2009), and studied their expression properties in response to different stimuli associated with viral infection. We further established their stimulatory or inhibitory effect on the salmon IFNa1 promoter in a Luciferase reporter assay. Our data suggests that AsIRF3 is the main regulator of salmon IFNa1 production along with AsIRF1, which is less potent. AsIRF2 is an inhibitor of IFNa1 transcription at high concentrations, being able to reverse the stimulatory effect of both AsIRF1 and AsIRF3. AsIRF7A and AsIRF7B seem to have a lesser role in salmon IFNa1 induction.

Table 1
Primers used in this study.

Name	Sequence 5′ → 3′	Purpose used
IRF1 fwd	ATGCCTGTGTCTAGGATGAGAATG	Cloning of ORF
IRF1 rev	TCAGAGTGGGCATGTGATCTGTTG	Cloning of ORF
IRF2 fwd	ATGCCCGTGGAGAGGATGCGA	Cloning of ORF
IRF2 rev	TCAGCAGGTCTTGACAGAGGAG	Cloning of ORF
IRF3 fwd	ATGTCTCAATCCAAACCGCTCCTAATC	Cloning of ORF
IRF3 rev	TTAGCACAAGTCCATCACCTCCTGCA	Cloning of ORF
IRF7A fwd	ATGCAAAGCTGCAAACCTCAGTTCGCT	Cloning of ORF
IRF7B fwd	ATGACAGAAGTACGTGGCTCAGCATTAA	Cloning of ORF
IRF7 rev	CTAGAAGTACTGCCCCATGGTG	Cloning of ORF
rtIRF-1F	AGGCTAATTTCCGCTGTGCA	Q-RT-PCR
rtIRF-1R	TTTTGTAGACGCGCACTGCT	Q-RT-PCR
rtIRF-2F	TCCCCTGTGTCCTCGTATGG	Q-RT-PCR
rtIRF-2R	GAAACTTGATTCCCACAGCCC	Q-RT-PCR
rtIRF-3F	TGGACCAATCAGGAGCGAAC	Q-RT-PCR
rtIRF-3R	AGCCCACGCCTTGAAAATAA	Q-RT-PCR
rtIRF-7AF	CTGAACTTTAGCAACCTAACCTT	Q-RT-PCR
rtIRF-7AR	ATTAGGACTGGTGGCACTGG	Q-RT-PCR
rtIRF-7BF	GAGGAGTGGGCAGAGAACTA	Q-RT-PCR
rtIRF-7BR	TTCTGGGAGACTGGCTGGG	Q-RT-PCR
rt18S-F	TGTGCCGCTAGAGGTGAAATT	Q-RT-PCR
rt18S-R	GCAAATGCTTTCGCTTTCG	Q-RT-PCR

2. Materials and methods

2.1. Fish, cells and viruses

Atlantic salmon presmolts (40–50 g) were obtained from and kept at the Aquaculture Research Station (Tromsø, Norway) in 300 L tanks containing fresh water at 10 °C.

TO cells originating from head kidney of Atlantic salmon were obtained from Dr. Heidrun Wergeland (University of Bergen) (Wergeland and Jakobsen, 2001). CHSE cells originate from Chinook salmon (*Oncorhynchus tschawytscha*) embryo cells (ATTC CRL 1681). TO and CHSE cells were cultivated in Eagles minimum essential medium (EMEM) with GlutaMax™-1 (Gibco, Invitrogen, Paisley, UK) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin and 1% nonessential amino acids (aa) and either 5% (TO) or 7.5% (CHSE) fetal bovine serum (FBS).

The ISAV isolate was obtained and propagated as described (Kileng et al., 2009).

2.2. Cloning of Atlantic salmon IRF1, IRF2, IRF3 and IRF7B

The different IRF-genes were cloned from a RACE-library derived from Atlantic salmon TO cells stimulated with recombinant salmon IFNa1 as described (Robertsen et al., 2003). Initial and nested IRF primers were designed from EST sequences obtained from GenBank. See Table 1 for primer sequences.

2.3. Stimulation of TO cells with poly I:C, recombinant salmon IFNa1 and ISAV

TO cells were transfected with polyinosinic polycytidylic acid (poly I:C) from GE healthcare Amersham Biosciences (Uppsala, Sweden) complexed with Fugene HD (Roche, Basel, Switzerland) in serum-free Optimem with GlutaMax™-1 (Gibco). TO cells (5×10^5 per well) were seeded in 6-well plates, grown for 24 h and transfected with 1 μg poly I:C complexed with 3 μL Fugene HD in 100 μL serum-free Optimem. Cells were harvested for RT-PCR assays 0, 12, 18, 24, 36, 48 and 60 h post transfection.

Recombinant Atlantic salmon IFN-a1 (rSasaIFN-a1) was produced in HEK-293 cells as described (Robertsen et al., 2003). The activity of rSasaIFN-a1 was measured by a cytopathic reduction (CPE) assay and the activity was estimated to 66,400 U/mL as described (Kileng et al., 2009). TO cells (5×10^5 per well) were seeded in 6-well plates, grown for 24 h and stimulated with