Short communication

Molecular cloning and expression analysis of interferon regulatory factor 8 (IRF8) in turbot, *Scophthalmus maximus*

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ABSTRACT

Interferon regulatory factor 8 (IRF8) in mammals is known to be involved in antiviral response. In this study, the gene of IRF8 was cloned from the turbot (*Scophthalmus maximus*) fish and its expression in response to polynosinic:polycytidylic acid (poly I:C) and turbot reddish body iridovirus (TRBIV) challenges was studied. Turbot (Sm)IRF8 gene is 4363 bp long, comprises nine exons and eight introns and encodes a putative 420 amino acid (aa) protein. The predicted protein sequence possesses a DNA binding domain (DBD), an IRF association domain (IAD) and a nuclear localization signal (NLS). Constitutive expression of SmIRF8 was detectable in all tested organs, with higher levels observed in the spleen, kidney and head kidney. SmIRF8 transcript levels were up-regulated by both poly I:C and TRBIV treatments in the spleen, head kidney, gills and muscle in an early phase of a 7-day time course and the poly I:C was a quicker inducer. In both challenge cases, the highest and earliest inductions were detected in the spleen, while the induction in the muscle was quite faint. These results provide insights into the role of SmIRF8 in antiviral response.

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1. Introduction

Interferon regulatory factor (IRF) family transcription factors play essential roles in various life processes in vertebrates, such as hematopoietic cell development, early immune responses to pathogens, antiviral defense, immuno-regulation and growth control (Barnes et al., 2002a). They modulate and coordinate the inborn and acquired immune responses mediated by recognition of pathogen-associated molecular patterns (PAMPs) by cell surface and cytosolic pattern recognition receptors (Honda et al., 2006; Honda and Taniguchi, 2006), in which interferons (IFNs) and interferon stimulated genes (ISGs) are transcriptionally modulated through the binding of IFRs to DNA binding recognition sites within promoters of target genes (Stark et al., 1998). So far, ten IRF members (IRF1–10) have been reported in mammals (Huang et al., 2010). All IRF members share homology in their first 115 aa which encompasses a DNA binding domain (DBD) containing a characteristic repeat of five tryptophan residues spaced by 10–18 aa (Escalante et al., 1998). In addition, IRFs (except IRF1 and IRF2) possess an IRF association domain (IAD) at the C terminus, which mediates the formation of homodimers or the recruitment of other IRFs and transcription factors to target gene promoters (Eroshkin and Mushegian, 1999; Ozato et al., 2007).

Interferon regulatory factor 8 (IRF8), also known as interferon consensus sequence binding protein (ICSBP), is a key element for the differentiation of myeloid progenitor cells toward macrophages and for the development of lymphomyeloid cell lineages, including B and T cells, dendritic cells (Dcs) and macrophages (Dror et al., 2007; Gabriele and Ozato, 2007). IRF8 is found to form strong interactions with ISRE domains in target gene promoters.
Fig. 1. Multiple alignment of SmIRF8 (turbot IRF8) amino acid sequence with other IRF8 proteins. The putative DNA-binding domain (DBD) and IRF-association domain (IAD) are shaded in gray. The bipartite nuclear localization signal (NLS, KGKFKKGKRTL) is bolded. The conserved tryptophan (W) residues composing a “tryptophan cluster” are boxed in the DBD. The residues identical in all sequences are shown with asterisks (*), whereas those with strong homologies and weak similarities are marked by colons (:) and dots (.), respectively. The accession numbers of the sequences are listed in Table 2.

only when it presents as heterodimers with other IRFs, especially when bound to IRF1, IRF2 and IRF4 (Rosenbauer et al., 1999; Alter-Koltunoff et al., 2008). In addition, IRF8 plays a role to inhibit the procedure of gene transcription when bound to ISRE motifs in gene targets, but can stimulate the event when bound to composite sites, such as the Ets-IRFE motif (Lee et al., 2006; Rosenbauer et al., 1999). It is reported that mammalian IRF8 is an important regulator of cross-talk between the toll-like receptor (TLR) and IFN-γ signaling pathways with respect to LPS–TLR4 and polyinosinic-polycytidylic acid (poly I:C)–TLR3 ligations (Zhao et al., 2006). Moreover, the crucial role of IRF8 in the development of Dc subtypes and the amplification of IFN production in plasmacytoid Dcs (pDcs) makes it a likely target for poly I:C-activated signaling pathways (Gabriele and Ozato, 2007; Tailor et al., 2007).

Recently, tremendous progresses have been made in characterization of IRF family members in fish species known to be critical for innate immune defense in mammals. However, little data are available presently about
Fig. 2. Neighbor-joining phylogenetic tree of IRF family proteins in vertebrates. The SmIRF8 (turbot IRF8) is underlined in the tree. Accession numbers: IRF1 (human, X14454; mouse, M21065; chicken, L39766; African clawed frog, NP_001083250; zebrafish, ENSDARP00000048292; Atlantic salmon, NP_001117117; Japanese flounder, AB005883); IRF2 (human, X15949; mouse, BC006577; chicken, X05478; African clawed frog, NP_001087095; Atlantic salmon, BT044804; Japanese flounder, JF312911); IRF3 (human, NM_001571; mouse, U75839; African clawed frog, NM_001086119; zebrafish, NP_001137376; Atlantic salmon, NM_001172282; turbot, HQ424131); IRF4 (human, U52682; mouse, AK089319; chicken, AF320331; zebrafish-a, ENSDARP0000005897; zebrafish-b, ENSDARP00000119326; stickleback-a, ENSGACP0000021735; stickleback-b, ENSGACP0000006567; Atlantic salmon-b, NM_001139982; Japanese flounder-b, JF312909); IRF5 (human, NM_002200; cow, BT021607; X. laevis, BC082862; Atlantic salmon, BT044767; grass carp, FJ560094); IRF6 (human, AF027292; pig, AF127368; chicken, DQ250731; X. laevis, BC108585; zebrafish, BC056772); IRF7 (human, U73036; mouse, U73037; chicken, U73038; zebrafish, BC058298; Atlantic salmon, BT045216; turbot, HQ424129); IRF8 (Fugu, ENSTRUP00000046607; stickleback, ENSGACP00000021059; Nile tilapia, XM_003442332; the accession numbers of other IRF8 sequences are listed in Table 2); IRF9 (human, M87503; mouse, US1992; African clawed frog, BC070535; zebrafish, NP_091273; Atlantic salmon, NP_001167190); IRF10 (chicken, AF380350; zebrafish, NP_098044; Japanese flounder, A8359170); IRF11 (zebrafish, ENSDARP00000070128); IRF11 (zebrafish, ENSDARP00000070128).
IRF8 in fish when compared with its mammalian counterpart (Holland et al., 2010). Turbot (Scophthalmus maximus) is an important fish cultured widely in North China’s coastal areas, but highly vulnerable to the infection of turbot red-dish body iridovirus (TRBIV). The characterization of IRF8 in turbots will undoubtedly promote the knowledge of turbot immune defense system which is essential for the development of effective strategies for infectious disease control of this economically valued species. Previously, some work had been done to the IRF family molecules such as IRF3 (Hu et al., 2011a), IRF5 (Xia et al., 2012) and IRF7 (Hu et al., 2011b) in turbots in our lab. Here, we reported the study of turbot (Sm)IRF8. We firstly cloned SmIRF8 gene and then characterized its features including the mRNA tissue distribution and expression in response to poly I:C and TRBIV challenges.

2. Materials and methods

2.1. Fish and challenges of poly I:C and TRBIV

Turbot (S. maximus) juveniles (68.4 ± 4.5 g, n = 170) were purchased from a local fish farm and kept in aerated seawater tanks at 16 °C for 1 week before use. Two groups of turbots were intraperitoneally (i.p.) injected with poly I:C (Sigma, St Louis, MO, USA) (10 mg/ml, 100 μl per fish) or TRBIV (2 × 10^6 TCID50/ml, 120 μl per fish), respectively. Control fish were injected with the same volume (100 or 120 μl per fish, respectively) of phosphate-buffered saline (PBS). The intact healthy tissue were used for tissue distribution analysis, while the spleen, head kidney, gills and muscle of injected fish were collected at various days post injection (0, 0.5, 1, 2, 3, 4, 5 and 7 days after poly I:C injection or 0, 1, 2, 3, 4, 5 and 7 days after TRBIV injection) for gene expression assay.

2.2. RNA and genomic DNA extraction

Total RNA was extracted from brain, gills, stomach, intestine, heart, head kidney, kidney, liver, spleen, gonad, muscle and skin of turbots using an Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. RNA samples were treated with DNase I to remove genomic DNA contamination using the Turbo DNA-free kit (Ambion, Austin, TX, USA). The RNA concentration was determined by measuring absorbance at 260 nm, and its quality was monitored by A260nm/A280nm ratios > 1.8. The genomic DNA was isolated from the head kidney by the standard phenol/chloroform extraction procedure (Palumbi et al., 1991).

2.3. Cloning of SmIRF8 cDNA and gene

The SMART cDNAs were synthesized using a cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) by following the manufacturer’s protocol. On the basis of the conserved sequences of known vertebrate IRF8s, degenerate primers (Table 1) were designed. The 794-bp partial sequences of SmIRF8 were obtained by homology cloning. Both 3′- and 5′-rapid amplifications of cDNA ends (RACE) were performed by two rounds of PCR using two sets of gene-specific primer (Table 1) and adaptor primer (supplied by the kit) combinations. The genomic PCR was performed with the genomic DNA template and the gene-specific primers (Table 1) designed based on the full-length cDNA sequence. All sequences mentioned across this group were amplified using Ex Taq DNA polymerase (TaKaRa, Dalian, Liaoning, China) under the following condition: initial denaturation at 94 °C for 4 min, then 25–40 cycles of 94 °C for 40 s, 48.9–61 °C for 30 s and 72 °C for 40 s–2 min, and final extension at 72 °C for 7 min. The PCR products were isolated using an E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, Doraville, GA, USA), cloned into pMD18-T vector (TaKaRa) and sequenced with an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA). The exon/intron structure of the SmIRF8 gene was determined by alignment of the cDNA to the genomic sequence using Genetyx 7.0 software (GENETYX Corporation, Tokyo, Japan). The SmIRF8 sequence was analyzed for homology with other known sequences by the NCBI BLAST program. Multiple sequence alignment was generated by the Clustal W program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). The phylogenetic tree was depicted on the overall amino acid sequences using the neighbor-joining algorithm within MEGA version 3.1 (Kumar et al., 2004).

2.4. Quantitative real time PCR (qPCR)

qPCR analysis was employed to investigate SmIRF8 mRNA tissue distribution and expression in response to poly I:C and TRBIV challenges in specific organs. 1.0 μg of total RNA from each tissue (5 individuals for each time point) was reverse-transcribed into cDNA by random primers using Superscript First Strand Synthesis System (Invitrogen). qPCR was conducted in 20 μl volume containing 1 × SYBR Green Real Time PCR Master Mix (Toyobo, Osaka, Japan), 0.2 μM each of gene-specific forward and reverse primers (Table 1) and 1.0 μl diluted cDNA (50 ng/μl). PCR conditions were 94 °C for 4 min, followed by 40 cycles of 94 °C for 40 s, 61.5 °C for 30 s, 72 °C for 20 s, and final elongation at 72 °C for 7 min and 4 μl for 5 min. Turbot 18S rRNA (GenBank accession number: EF126038) was used as endogenous control. All samples were amplified in triplicates. Fluorescent detection was performed after each extension step. A dissociation protocol was performed after thermocycling to verify that a single amplicon of expected size was amplified. The expression levels of target genes were normalized to 18S rRNA and expressed as fold change relative to the expression level in control which was set to 1 in the analysis of gene expression upon immune challenges.

2.5. Statistical analysis

Statistical analysis was performed using SPSS13.0 software (SPSS Inc., Chicago, IL, USA). Differences in the data were compared by one-way analysis of variance (ANOVA) followed by Duncan’s post hoc test for multiple comparisons. Differences were considered significant at P < 0.05.
**Table 1**

Primer sequences used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5'–3')</th>
<th>Target gene</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRF8-hF1</td>
<td>ATACCTGGAARCGYCGNCTA</td>
<td>SmIRF8</td>
<td>First round homology PCR</td>
</tr>
<tr>
<td>IRF8-hF2</td>
<td>GATTAYAYACARGATGGGAYG</td>
<td>18S rRNA</td>
<td>Nested homology PCR</td>
</tr>
<tr>
<td>IRF8-gF1</td>
<td>CGAGGGAATGCAGTACCTGG</td>
<td>SmIRF8</td>
<td>First round 3'–RACE PCR</td>
</tr>
<tr>
<td>IRF8-gR2</td>
<td>GAGAGGGATGCTGTCGAC</td>
<td>SmIRF8</td>
<td>Nested 3'–RACE PCR</td>
</tr>
<tr>
<td>IRF8-5F1</td>
<td>AGAGGTCTGAGCTGGTCGAC</td>
<td>SmIRF8</td>
<td>Genomic PCR</td>
</tr>
<tr>
<td>IRF8-3F1</td>
<td>AGACATGTCCAGGATTGGGT</td>
<td>SmIRF8</td>
<td>Tissue PCR</td>
</tr>
<tr>
<td>18sRNA-R</td>
<td>ATTCCTGGCAATACTGCAC</td>
<td>SmIRF8</td>
<td>qPCR</td>
</tr>
</tbody>
</table>

Note: (1) N represents all four nucleotides; D, A, T or G; R, G or A; Y, C or T.

3. **Results**

3.1. **Molecular characterization of SmIRF8**

The cDNA sequence of SmIRF8 (GenBank accession number: JQ219663) is 2317 bp long and contains a 1263 bp coding region encoding a putative protein of 420 amino acid residues. The cDNA had a 125-bp 5'-untranslated (UTR) and a 929-bp 3'-UTR where three mRNA instability motifs (ATTTA) and two polyadenylation signals (AATAAA) were found. The predicted protein sequence shares the highest identity of 64.4–69.7% to fish IRF8 in a BLAST analysis (Table 2) and possesses three conserved domains: an N-terminal DNA-binding domain (DBD) characterized by a five tryptophan pentad-repeat, a C-terminal IRF association domain (IAD) and a bipartite nuclear localization signal (NLS) in the DBD (Fig. 1). Phylogenetic analysis separated the members of the IRF family into four subfamilies: IRF1 and IRF2 making up into the IRF1 subfamily, IRF3 and IRF7 into the IRF3 subfamily, IRF4, IRF8, IRF9 and IRF10 into the IRF4 subfamily, IRF5 and IRF6 into the IRF5 subfamily. In the tree, SmIRF8 exhibited the closest phylogenetic distance to stickleback IRF8 (Fig. 2).

3.2. **Genomic structure of SmIRF8**

The SmIRF8 gene sequence (GenBank accession number: JQ219665) is 4363 bp long, consisting of 9 exons and 8 introns, with one intron existing in the 5'-UTR (Fig. 3). All the 5'- and 3'-ends of the introns show canonical splicing motifs (GT/introns/AG) (Breathnach and Chambon, 1981).

![Fig. 3](image)

Fig. 3. Schematic diagram of the exon–intron arrangement of SmIRF8 gene. Exons are shown by boxes and introns by straight or concave lines. Untranslated regions are indicated by white boxes. The number of nucleotides in each exon and intron was shown above and below the corresponding element, respectively.

3.3. **Tissue distribution of SmIRF8 mRNA**

The tissue distribution of SmIRF8 mRNA was examined by qPCR in twelve tissue types including brain, gills, stomach, intestine, heart, head kidney, kidney, liver, spleen, gonad, muscle and skin of healthy turbots (Fig. 4). The constitutive expression of SmIRF8 was observed in all tissues examined although at a low level in the skin, muscle and liver. The highest level was found in the spleen, followed by the kidney and head kidney. In other tissues, a similar moderate level was seen.

3.4. **Induction of SmIRF8 transcripts by poly I:C and TRBIV**

In order to obtain further insights into the roles of SmIRF8 in immune response, its gene expression was investigated by qPCR in the four selected tissues, gills, head kidney, spleen and muscle, of poly I:C or TRBIV-challenged turbots. As shown in Fig. 5, the SmIRF8 was up-regulated by as shown in Table 3. The coding sequences are 99.9% matched with the mRNA sequence.
both poly I:C stimulation and TRBV infection. Upon challenge of poly I:C, SmIRF8 was markedly induced in the spleen with a peak transcript level of 8.0-fold over control arising at day 0.5 post-stimulation and less induced in the head kidney and gills where the maximum induction is 5.1- and 3.3-fold arising at day 0.5 and 1 post-stimulation, respectively. The induction in the muscle was weak with a peak level of 2.8-fold arising at day 1 post-stimulation. Upon challenge with TRBV, the maximum induction levels of SmIRF8 were 6.9-, 3.8-, 2.9- and 2.5-fold and appeared at day 1, 2, 2 and 2 post-infection in the spleen, head kidney, gills and muscle, respectively.

4. Discussion

In the present study, we cloned the cDNA and genomic sequences of an IRF8 homologue from head kidney of turbot. The cDNA contains a 1263 bp open reading frame (ORF) that encodes 420 aa. In accordance with other inflammatory mediators, the 3′-UTR of the sequence possesses three ATTTA instability motifs, suggesting that it may be transiently expressed. The SmIRF8 gene possesses the same structure of 9-exon and 8-intron as other vertebrate IRF8s (Huang et al., 2010). According to the IRF-like structure of the inferred peptide and homology with fish IRF8 orthologs, the cloned sequence was identified as SmIRF8. Furthermore, the phylogenetic analysis confirmed the credibility of the nomenclature for SmIRF8 by grouping it with fish IRF8s in the vertebrate IRF8 clade. The SmIRF8 protein has three conserved domains, an N-terminal DBD, a C-terminal IAD and a NLS in the DBD, which were also found in other known IRF8s. The DBD is typical of all IRF members and possesses five tryptophan repeats, being located in the 13, 28, 40, 60 and 79 residues (Fig. 1), which are highly conserved throughout the IRF family (Eroshkin and Mushegian, 1999; Nehyba et al., 2002). The tryptophan pentad-repeat forms a helix–turn–helix motif that binds to ISRE consensus sequences found in target promoters (Honda and Taniguchi, 2006; Paun and Pitha, 2007). The IAD is another conserved domain in IRF family members except IRF1 and IRF2 and plays an important role in the formation of IRF homo/hetero-dimers and associations with other transcription factors (Eroshkin and Mushegian, 1999). The NLS is related to nuclear translocation and reservation of IRFs. It has been identified in IRF1, IRF3, IRF4, IRF5, IRF8 and IRF9, most of which have a single NLS in the amino-terminal domain just like SmIRF8, only IRF5 contains two NLSs (Barnes et al., 2002b).

The SmIRF8 transcripts were detectable in all examined tissues of healthy fish by a qPCR analysis. In mammals, IRF8 is usually expressed in immune tissues. Similar with the expression profile of mammalian IRF8, SmIRF8 was found to be expressed more abundantly in known lymphomyeloid-rich tissues, such as the spleen, kidney and head kidney tissues, but less abundant in the skin.
Fig. 5. qPCR analysis of SmIRF8 expression profiles in turbot challenged with 2.4 × 10^9 TCID_{50} or 1.0 mg poly I:C per fish during a 7-day time course. (A–D) Fold changes of SmIRF8 expression in the gills, head kidney, spleen and muscle with poly I:C stimulation, respectively; (E–H) Fold changes of SmIRF8 expression in the gills, head kidney, spleen and muscle with TRBIV infection, respectively. Each data point is expressed as the mean of five replicates ± standard error. (S.E.)

muscle and liver. Thus, it suggests that SmIRF8 plays a role predominantly in the immune system.

Previous studies have demonstrated that both poly I:C and TRBIV can cause antiviral responses in turbots where IRF3, IRF5 and IRF7 play a role (Hu et al., 2011a,b; Xia et al., 2012). In this study, we investigated whether SmIRF8 is involved in this process by a gene expression analysis. We selected the gills, head kidney and spleen to perform the study due to their immune importance in fish species; we also performed the study in the muscle in order to determine whether SmIRF8 expression alters in this non-immune organ. Although the SmIRF8 was found to be up-regulated by the stimulation of both poly I:C and TRBIV, the induction by poly I:C was earlier and a bit stronger. Poly I:C is a synthetic mimic of dsRNA and recognized by TLR3 and TLR22 (Matsuo et al., 2008). It is also a well-established inducer of fish type I IFNs and ISGs (Bergan et al., 2006). On the contrary, TRBIV is an enveloped dsDNA virus, a virus type which can mimic the ability of bacterial DNA to activate immune cells such as macrophages, B-cells, NK-cells and Dcs to produce cytokines through TLR9 signaling pathway (He et al., 2010; Krug et al., 2004). Therefore, it is not strange that the induction kinetics by the two stimuli are different. The up-regulation of IRF8 by poly I:C treatment was also reported in rainbow trout (Holland et al., 2010) and mammals (Démoulins et al., 2009). Further, mammalian studies showed that IRF8 stimulates the IFN response mainly through the retinoic-acid inducible gene-1 (RIG-I) and TLR3 pathways (Alexopoulos et al., 2001). In addition, our study showed that in both poly I:C and TRBIV challenge cases, the spleen exhibited the highest and earliest increase for SmIRF8, suggesting an immune importance of this organ and its easy contact with the i.p. injected stimuli, whereas the muscle had the lowest induction of SmIRF8 which may be explained by a non-immune property of the muscle and the fact that the target tissues of the virus are limited to the epithelial and connective tissues (Shi et al., 2004), but it suggests a role of SmIRF8 in the non-immune tissues. Compared with other known molecules in turbot IFN system, SmIRF8 has a lower inducibility than IRF3 or
IRF7, but the initiation and peak time points of its induction are very close to those of IRF3 and prior to Mx, a known ISG (Hu et al., 2011a; Xia et al., 2012). Collectively, these results provide a possibility that SmIRF8 plays a role in antiviral IFN response as reported for mammalian IRF8.

In summary, we identified and characterized the structure and expression pattern of an IRF8 gene in turbot in the present study. We demonstrated that SmIRF8 expression is induced by both poly I:C and TRBV1 in an early phase of the treatment. Our findings may help a further understanding of the functions and evolution of vertebrate IRF8.

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