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IFN Regulatory Factor 10 Is a Negative Regulator of the IFN Responses in Fish

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IFN regulatory factor (IRF) 10 belongs to the IRF family and exists exclusively in birds and fish. Most IRFs have been identified as critical regulators in the IFN responses in both fish and mammals; however, the role of IRF10 is unclear. In this study, we identified IRF10 in zebrafish (Danio rerio) and found that it serves as a negative regulator to balance the innate antiviral immune responses. Zebrafish IRF10 (DrIRF10) was induced by intracellular polyinosinic:polycytidylic acid in ZF4 (zebrafish embryo fibroblast-like) cells. DrIRF10 inhibited the activation of zebrafish IFN1 (DrIFN1) and DrIFN3 promoters in epithelioma papulocystis cells in the presence or absence of polyinosinic:polycytidylic acid stimulation through direct interaction with the IFN promoters, and this inhibition was also shown to block IFN signaling. Overexpression of DrIRF10 was able to abolish the induction of DrIFN1 and DrIFN3 mediated by the retinoic acid–inducible gene I–like receptors. In addition, functional domain analysis of DrIRF10 showed that either the DNA binding domain or the IRF association domain is sufficient for its inhibitory activity for IFN signaling. Lastly, overexpression of DrIRF10 decreased the transcription level of several IFN-stimulated genes, resulting in the susceptibility of host cells to spring viremia of carp virus infection. Collectively, these data suggest that DrIRF10 inhibits the expression of DrIFN1 and DrIFN3 to avoid an excessive immune response, a unique regulation mechanism of the IFN responses in lower vertebrates. The Journal of Immunology, 2014, 193: 000–000.

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

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Address correspondence and reprint requests to Prof. Yong-An Zhang, State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, 7 Donghu South Road, Wuhan 430072, China. E-mail address: yzhang@ihb.ac.cn

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Abbreviations used in this article: CaRIR, RLR of crucian carp; ChIP, chromatin immunoprecipitation; Co-IP, coinmunoprecipitation; DBD, DNA binding domain; DrIFN, zebrafish (Danio rerio) IFN; DrIRF, zebrafish (Danio rerio) IRF; EPC, epithelioma papulocystis cyprinid; IAD, IRF association domain; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN stimulatory response element; LGP2, laboratory of genetics and physiology 2; MITA, mediator of IRF3 activation; LGP2, lacking the two caspase recruitment domains in the N terminus, is believed to act as a negative regulator of the RLR signaling pathway (3–7). Recently, several reports demonstrated that a functional RLR signaling pathway also exists in fish, suggesting that the RLR-mediated antiviral pathway is conserved from lower vertebrates to mammals (8–13).

Although production of IFN activated by RLR molecules is necessary for the host to defend against a virus infection, unrestricted expression of IFN can be hazardous to the host, resulting in cellular toxicity and autoimmune diseases (14). Compared with the proteins that upregulate IFN expression, there is another molecular system that negatively regulates the RLR signaling response to prevent the host from unnecessary IFN activation under a steady-state situation (15). Besides LGP2, several other proteins were described as supporting the negative regulation of RLR signaling. For example, IFN-stimulated gene 15 (ISG15) and dihydroxyacetone kinase sequester RIG-I and MDA5, respectively, to reduce IFN expression (16, 17). In addition, three proteins have also been identified to balance MITA-mediated IFN response in mammals, the E3 ubiquitin ligase RING-finger protein 5, E3 ubiquitin ligase tripartite motif protein 32, and ISG56. All of them were found to interact and disrupt MITA activation after viral infection (18–20). However, to date, none of IRFs has been identified as a negative regulator targeting MITA to block RLR signaling.

The IRF family proteins are involved in regulation of IFN gene expression in innate and adaptive immune responses (2, 21). To date, nine members of the IRF family have been found in mammals. Most IRF proteins contain a DNA binding domain (DBD) at the N terminus and an IRF association domain (IAD) at the C terminus. The DBD is critical for interaction of IRF with IFN promoters that upregulate IFN expression, there is another molecular system that negatively regulates the RLR signaling response to prevent the host from unnecessary IFN activation under a steady-state situation (15). Besides LGP2, several other proteins were described as supporting the negative regulation of RLR signaling. For example, IFN-stimulated gene 15 (ISG15) and dihydroxyacetone kinase sequester RIG-I and MDA5, respectively, to reduce IFN expression (16, 17). In addition, three proteins have also been identified to balance MITA-mediated IFN response in mammals, the E3 ubiquitin ligase RING-finger protein 5, E3 ubiquitin ligase tripartite motif protein 32, and ISG56. All of them were found to interact and disrupt MITA activation after viral infection (18–20). However, to date, none of IRFs has been identified as a negative regulator targeting MITA to block RLR signaling.

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stimulatory response elements (ISREs) in the promoter region of most ISGs (22, 23). In contrast, the IADs, except for that of IRF1 and IRF2, form a homodimer that recruits other transcription factors (21). Most IRFs are positive regulators of IFN expression (22). For example, IRF3 is central in triggering the expression of IFN and other antiviral genes when activated by RLRs (24, 25). However, not all IRFs induce IFN expression. IRF2 and IRF4 are known negative regulators. IRF2 shares high sequence homology with IRF1 and serves as a transcriptional repressor of expression of IFN and ISGs by competing with IRF1 (26–28). IRF4 interacts with IRF5 and balances the antiviral responses when host cells were infected by virus (29, 30).

In fish, 10 members of the IRF family have been identified and characterized (31–37). IRF10 is present in nonmammals including birds and fish (34, 38, 39). The expression of IRF10 was analyzed in chicken (Gallus gallus; GgIRF10). In contrast with other proteins that have abundant basic expression for immediate responses after virus infection, synthesis of GgIRF10 was delayed and shown to require cellular signal transduction, indicating that it plays an important role at later stage of the host antiviral defense. Specifically, GgIRF10 was found to be involved in the upregulation of the MHC class I by binding to the ISRE site in the promoter, suggesting that GgIRF10 also plays a role in adaptive immunity (40). More recently, the expression pattern of IRF10 has been analyzed in Japanese flounder (Paralichthys olivaceus; PohlIRF10), and the data indicated that PohlIRF10 is involved in innate immune responses (41). However, the biological function and signaling pathway of IRF10 have not been characterized in fish.

In this study, we report the functional characterization of zebrafish (Danio rerio) IRF10 (DrIRF10) in the regulation of IFN transcription. The expression of DrIRF10 was studied in zebrafish embryo fibroblast-like (ZF4) cells in response to polyinosinic-polycytidylic acid (poly I:C) stimulation. Overexpression of DrIRF10 in epithelioma papulosum cyprinid (EPC) cells was able to inhibit the activations of DrIFN1, DrIFN3, and EPC IFN promoters directly. Moreover, DrIRF10 was shown to repress the activation of DrIFN1 and DrIFN3 promoters by the RLR signaling molecules such as RIG-I, MDAs, MTA, TBK1, and IRF3. Further domain analysis demonstrated that the DBD is sufficient for the suppressive effect of the DrIRF10, whereas the IAD inhibits the activation of DrIFN1 and DrIFN3 promoters by interacting with DrMTA. Lastly, overexpression of DrIRF10 decreased the expression of several IFN-related genes, resulting in a weakened antiviral resistance of EPCs to spring viremia of carp virus (SVCV) infection. Taken together, our findings demonstrate that DrIRF10 is a negative regulator of IFN production in fish.

Materials and Methods

Cells and transfection

ZF4 cells were cultured at 28°C in DMEM/F12 medium supplemented with 10% heat-inactivated FBS. EPC cells were cultured at 28°C in M199 medium supplemented with 10% FBS. HEK 293T cells were maintained at 37°C, 5.0% CO2 in DMEM medium supplemented with 10% FBS. Transfection was performed according to a previous report (42). In brief, EPC cells were seeded in 24-well plates overnight and transfected with 0.5 or 0.75 μg plasmids suspended in 1 μl Lipofectamine 2000 (Invitrogen) and 100 μl OPTI-MEM I Reduced Serum Medium (Invitrogen). At 6 h after transfection, the cell medium was replaced by fresh M199 medium with 10% FBS. For stimulation experiments, ZF4 cells seeded in six-well plates were transfected with 2 μg/ml poly I/C for 3, 6, 12, 24, and 48 h, then harvested and resolved in TRIZol Reagent (Invitrogen) for RNA extraction. In chromatin immunoprecipitation (ChIP) and coimmunoprecipitation (Co-IP) assays, 293T cells seeded in 10-cm dishes were cotransfected with 5 μg HA-tag plasmids and 5 μg Flag-tag plasmids or relative promoter constructions. Forty-eight hours later, the cell lysates were immunoprecipitated with anti-HA-Agarose beads (Sigma). Then the immunoprecipitates were analyzed by semiquantitative PCR or Western blotting.

Gene cloning and plasmids

The open reading frame (ORF) of DrIRF10 and the DBD (aa 7–116) deletion mutants were generated by PCR and cloned into the Nhel/Xhol sites of pcDNA3.1/myc-His(+)/A vector (Invitrogen). The ORF sequences of the genes in the RLR signaling pathway of zebrafish including DrRIG-I-Nter (the N terminus of zebrafish RIG-I), DrMDA5, DrMTA, DrTBK1, DrIF3, and the EPC IFN promoter were obtained from public database and cloned into pcDNA3.1/myc-His(+)A vector (Invitrogen) and pG3-Basic luciferase (LUC) reporter vector (Promega) (9). The ISRE LUC reporter construct containing five ISRE motifs in series was purchased from Stratagene. In ChIP experiments, the wild type DrIFN1 promoter and ISRE motif mutations were cloned and inserted into the KpnI/Xhol sites of pGL3-Basic LUC reporter vector. The ORF of DrIF3 and DrIF3 with an N-terminal HA-tag sequence were cloned into the Nhel/Xhol sites of pcDNA3.1(+)/vector. In Co-IP experiments, the PCR-based mutagenesis method was used for plasmid construction. PCR fragments encoding DrIRF10 and its truncated mutants (the DBD and IAD) with an N-terminal HA-tag sequence were cloned into the Nhel/Xhol sites of pcDNA3.1(+)/vector. The ORF of DrMTA with a C-terminal Flag-tag sequence was cloned into the EcoRI/Xhol sites of pcDNA3.1(+)/vector. The crucian carp (Carassius auratus L.) RLR molecules and zebrafish IFN promoters were described previously (11). All constructs were verified by sequencing, and primers used in this study are listed in Supplemental Table I.

RNA extraction and real-time PCR

Total RNA of ZF4 and EPC cells, as well as tissues from healthy zebrafish, were extracted by TRIZol Reagent (Invitrogen). cDNA was synthesized using random primers (NNNNNN, N means random nucleotide) and the M-MLV reverse system (Promega). Real-time PCR was performed in a DNA Engine Chromo 4 real-time system (BioRad) with SYBR green real-time PCR master mix (BioRad). Real-time PCR conditions were as follows: 94°C for 5 min, then 94°C for 30 s, 60°C for 30 s, and 72°C for 10 s for 30 cycles, followed by 72°C for 5 min. Reactions were performed in a 20-μl volume containing 10 μl SYBR green real-time PCR master mix (BioRad), 7 μl double-distilled water, 1 μl of each forward and reverse primers, and 1 μl cDNA template. The expression of the test genes was calculated as relative expression to β-actin using the 2−ΔΔCt method, and samples were analyzed in triplicates (43). The primers are listed in Supplemental Table I.

LUC activity assay

For LUC activity assays, EPC cells were seeded in 24-well plates overnight before transfection. The PRL-TK vector (Promega) was used to normalize the expression level. Cells were harvested and lysed according to the manual of Dual-Luciferase Reporter Assay System (Promega). LUC activities were measured by Junior LB9509 Luminometer (Berthold) and normalized to the amount of Renilla LUC activities. The mean of three independent experiments was used for statistical analysis.

Co-IP assay

The HEK 293T cell lysates were prepared using lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, with protease inhibitors [Roche], pH 7.5). The cellular debris was removed by centrifugation at 13,000 × g for 10 min at 4°C. The supernatant was transferred to a fresh tube and incubated with anti–HA-Agarose beads (Sigma) overnight at 4°C. The target proteins bound to the HA-tagged proteins were precipitated, washed, and resuspended in 2× SDS sample buffer, then boiled and resolved on SDS-PAGE in 12% polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes (BioRad), which were probed with an anti-Flag (1:2000; Sigma) or anti-HA mAb (1:2000; Sigma). The membrane was incubated with a HRP-conjugated goat anti-mouse IgG (1:5000; Thermo). After washing with TBST, the membrane was stained using the ECL system according to the manufacturer’s instructions.

ChIP assay

For the ChIP assay, intracellular protein–DNA complexes were cross-linked in situ by addition of 1% formaldehyde at room temperature for 10 min, and the reaction was stopped by adding glycine to 0.125 M. After lysis, the cell lysates were clarified by centrifugation at 12,000 × g for 10 min. Five percent of the clarified cell lysates, as set the input sample, were saved as a control in semiquantitative PCR to relatively quantify the DNA concen-
tation. The resulting cell lysates were incubated with anti–HA-Agarose beads overnight at 4°C. Immunoprecipitated HA-tagged protein–DNA complexes were washed four times with RIPA buffer (Sigma). The precipitated DNA was treated with RNase (Sigma) for 30 min at 37°C and with Proteinase K (Sigma) for 6 h at 65°C. The DNA was purified using a PCR purification kit (Omega). Purified DNA was analyzed by semiquantitative PCR using specific primers (Supplemental Table I).

Antiviral effect evaluation

EPC cells seeded in 24-well plates were transfected with 0.5 μg DrIRF10 construct or empty vector. At 24 h after transfection, the EPC cells were washed and infected with 10 tissue culture infective dose 50 (TCID₅₀) SVCV per well, respectively. SVCV was propagated in EPC cells using methods reported previously (44).

Results

Molecular characterization of DrIRF10

The ORF of DrIRF10, amplified from a cDNA library of ZF4 cells stimulated with poly I:C, encodes a protein of 392-aa residues. Multiple sequence alignment revealed that DrIRF10 is highly homologous to the IRF10s in other fish and chicken, which possesses a conserved N-terminal DBD, a C-terminal IAD, and a putative nuclear localization signal within the DBD (Supplemental Fig. 1).

Expression pattern and tissue distribution of DrIRF10

To investigate the induction pattern of DrIRF10 in vitro, we performed real-time PCR to assess the transcription of DrIRF10 in ZF4 cells transfected with poly I:C. The DrIRF10 expression was induced at 3 h after poly I:C transfection and reached a peak at 6 h, suggesting it is likely a typical ISG (Fig. 1A). DrIFN1, DrIFR3, and DrIRF7, known as antiviral genes and IFN regulators (45, 46), were also upregulated (Fig. 1B–D). The tissue distribution of DrIRF10 was examined in 10 different tissues of healthy zebrafish. As shown in Fig. 1E, the transcripts of DrIRF10 were detected in the trunk kidney, heart, and testis, with a relatively lower level in the brain, eye, gill, and spleen, but little in the intestine, liver, and ovary.

DrIRF10 inhibits the activation of DrIFN1, DrIFN3, and EPC IFN promoters

For higher transfection efficiency, EPC cells, instead of ZF4 cells, were chosen for overexpression plasmid transfection. Three constructs of zebrafish IFN promoters (DrIFN1pro, DrIFN2pro, and DrIFN3pro) representing group I and group II IFNs described in a previous report (11) were used to analyze the biological function of DrIRF10. Initially, the activation of DrIFN1pro and DrIFN3pro was confirmed by transfection of poly I:C in EPCs where DrIFN1pro and DrIFN3pro activities were significantly induced by poly I:C (7- and 20-fold compared with the nonstimulated group, respectively). However, no induction was observed for DrIFN2pro (Fig. 2A). Subsequently, the effect of DrIRF10 on the DrIFN1pro, DrIFN2pro, and DrIFN3pro activities was examined after transient transfection of DrIRF10. The LUC activities driven by DrIFN1pro and DrIFN3pro, but not DrIFN2pro, were severely inhibited by DrIRF10 (Fig. 2B), whereas the LUC activities were enhanced by DrIRF9 (Fig. 2C). In addition, overexpression of DrIRF10 abrogated poly I:C–induced activation of DrIFN1pro and DrIFN3pro (Fig. 2D).

To further clarify the inhibition mechanism of DrIRF10, we analyzed the activation of ISRE-Luc by overexpression of DrIRF10 in EPC cells. As predicted, the ISRE-Luc activity was strongly inhibited in DrIRF10 transfected cells compared with the cells transfected with vector plasmid (Fig. 2F).

To further clarify the inhibition mechanism of DrIRF10, the ChIP assay was used to analyze the interaction between DrIRF10 and DrIFN1pro or DrIFN3pro. As shown in Fig. 2G and 2H, when 293T cells were cotransfected with the empty vector or DrIRF10 and DrIFN1pro or DrIFN3pro, DrIRF10 was found to bind to the DrIFN1 or DrIFN3 promoter directly. These results illustrate that DrIRF10 negatively regulates the expression of DrIFN1, DrIFN3, and EPC IFN in EPC cells by interacting with their promoter regions.
FIGURE 2. Inhibition of DrIFN1 and DrIFN3 by overexpression of DrIRF10. (A) Induction of DrIFN1, DrIFN2, and DrIFN3 promoters by poly I:C. EPCs seeded in 24-well plates overnight were transfected with DrIFN1pro-Luc, DrIFN2pro-Luc, or DrIFN3pro-Luc; pRL-TK was used as an internal control. At 24 h after transfection, cells were treated with poly I:C or left untreated. The LUC activities were monitored at 24 h after stimulation. (B and C) Activation of DrIFN1, DrIFN2, and DrIFN3 by overexpression of DrIRF10 or DrIRF9. EPC cells were cotransfected with DrIRF9 or DrIRF10 and DrIFN1pro-Luc, DrIFN2pro-Luc, or DrIFN3pro-Luc at the ratio of 1:1. The LUC activities were assessed at 48 h posttransfection. (D–F) Inhibition of DrIFN1, DrIFN3, EPC IFN, and ISRE by overexpression of DrIRF10 in poly I:C-stimulated cells. EPC cells were cotransfected with DrIFN1pro-Luc, DrIFN3pro-Luc, EPC IFNpro-Luc, or ISRE-Luc and DrIRF10, then stimulated with poly I:C at 24 h posttransfection. LUC activities were monitored at 24 h after stimulation. Error bars are the SDs obtained by measuring each sample in triplicate. Asterisks indicate significant differences between control and treatment groups (*p < 0.01). The p values were calculated by Student t test. (G and H) ChIP semiquantitative PCR analysis of DrIRF10 binding to DrIFN1 and DrIFN3 promoters in 293T cells. 293T cells seeded in 10-cm dishes were cotransfected with 5 μg HA-DrIRF10 and 5 μg DrIFN1pro or DrIFN3pro. Empty vector pcDNA3.1 (5 μg) was transfected in parallel as control. After 48 h, cell lysates were immunoprecipitated with anti–HA-Agarose beads. Then the DNA binding to DrIRF10 was checked by semiquantitative PCR. The DrIFN1 promoter sequences amplified by the primer pairs 1, 2, and 3 are located at −549 to −427, −342 to −252, and −179 to −55 bp, respectively. The DrIFN3 promoter sequences amplified by the primer pairs 1, 2, and 3 are located at −1422 to −1258, −1394 to −1265, and −1244 to −1109 bp, respectively. The input was used as a control in semiquantitative PCR to quantify the DNA concentration.
FIGURE 3. DrIRF10 represses the activation of DrIFN1 and DrIFN3 induced by RLRs. DrIFN1 (A) and DrIFN3 (B) activities were downregulated by DrIRF10 in RLR signaling pathway. EPC cells were cotransfected with DrRIG-I-Nter, DrMDA5, DrMITA, DrTBK1 or DrIRF3, and DrIRF10 plus DrIFN1pro-Luc or DrIFN3pro-Luc at the ratio of 1:1:1. At 48 h posttransfection, cells were collected for detection of LUC activities. The data represented the average of three independent experiments. Error bars are the SDs obtained by measuring each sample in triplicate. (C) Schematic representation of mutated DrIFN1 promoter-driving LUC constructs. The base substitution mutations of each ISRE site are shown. (D) EPC cells were transfected with DrIFN1 or three mutants, then stimulated with poly I:C at 24 h posttransfection. (E and F) EPC cells were cotransfected with DrIRF3, vector or DrIRF10, and DrIFN1 or three mutants; LUC activities were monitored at 48 h after transfection. (G and H) ChIP semiquantitative PCR analyses of DrIRF3 and DrIRF10 binding to DrIFN1 promoter in 293T cells. The cells seeded in 10-cm dishes were cotransfected with 5 μg HA-DrIRF3 or HA-DrIRF10 and 5 μg DrIFN1pro, Mut1pro, Mut2pro, or Mut3pro. After 48 h, cell lysates were immunoprecipitated with anti-HA-Agarose beads. Then the DNA (upper bands) binding to HA-DrIRF3 (G) or HA-DrIRF10 (H) was checked by semiquantitative PCR. The input (lower bands) was used as a control in semiquantitative PCR to quantify the DNA concentration.
**DrIRF10 abolishes RLR-mediated DrIFN1 and DrIFN3 induction**

The fish RLR signaling cascade has been reported as an important pathway to activate the expression of IFN and downstream antiviral genes (8). To determine whether DrIRF10 is involved in negative regulation of the IFN response induced by RLRs, ORFs of the genes involved in the RLR signaling pathway, including DrRIG-I-Nter, DrMDA5, DrMITA, DrTBK1, and DrIRF3, were cloned according to the sequences in a public database. As shown in Fig. 3A, overexpression of key molecules of the RLR cascade led to a significant induction of DrIFN1pro activity, and such activation was severely repressed in cells cotransfected with DrIRF10 expression plasmid. Similarly, the expression of DrIFN3 induced by the RLRs (except DrIRF3) was also inhibited by overexpressed DrIRF10 (Fig. 3B). At the same time, the RLRs of crucian carp (CaRLRs) described in a previous report (11) were also used to analyze the suppression of DrIFN1 and DrIFN3 by DrIRF10. Consistent with zebrafish RLRs, the activation of DrIFN1 and DrIFN3 induced by CaRLRs were also abolished by DrIRF10 (Supplemental Fig. 2A and 2B).

To determine whether DrIRF10 directly or indirectly suppresses IFN expression, three ISRE motif mutants of DrIFN1 were constructed and analyzed using the LUC assay (Fig. 3C). For DrIFN1, the second (located from -2110 to -2101 bp in the 5′UTR) and third (-72 to -63 bp) ISRE motifs were necessary for promoter activity when treated with poly I:C (Fig. 3D). In the cells overexpressing DrIRF3 and a mutant for the third ISRE motif, LUC activity was not elevated, suggesting that the third ISRE motif is required for DrIRF3-induced IFN expression (Fig. 3E). Overexpression of DrIRF10 abolished the activations of DrIFN1 promoter and its mutants induced by DrIRF3 (Fig. 3F). To identify whether DrIRF3 activates DrIFN1 promoter by interacting with...
FIGURE 6. Overexpression of DrIRF10 increases virus replication in SVCV-infected EPCs and inhibits expressions of IFN-related genes. (A and B) Increase of virus replication by overexpression of DrIRF10. EPC cells seeded in 24-well plates overnight were transfected with 0.5 μg DrIRF10 or empty vector. At 24 h posttransfection, EPC cells were infected with SVCV at a dose of 10 TCID<sub>50</sub> per well for 5 d at 28˚C. Then cell monolayers were stained with crystal violet. The culture supernatants from cells infected with SVCV were collected and the viral titer was measured by standard TCID<sub>50</sub> method. Results are representative of two independent experiments. (C) EPC cells seeded in six-well plates were transfected with Flag-DrIRF10 or empty vector. After 24 h, the cells were infected with 10 TCID<sub>50</sub> of SVCV per well for 24 h; then the cells were fixed, permeabilized, and immunoblotted with anti-Flag Ab. The cell monolayers were further stained with FITC and DAPI. Mock-infected EPC cells were used as a control. Green staining represents DrIRF10 protein signal, and blue staining indicates nucleus region (original magnification 3×100; oil immersion objective). Scale bar, 10 μm. (D) Real-time PCR detection of the transcript levels of IFN-related genes by overexpression of DrIRF10 in poly I:C- or SVCV-stimulated cells. EPC cells seeded in six-well plates overnight were transfected with DrIRF10 or empty vector, and stimulated with 2 μg/ml poly I:C or with SVCV at a dose (Figure legend continues)
**FIGURE 7.** Mechanisms of suppression of DrIFN1 and DrIFN3 by DrIFR10. Upon RNA virus infection, viral RNA is recognized by DrRIG-I or DrMDA5, signal of infection transfers to the nuclear through RLR pathway, and then DrIFN1 and DrIFN3 transcriptions are launched to set up an antiviral state. Two mechanisms are found for DrIFR10 to balance this immune reaction. Mainly, DrIFR10 inhibits DrIFN1 and DrIFN3 induction by binding to the ISRE motif in their promoter regions via DBD. In addition, DrIFR10 also interacts with DrMITA via IAD to block DrIFN1 and DrIFN3 activation.

The DBD domain is essential for the inhibitory activity of DrIFR10

The DBD domain of IRFs has been identified as the specific site for binding to the ISRE motif in the promoters of IFNs and ISGs (47). As shown in Fig. 1B, this domain is well conserved in fish. In this study, two truncated mutants, DrIFR10-ΔN lacking DBD domain and DrIFR10-ΔC lacking IAD domain (Fig. 4A), were generated for characterizing the involvement of the domains in regulating the IFN response. DrIFR10-ΔC was shown to suppress the expression of DrIFN1 and DrIFN3 induced by poly I:C, whereas DrIFR10-ΔN had little effect (Fig. 4B and 4D). Similar to wild type DrIFR10, the activation of DrIFN1 induced by DrRIG-I-Nter, DrMDA5, DrMITA, DrTBK1, and DrIFR3 was significantly repressed by DrIFR10-ΔC (Fig. 4C). Not surprisingly, the activation of DrIFN3 induced by these stimulators was also suppressed by DrIFR10-ΔC, except for DrIFR3, because it was incompetent to induce the expression of DrIFN3 as demonstrated in a previous study (11) (Fig. 4E). In contrast, such inhibition was not observed in most DrIFR10-ΔN groups, indicating that the DBD domain is indispensable for DrIFR10 function through its direct binding to the promoter region of DrIFN1 and DrIFN3. In addition, DrIFR10-ΔN had a moderate inhibitory effect on the DrIFN1 and DrIFN3 promoters after induction by DrRIG-I-Nter and DrMITA, suggesting that the IAD of DrIFR10 may be involved in the signaling pathway via protein–protein interaction. Similarly, DrIFR10 also suppressed the induction of DrIFN1 and DrIFN3 induced by CoRLRs; the only difference was that both DrIFN1 and DrIFN3 activated by CoR-I were inhibited by DrIFR10-ΔN, suggesting that such inhibition of IFN production by DrIFR10 is conserved in fish (Supplemental Fig. 3A and 3B).

DrIFR10 blocks DrMITA-mediated DrIFN promoter activities via IAD

Fish MITA has been identified as a positive regulator for the IFN response via the MITA-TBK1-IRF3 signaling pathway (11). Interestingly, DrIFR10-ΔN lacking the N terminal DBD was able to repress the induction of DrIFN1 and DrIFN3 activated LUC activities by both DrRIG-I-Nter and DrMITA, but not DrTBK1. Because fish MITA has been shown to be involved in signaling events between activation of RIG-I and TBK1, we speculated that DrIFR10 may interact with DrMITA via its IAD to suppress DrIFN expression. As shown in Fig. 5, in HEK 293T cells co-transfected with Flag-DrMITA and wild type DrIFR10, DrIFR10-ΔC, or DrIFR10-ΔN, DrMITA was found to interact with wild type DrIFR10 or DrIFR10-ΔN, but not DrIFR10-ΔC, confirming that the C terminal IAD is the domain for interaction with DrMITA.

Overexpression of DrIFR10 suppresses host antiviral response against SVCV infection in EPC cells

SVCV is an ssRNA virus and infects EPC cells efficiently (42). EPC cells were transfected with DrIFR10 and infected with SVCV, to determine the role of DrIFR10 in the host immune response against viral infection. At 5 d postinfection, apparent cytopathic effect was observed in DrIFR10-overexpressed cells, although it was not visible in the control cells (Fig. 6A). This was confirmed by the titer of SVCV, which significantly increased (16-fold) in DrIFR10-overexpressed cells compared with that in the control cells (Fig. 6B). Cellular location analysis demonstrated that DrIFR10 distributes in both nucleus and cytoplasm with or without virus infection (Fig. 6C), which indicated that DrIFR10 exerts inhibition by two mechanisms, DNA binding and protein interaction, to suppress DrIFN expression (Fig. 7). In addition, we analyzed the expression of several IFN-related genes in DrIFR10-transfected EPCs when stimulated with poly I:C. As shown in Fig. 6D, upon infection with SVCV or stimulation with poly I:C, the transcript levels of DrIFN1, RIG-I, and VIG-1 were decreased by over-expression of DrIFR10, whereas the transcripts of β2M (L chain of MHC I) were not affected in EPC cells.

Discussion

Previous studies have shown that the IRF family provides a basis for host innate and adaptive immune responses in both mammals

of 10 TCID₉₀ per well at 24 h posttransfection for 3, 6, 12, and 24 h. Then total RNAs were extracted to examine the mRNA levels of IFN, RIG-I, VIG-1, and β2M transcripts in EPC cells by real-time PCR. The relative transcription levels were normalized to the transcription of β-actin and represented as fold induction relative to the transcription level in the control cells, which was set to 1. Error bars represent SDs obtained by measuring each sample in triplicate.
and fish (13, 21, 48). Compared with the nine mammalian IRF members, fish possess an additional IRF (IRF10), belonging to the IRF4 subfamily, whose function in the immune responses is still unclear. In this article, we demonstrate for the first time, to our knowledge, that fish IRF10 negatively regulates the expression of IFNs for a balanced immune response. Although IRF10 shares highly conserved functional domains with other IRF members, the functional mechanisms of them to regulate IFN responses are totally different (49).

The tissue distribution analysis of DrIRF10 showed that it was expressed in various tissues of zebrafish, and that DrIRF10 possesses the consistent expression pattern with Japanese flounder IRF10 (PoIRF10) in most tissues, such as brain, eye, heart, ovary, as well as lymphoid tissues, such as the kidney and spleen, but not in the gill, intestine, and liver, in which the transcriptions of PoIRF10 were higher (41). These results suggest that IRF10 may possess conserved functions in teleost fish.

As in mammals, fish IFNs exert a pivotal role in the host innate immune system (50, 51). Four zebrafish IFN genes termed DrIFN1 to DrIFN4 can be induced by virus infection. According to the presence of cysteines, they are divided into group I IFN (DrIFN1 and DrIFN4) and group II IFN (DrIFN2 and DrIFN3) (50). In our study, poly I:C, a mimic of virus dsRNA, was able to upregulate DrIFN1 and DrIFN3, indicating that both group I and group II DrIFNs are involved in host antiviral defense. Overexpression of DrIRF10 downregulated the DrIFN1 and DrIFN3 responses induced by poly I:C, whereas the effect of DrIRF10 on DrIFN2 was opposite. Because the DrIFN2 promoter was not upregulated by poly I:C induction, we presumed that it is not involved in the poly I:C-mediated signaling pathway, which could activate the DrIFN1 and DrIFN3 promoters. It appeared as though DrIRF10 inhibits the signaling pathway of poly I:C-mediated DrIFN1 and DrIFN3 induction, but it also plays a positive role in the signaling pathway associated with the regulation of DrIFN2. Previously, the ISRE sites have been identified in the promoter region of DrIFNs (11), which appear to be the binding site for DrIRF10, as shown in this study. Possibly, DrIRF10 competes with other IFN-responsive transcriptional factors for the ISRE sites, and hence antagonizes the cellular response elicited by IFNs. Furthermore, our results demonstrated that the third ISRE site adjacent to the TATA box is responsible for DrIRF10-mediated inhibition of gene expression of IFNs and ISGs.

IRF10 contains two functional domains: N-terminal DBD and C-terminal IAD. This study confirms that the DBD is the core unit that interacts with the promoter of target genes involved in the IFN pathway. These include IFNs because overexpression of the DBD of DrIRF10 blocked DrIFN expression induced by poly I:C or RLRs. The IAD is shown to be engaged in protein–protein interaction with other IRF members or related molecules. To determine what signaling event is interfered by DrIRF10, some known adaptor proteins regulating RLR activated IFN expression, including DrRIG-I-Nter, DrMDA5, DrMITA, DrTBK1, and DrIRF3, were coexpressed in EPC cells with the IAD of DrIRF10. It is interesting that the IAD of DrIRF10 inhibited IFN expression in DrRIG-I-Nter or DrMITA transfected cells but had no effect in DrTBK1 transfected cells. Although the exact roles of MITA have not been fully elucidated, a previous study has shown that MITA is a signaling molecule downstream of RIG-I in the RLR signaling pathway (11). Therefore, DrIRF10 could target one or more of the signaling events before involvement of DrTBK1.

Mammalian IRF2 and IRF4 have been identified as negative regulators in the IFN response. IRF2 and IRF1 both contain a conserved N-terminal DBD, which recognizes the positive ISRE motif in the promoter region of IFNs. However, the regulatory effects of IRF1 and IRF2 on IFN transcription are completely opposite. IRF1 is found to upregulate the expression of IFN-β and IFN-γ, whereas IRF2 inhibits this induction, acting as an antagonist for IRF1 (26, 28, 34). Similarly, IRF4, competing with IRF5 for MyD88 interaction, markedly decreases the induction of proinflammatory cytokines and ISRE-Luc (52). In fish, Atlantic salmon IRF2 could not activate the IFN promoter activity (34); however, zebrafish IRF4 induced the IFN promoter (data not shown), indicating that the modulating mechanisms of fish IRFs on IFN regulation could be different from their mammalian counterparts. In our study, DrIRF10 is functionally similar to mammalian IRF2, negatively regulating IFN production, and such inhibition is performed via competition with DrIRF3 on IFN transcription. During evolution, IRF10, a negative regulator of IFN expression, was eliminated in mammals (40), whose role was replaced by IRF2 in mammals to balance the immune reaction after virus clearance.

Besides fish, IRF10 also exists in birds (40). The transcription of GgIRF10 was not increased within 6 h after poly I:C treatment in chicken embryonic fibroblasts, whereas both mRNAs of PoIRF10 and DrIRF10 were significantly elevated within 6 h after stimulation by poly I:C. In addition, GgIRF10 upregulated the expression of MHC I when treated with IFN, but DrIRF10 could not induce the transcription of MHC I in poly I:C- or SVCV-stimulated EPCs. Although GgIRF10 and DrIRF10 share highly conserved DBD and IAD, with ~76.1% amino acid identity, our results suggest that the immune function of IRF10 is totally distinct between birds and fish, resembling the function of mammalian IRF1 and IRF2, respectively.

This study has identified DrIRF10 as a novel negative regulator of IFN transcription that functions by obstruction of MITA-mediated IFN activation or competitive binding to the ISRE site of the IFN promoter (Fig. 7). The results of this study also illustrate the regulating role of DrIRF10 in the hosts’ innate immune response against virus infection.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Fig. 1. Multiple sequence alignment of IRF10s. The DBD and IAD are lined above amino acid sequences. The nuclear localization signal is lined under amino acid sequences. The identical and similar residues are shaded black and grey, respectively. The sequence gaps are indicated with hyphens. GenBank accession numbers of the amino acid sequences used in the multiple alignment are shown as follows: zebrafish, NP_998044; grass carp, ACT83676; tilapia, XP_005448898; flounder, BAI63220; fugu, XP_003963449; chicken, NP_989889.
Supplemental Fig. 2. *DrIRF10* represses the activation of *DrIFN1* and *DrIFN3* induced by crucian carp RLRs. EPC cells were cotransfected with *DrIRF10* and *CaRIG-I, CaMDA5, CaMITA, CaTBK1* or *CaIRF3* plus *DrIFN1pro-Luc* (A) or *DrIFN3pro-Luc* (B) at the ratio of 1:1:1. At 48 h post-transfection, cells were collected for detection of luciferase activities. The data represented the average of three independent experiments. Error bars are the SDs obtained by measuring each sample in triplicate.
Supplemental Fig. 3. DBD is essential for DrIFR10 to inhibit the activation of DrIFN1 and DrIFN3 induced by crucian carp RLRs. EPC cells were cotransfected with DrIFR10, DrIFR10-ΔN or DrIFR10-ΔC and CaRIG-I, CaMDA5, CaMITA, CaTBK1 or CaIRF3 plus DrIFN1pro-Luc (A) or DrIFN3pro-Luc (B) at the ratio of 1:1:1. At 48 h post-transfection, cells were collected for detection of luciferase activities. The data represented the average of three independent experiments. Error bars are the SDs obtained by measuring each sample in triplicate.
## Supplemental Table I  Primers used in this study

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