Identification of Drel as an Antiviral Factor Regulated by RLR Signaling Pathway

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Abstract

**Background:** Retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs) had been demonstrated to prime interferon (IFN) response against viral infection via the conserved RLR signaling in fish, and a novel fish-specific gene, the grass carp reovirus (GCRV)-induced gene 2 (Gig2), had been suggested to play important role in host antiviral response.

**Methodology/Principal Findings:** In this study, we cloned and characterized zebrafish Gig2 homolog (named *Danio rerio* Gig2-I, DreI), and revealed its antiviral role and expression regulation signaling pathway. RT-PCR, Western blot and promoter activity assay indicate that DreI can be induced by poly I:C, spring viremia of carp virus (SVCV) and recombinant IFN (rIFN), showing that DreI is a typical ISG. Using the pivotal signaling molecules of RLR pathway, including RIG-I, MDA5 and IRF3 from crucian carp, it is found that DreI expression is regulated by RLR cascade and IRF3 plays an important role in this regulation. Furthermore, promoter mutation assay confirms that the IFN-stimulated regulatory elements (ISRE) in the 5′ flanking region of DreI is essential for its induction. Finally, overexpression of DreI leads to establish a strong antiviral state against SVCV and *Rana grylio* virus (RGV) infection in EPC (Epithelioma papulosum cyprinid) cells.

**Conclusions/Significance:** These data indicate that DreI is an antiviral protein, which is regulated by RLR signaling pathway.

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Introduction

In mammals, recognition of viral components through pattern recognition receptors (PRRs) triggers several signaling cascades, eventually converging on the transcription activation of IFNs and ISGs against virus infection [1]. Three classes of PRRs have been identified: retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs), Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). RLR family, the cytosolic recognition receptors (PRRs) triggers several signaling cascades, leading to the activation of interferon regulatory factor 3/7 (IRF-3/7) also known as IPS-1/VISA/Cardif) [6,7,8,9]. Subsequently, MAVS, the mitochondrial antiviral signaling protein (MAVS, also presumed that RLR family might be conserved in vertebrates and the core function domains diversification might be essential to the function divergence of recognition of viruses [12]. A RIG-I-like molecule was identified in salmonid and cyprinid cell lines. Overexpression of RIG-I N terminal CARDs was able to establish a strong antiviral state against several DNA and RNA viruses by inducing the expression of ISGs [13]. MDA5 has been demonstrated to play virus recognition role in rainbow trout and Japanese flounder [14]. In crucian carp, overexpression either RIG-I or MDA3 was able to strongly upregulate fish virus-induced IFN production [15]. The third member of RLR family, LGP2, has been identified from rainbow trout and Japanese flounder, and its overexpression confers host cells powerful antiviral activity by induction of ISGs. However, crucian carp LGP2 was demonstrated to negatively regulate the RLR signaling pathway [14,15,16]. MAVS, downstream adaptor protein of RLRs, has also been cloned from several fish species including Japanese flounder, Atlantic salmon and zebrafish. Further experiments showed that the MAVS-overexpressed cells exhibited a severe inhibition of the replication of both DNA and RNA viruses [13,17]. As mentioned above, the critical molecules of RLR signaling...
pathway in fish and mammals share the similar structure and function, however, several differences also exist. Fish mediator of IFR3 activation (MITA) is able to link the signal from RIG-I/MDA5 to downstream kinase TBK1 and IFN transcription factor IFR3, but failed to induce the activation of NF-kB [13]. The transcription factor IFR3 is a critical protein downstream of RLR signal pathway, and regulates the expression of type I IFN and ISGs. A recent study shows that fish IFR3, unlike its mammalian orthologs, is a typical ISG which is significantly upregulated by rIFN, poly I:C, B-DNA and Z-DNA. Besides that, the phosphorylation and nuclear translocation of fish IFR3 were also observed in the condition of rIFN treatment, which did not occur in mammals [18]. Furthermore, grass carp reovirus (GCRV)-induced gene 2 (Gig2) has been identified as a novel fish-specific gene from the UV-inactivated GCRV-treated Carassius auratus blastulae embryonic (CAB) cells [19], and its transcription is upregulated by viral infection and IFN treatment. Subsequently, CaGig2 was presumed to play an important role in fish innate antiviral response because overexpression of IFR7 led to a strong activation of CaGig2 promoter [20]. These studies have revealed that CaGig2 is an ISG that is regulated by IFR7, however, the subtle signal cascade for its induction and role in antiviral response are still unknown.

As a powerful vertebrate model for infectious disease and immune function, zebrafish has risen tremendous interests in fish antiviral response are still unknown. IRF7, however, the subtle signal cascade for its induction and role in antiviral response are still unknown. IRF7, however, the subtle signal cascade for its induction and role in antiviral response are still unknown. IRF7, however, the subtle signal cascade for its induction and role in antiviral response are still unknown.

Results

Identification of zebrafish Gig2 homolog

By searching zebrafish genome database with CaGig2 sequence (accession No.GQ161131), several homologous CaGig2 genes were found and the most identical one was named as DreI (accession No. HQ269376). Using PCR method, we obtained the full-length cDNA of DreI from a cDNA library of zebrafish liver (ZFL) cells stimulated with poly I:C. The open reading frame consists of 474 nucleotides (nt) and encodes 158 amino acids with approximately 76.1% identity to CaGig2. Additionally, neither nuclear localization signal nor transmembrane motif (TM) was found in DreI protein (Fig. 1).

Inducible expression of DreI by poly I:C in vitro

To investigate the expression pattern of DreI, real-time PCR was applied to detect its mRNA level. As shown in Fig. 2A, transient transfection with poly I:C was able to induce the transcription of DreI in ZFL cells at 12 h, and to reach the peak at about 72 h. To further clarify the expression of DreI at protein level, the full-length ORF of DreI was expressed in E. coli as a His tagged fusion protein with about 39 kD (Fig. 2B, lane 2), and the purified DreI-His protein (Fig. 2B, lane 3) was used to immunized white rabbit to generate a polyclonal anti-DreI antiserum. For specificity detection, the poly I:C treated ZFL cell lysate was analyzed with this antiserum by Western blotting. As shown in Fig. 2C, the anti-DreI antiserum from the pre-immunized rabbit rather than the serum from the pre-immunized rabbit could detect the endogenous DreI protein with a molecular weight of appropriate 22 kDa which is different with that of the fusion protein (about 39 kDa). Moreover, when the anti-DreI antiserum was preabsorbed with the fusion protein DreI-His, it could not recognize the 22 kDa protein band. Therefore, the produced antiserum was subsequently used to monitor the expression of DreI protein. In accordance with the induction kinetics at mRNA level, DreI protein was also significantly induced by transfection of poly I:C in ZFL cells since 24 h (Fig. 2D). Furthermore, ZFL cells were treated with various concentrations of poly I:C from 1 μg/ml to 100 μg/ml for 24 hours. As shown in Fig. 2E, the DreI protein was greatly upregulated by poly I:C treatment in a dose-dependent manner. For the limitation on the highly related protein family recognition of polyclonal antiserum, other member(s) besides DreI might contain in the band. These results indicate that DreI can be induced by poly I:C both at mRNA and protein levels.

Analysis of DreI promoter

To further characterize the expression of DreI, we searched 5′ flanking region about 700 bp upstream of the start codon of DreI in zebrafish genome database. As shown in Fig. 3A, the putative transcription start site (A) is localized at 388 bp upstream from the translation start codon of DreI and determined as position +1. An intron of 319 bp (+71 to +390) was found to exist in the 5′ UTR, which contains the conserved flanking dinucleotides GT-AG in the exon/intron structure. Moreover, promoter sequence characterization analysis revealed several putative transcription factor binding sites in the 5′ flanking regulatory region. A putative ISRE motif, present in the promoters of most ISGs, was identified in the region from −48 to −39, which matches the consensus sequence of (G/A/T)GAAA(1–2)GAAA(G/C)(A/T/C) [22]. A gamma IFN activated sequence (GAS) site (TTNCNNNAA) and eight GAAA/TTTC motifs were also found in the DreI promoter, which are the characteristics of genes responsive to both type I IFN and type II IFN (Fig. 3A).

To analyze the promoter activity, about 200 bp minimal promoter sequence from −182 to +36 containing the putative GAS site and ISRE motif was cloned in front of luciferase reporter gene to generate the DreI promoter-driven luciferase construct, which was designated DreP-Luc (Fig. 3B). In comparison with pGL3-basic vector, the relative luciferase activities were greatly elevated in the DreP-Luc transfected EPC cells, indicating that the cloned DreI promoter is a strong promoter. As anticipated, when the DreP-Luc transfected EPC cells were treated or infected by poly I:C, rIFN, or SVCV, the relative luciferase activities were further induced. As shown in Fig. 4A, compared to the untreated (null) cells, about 3.07-fold, 2.86-fold and 1.58-fold activation of DreI promoter were induced in the presence of poly I:C transfection, rIFN treatment and SVCV infection, respectively. However, none inducible activities were observed in the transfected cells with control pGL3-basic vector. Intriguing, RGV infection did not induce the activation of DreP-Luc, and even inhibited its basal luciferase activity. It was likely that RGV had evolved some mechanisms to avoid or impede host immune response. These results together indicate that
DreI promoter was significantly activated by poly I:C, rIFN and SVCV.

Regulation of DreI expression via RLR signaling

Recently, great progress has been made in characterization of fish RLR signaling pathway response to virus infection. In crucian carp, a functional RLR-activated signaling cascade has been found to be essential for IFN response against viral infection [20]. To determine whether DreI was regulated through RLR signaling pathway, CaRIG-1, CaMDA5 and CaIRF3 were employed in the current study. As shown in Fig. 4B, overexpression of CaRIG-1 and CaMDA5 led to a significant activation of DreI promoter (2.31 and 2.47 fold relative to the empty vector respectively). Moreover, a 4.62-fold luciferase activity of DreI pro-Luc was induced by

Figure 1. Alignment of amino acid sequences of DreI and CaGig2. The amino acid sequences of DreI and CaGig2 are deduced from the cDNA sequences (Accession numbers: DreI, HQ269376; CaGig2, No. GQ181131). The identical (*) and similar (.) amino acids identified by Clustal X program are indicated.
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Figure 2. Inducible expression pattern of DreI. (A) ZFL cells seeded on 6-well plates overnight were transfected with 2 µg/ml poly I:C for 2, 6, 12, 48, 72, 96, and 120 h. Then total RNAs were extracted to examine the expression level of DreI transcripts by real-time PCR. β-actin was introduced as endogenous control. (B) Prokaryotic expression of the fusion protein DreI-His and generation of anti-DreI polyclonal antibody. Lane 1: lysate of normal bacteria; lane 2: lysate of IPTG-induced bacteria; lane 3: the purified protein by Ni²⁺-NTA affinity chromatography. (C) Transfection of ZFL cells with 2 µg/ml poly I:C for 48 h, the lysate was immunoblotted by polyclonal anti-DreI antiserum (lane 4), normal rabbit serum (lane 5) or anti-DreI antiserum pre-adsorbed with purified prokaryotic protein (lane 6). (D) ZFL cells were stimulated with 2 µg/ml poly I:C plus 4 µl/ml Lipofectamine 2000 for 12, 24, and 48 h, then lysed and detected by anti-DreI antiserum. β-actin served as an internal control. (E) For dose-dependent analysis, ZFL cells were treated with 1, 10, and 100 µg/ml poly I:C for 24 h. β-actin served as an internal control.
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overexpression of CaIRF3 in comparison with that of control vector. These data indicate that the expression of DreI was upregulated by CaRIG-I, CaMDA5 and CaIRF3.

In a previous study, fish IRF3 has been demonstrated as a crucial transcription factor for IFN and ISG expression downstream of RIG-I and MDA5 [15]. To delineate the role of CaIRF3 in the regulation of DreI expression, CaIRF3-DN, a dominant negative form of CaIRF3, was used in this study. As shown in Fig. 4C, the induction of DreI promoter by SVCV, poly I:C and rIFN was severely impaired by overexpression of CaIRF3-DN, indicating that CaIRF3 was indispensable for DreI induction by such mentioned stimuli. Furthermore, the activation of DreI promoter induced by CaRIG-I, CaMDA5 and CaIRF3 (3.28, 4.76 and 9.13 fold respectively) was also significantly inhibited by overexpression of CaIRF3-DN, resulting in 1.43-, 2.01- and 3.89-fold reduction, respectively (Fig. 4D). These results together indicated that RLR-activated signaling cascade was essential for DreI expression upon viral infection and IRF3 was probably the master transcription factor for the inducible transcription of DreI.

ISRE motif is indispensable for DreI induction

Since ISRE motif in promoter of ISGs is essential for its transcriptional activation upon stimulation, an ISRE mutant DreI promoter-driven luciferase construct (designated as DreIpro-mut-Luc) was made to clarify whether the ISRE motif was necessary for DreI induction. EPC cells were transfected with wild type or ISRE mutant DreI promoter construct followed by treatment with poly I:C or rIFN and by infection with SVCV or RGV. Similar to previous experiments, wild type DreI promoter was significantly activated by poly I:C, rIFN, and SVCV, whereas no significant activation of DreIpro-mut-Luc was observed in the same condition (Fig. 5A). These results suggested that the ISRE motif was indispensable for DreI transcription activation induced by several stimuli.

Furthermore, we also determined the role of ISRE motif in the activation of DreI promoter triggered by CaRIG-I, CaMDA5 or CaIRF3. As shown in Fig. 5B, consistent with previous results, overexpression of CaRIG-I, CaMDA5, and CaIRF3 led to a remarkable activation of DreI promoter (2.14, 1.66, and 4.62 fold, respectively), but such activation was severely inhibited when it came to DreIpro-mut-Luc (1.63, 1.21, and 1.68 fold, respectively). Collectively, these results demonstrated that the ISRE motif was the major cis-element indispensable for DreI induction.

Antiviral activity of DreI protein

Since DreI is indeed a typical ISG, the role of DreI protein should be determined in cellular antiviral response against viral...
Figure 4. Activation of DreI promoter by several stimuli via RLR signaling. (A) Induction of DreI promoter by SVCV, RGV, rIFN and poly I:C for 24 h. EPC cells seeded in 24-well plates overnight were transfected with DreIpro-Luc, and pRL-TK was used as an internal control. At 24 h after transfection, cells were treated with several stimuli or left untreated. The luciferase activities were monitored at 24 h after stimulation. (B) EPC cells were cotransfected with pcDNA-CaRIG-I, pcDNA-CaMDA5, or pcDNA-CaIRF3 and DreIpro-Luc at the ratio of 1:1. The luciferase activity was assessed at 48 h post-transfection. (C) EPC cells were cotransfected with DreIpro-Luc and pcDNA-CaIRF3-DN and stimulated with several stimuli at 24 h posttransfection. Luciferase activities were monitored at 24 h after stimulation. (D) EPC cells were cotransfected with pcDNA-CaRIG-I, pcDNA-CaMDA5, or pcDNA-CaIRF3 and DreIpro-Luc plus pcDNA-IRF3-DN at the ratio of 1:1:1. At 48 h posttransfection, cells were collected for detection of luciferase activities. The results represent three independent experiments and error bars are the SDs obtained by measuring each sample in triplicate. The significant differences between control and treatments groups are determined by T-TEST. *p<0.01.

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Figure 5. ISRE is essential for the induction of DreI promoter. (A) EPC cells seeded in 24-well plates overnight were transfected with DreIpro-Luc or DreIpro-mut-Luc plus pRL-TK and induced by SVCV, RGV, rIFN and poly I:C for 24 h. Cells were collected for measuring the luciferase activities. (B) EPC cells were cotransfected with pcDNA-CaRIG-I, pcDNA-CaMDA5, or pcDNA-CaIRF3 and DreIpro-mut-Luc at the ratio of 1:1. In the control group, the EPC cells were cotransfected with indicated expression constructs and DreIpro-Luc at the ratio of 1:1. At 48 h posttransfection, the cells were collected to assess the luciferase activates. The results are the representative of three independent experiments and error bars are the SDs obtained by measuring each sample in triplicate. *p<0.01.

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infection. EPC cells were transfected with DreI or empty vector as a control followed by infection with SVCV or RGV, which belong to RNA or DNA virus, respectively. At five-day postinfection with SVCV, an obvious broad CPE was observed in control cells, whereas overexpression of DreI fully protected cells against SVCV infection (Fig. 6A). Consistently, the SVCV virus titer was remarkably decreased about 100-fold (10^9 TCID_{50}/ml versus 10^4 TCID_{50}/ml) in the DreI-transfected cells compared to that of control cells (Fig. 6B). On the other hand, overexpression of DreI was able to greatly delay the appearance of CPE resulted from RGV infection (Fig. 6C). Moreover, a viral titer of 4.2×10^4 TCID_{50}/ml was detected in the supernatant from the DreI-overexpressed cells, which was a 1740-fold reduction compared to that of control cells (7.3×10^7 TCID_{50}/ml; Fig. 6D). Finally, a real-time PCR was performed to detect the induction of IFN in EPC cells by overexpression of DreI. As shown in Fig. 6E, DreI indeed functional as a final effector. Therefore, the data indicate that DreI is an antiviral protein against both RNA and DNA viruses in fish cells.

DreI is localized in the cytoplasm

To determine the cellular localization of DreI, immunofluorescence microscopy was used to examine the localization of DreI in ZFL cells. Since DreI protein did not express in normal ZFL cells as described above, ZFL cells plated on microscobic glass overnight were stimulated with poly I:C, SVCV, RGV or not. At 72 h poststimulation, the DreI protein was detected by polyclonal anti-DreI rabbit serum. As anticipated, no signal was monitored in the untreated ZFL cells, however, the red signals of DreI were markedly observed to be widespread in the cytoplasm when the cells were stimulated with poly I:C, or infected with SVCV or RGV (Fig. 7A). Because of the discrepancy between positive staining of DreI protein and negative effect of DreI promoter in the context of RGV infection, a real-time PCR was performed to monitor the expression of DreI mRNA by RGV infection. As shown in Fig. 7B, DreI transcription is intensively upregulated from 48 h after RGV infection. These results indicate that DreI is a cytoplasmic protein.

Discussion

In the past two decades, an immense progress has been made in identification of IFNs and ISGs in several fish species, and lots of findings indicate that fish, similar to mammals, also possesses a functional conserved IFN system to inhibit viral infection [21]. Although a great number of ISGs had been identified in response to IFN from several fish species, only a few of them have been characterized in the expression regulation and antiviral function. Herein, we have identified DreI as a typical ISG protein, which is regulated by the conserved RIG-I/MDA5 signaling through transcription factor IRF3, and demonstrated its antiviral activity against both DNA and RNA viruses. This is the first study to determine the expression regulation and antiviral function of DreI.

Fish type I IFN system exerts a pivotal role in host defense against virus infection, and the induction of IFNs and ISGs is considered as the hallmark of antiviral innate immune response [23]. Upon viral infection, the cytosolic receptors RIG-I and MDA5 can detect viral genome RNA or replication byproduct dsRNA to induce multiple signaling pathways, and eventually activate transcription factors IRF3 and NF-kB, which subsequently translocate into nucleus and initiate IFN transcription. Through an autocine/paracrine loop, a large number of ISGs are activated by IFN [24]. In the present study, the expression of DreI was shown to be significantly unregulated by poly I:C at mRNA level. Furthermore, a specific polyclonal antibody against DreI was generated and used to confirm the expression profile of DreI protein. Western blotting analysis showed that DreI protein was induced by poly I:C treatment in both time- and dose-dependent manners. It is worthy of note that no signal of DreI mRNA or protein was detected in untreated ZFL cells. Therefore, DreI seems to be an ISG gene induced by poly I:C and IFN. On the other hand, characterization of 5' flanking regulatory sequence of DreI reveals multiple transcription factor binding sites including ISRE and GAS. Luciferase reporter assay exhibits that the activity of DreI promoter is remarkably upregulated by poly I:C, IFN and SVCV infection. Collectively, these results together indicate that DreI is a typical ISG gene. Recently, the cytosolic viral RNA sensors RIG-I/MDA5-mediated signaling pathway responsible for type I IFN activation was functionally demonstrated in some fish species. Some pivotal signaling molecules, including RIG-I, MDA5, MITA, TBK1 and IRF3, have been cloned and characterized in crucian carp [15]. To elucidate subtile signaling pathway responsible for DreI induction, several crucian carp molecules, such as RIG-I, MDA5 and IRF3, were used in the current study. In reporter assays, a strong activation of DreI promoter was observed when the cells were transfected with any molecule among CaRIG-I, CaMDA5 and CaIRF3 even in the absence of poly I:C or rIFN stimulation. CaIRF3 is an important transcription factor for the induction of IFN and ISGs, whereas CaIRF3-DN, devoid of DNA binding domain, exhibits a dominant negative effect. This is probably due to its ability to interact with endogenous TBK1, IRF7 or itself, and thus abolishes the function of endogenous IRF3. As anticipated, the activation of DreI promoter induced by overexpression of CaRIG-I, CaMDA5 and CaIRF3 was severely abrogated by CaIRF3-DN overexpression. It means that the expression of DreI is IRF3-dependent and is regulated by RLR pathway. In a previous study, CaIRF7 was also demonstrated as a strong transcription factor for CaGig2 expression [20]. Taken together, these date verify the role of the conserved RLR signaling cascade in the activation of IRF3/7-dependent IFN and ISGs. Although the expression of ISGs activated by virus-induced IFN through the conserved Jak-Stat signaling cascade was also confirmed in fish [23], the further study is needed to confirm that the Jak-Stat pathway also is also involved in CaGig2 induction.

Previous studies have shown that more than 300 ISGs like DreI have been identified in response to IFN. However, only a few of them have been characterized for the function to resistant to virus invasion by affecting virus transcription, replication or cell apoptosis. For example, Mx, an IFN-inducible large GTPase belonging to the dynamin family, can inhibit various viruses by blocking capsid protein transposition and viral RNA synthesis [25,26]. PKR encodes a protein that contains a dsRNA binding region and a protein translation inhibition region. Overexpression of PoPKR increases phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) to inhibit viral protein synthesis [27,28]. Viperin, an IFN-inducible protein, exerts an antiviral effect by inhibiting human cytomegalovirus (HCMV) maturation and assembly [29]. SVCV and RGV distribute worldwide and belong to RNA and DNA aquatic viruses respectively, and both of them could induce cell apoptosis in vitro and lead to high mortality in the infected fish [30,31]. In the current study, overexpression of DreI conferred host cells to establish a strong antiviral state to inhibit the replication of both RNA and DNA viruses. Although DreI displays a powerful antiviral activity against viral infection, its underlying mechanisms are still elusive. Since subcellular localization plays an important role, we subsequently detected the distribution of DreI in vitro under the condition of viral infection. In line with results of DreI induction at mRNA and protein levels, immunofluorescence
localization revealed cytoplasm distribution of DreI upon viral infection, and no signal was observed in normal cells, although its exact localization, such as mitochondrion or endoplasm reticulum, remained unknown. Collectively, it would be concluded that DreI inhibits viral infection in cytoplasm, and future study is dedicated to identify the viral protein(s) interacted with DreI.

Recently, the major components of type I IFN system, ISGs and its regulatory element ISRE, were identified to be conserved in teleost fish. The ISRE motif was found to be crucial for the induction of most fish ISGs, such as Mx. Deletion mutational analysis suggested that the ISRE motif closest to the transcription start site might be necessary for promoter activity of Mx gene [22,32,33,34]. Similar situations were also found in the ISRE motif within promoters of Japanese flounder ISG15 and crucian carp IFN [17,35]. Consistent with other ISGs promoter, the 5′ flanking region of DreI also contains a typical ISRE motif. Mutation assays showed that the ISRE motif disruption completely abolished the activation of DreI promoter induced by SVCV, poly I:C and rIFN, confirming its essential role within DreI promoter. Furthermore, a high level of DreI promoter activity was monitored even without poly I:C transfection in luciferase assay, but no any signal of DreI mRNA or protein was detected in absence of poly I:C stimulation. These results indicate that DreI promoter is a strong promoter and seems to lack the negative

Figure 6. Overexpression of DreI induces powerful antiviral immunity. (A, C) EPC cells seeded in 24-well plates overnight were transfected with 0.5 μg DreI plasmid or empty vector as a control. At 24 h posttransfection, EPC cells were infected with SVCV or RGV at a dose of 10 TCID50 per well for 5 days at 25°C. Then Cell monolayers were stained with crystal violet. (B, D) The culture supernatants from cells infected with SVCV and RGV were collected and the viral titers were measured by standard TCID50 method. The CPE caused by SVCV or RGV in viral titer measurement assays was also presented. The results are the representative of two independent experiments. (E) EPC cells were transfected with empty vector or DreI plasmid and were sampled at the indicated times. The relative transcript level of IFN was detected by real-time PCR and normalized to the expression of β-actin. Error bars represent SDs obtained by measuring each sample in triplicate.

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regulatory elements. Such situation of high basal expression level also occurred in CaGig2 promoter and zebrafish IFN3 promoter [15,20]. Interestingly, in luciferase assay, DreI promoter activity was not activated, but rather inhibited severely by RGV infection at 24 h postinfection. However, immunofluorescent localization experiments showed that the expression of DreI protein was significantly induced by RGV infection at 72 h postinfection. There are three explanations for such conflicting phenomena mentioned above. First, compared with ssRNA virus SVCV, a delayed appearance of host innate antiviral IFN response was probably induced by dsDNA virus RGV. Since poly dAT:dAT (B-DNA) and poly dG(dC):dG(dC) (Z-DNA) induced the expression of IFN and ISG with slower kinetics than poly I:C [10]. Secondly, the DreI promoter we cloned was not long enough, and seemed to contain additional positive regulatory elements, such as IRF- or NF-kB-binding sites. The activity of DreI promoter containing only one ISRE motif could be easily inhibited by viral proteins synthesized by RGV. Finally, it was likely that the antiviral IFN response was blocked by RGV in the early stage of infection, but activated by subsequently synthesized viral products in the late stage. There are a lot of examples that, during the infection, many viruses synthesize some viral proteins to antagonize the innate immune response. Paramyxoviruses V protein was demonstrated to combine with cytosolic RNA sensor MDA5 to inhibit viral recognition and virus-triggered type I IFN signaling pathway [36]. NS3/4A encoded by hepatitis C virus was revealed to degrade the adaptor protein Cardif to block the RIG-I/MDA5-activated antiviral response [37,38,39].

Most of the ISGs identified in fish are conserved in vertebrates, but some are restricted to vertebrate subsets. CaGig2, a gene identified from subtractive suppression hybridization cDNA library stimulated with UV-inactivated GCRV, is a good case of such ISG restricted to some primitive vertebrates. By searching the EST and genome databases available at NCBI and Ensembl, several predicted genes homologous to DreI were found only in some aquatic vertebrate species, such as Tetraodon nigroviridis (accession No., CR719319), Sebastes schlegelii (accession No., AB190858), and Xenopus (accession No., XM_002932940). Considering the antiviral activity of DreI, primitive vertebrates might possess specific mechanism(s) to inhibit viral infection, which is probably lost in higher vertebrates during immune system evolution. It is interesting to clarify why Gig2 gene is absent in reptiles, birds and mammals and whether the absence reduces the chance of aquatic virus infection when they transit their habitat from water to land [40]. Therefore, Gig2 may be an antiviral protein specific to aquatic viruses, and may have been lost during the evolution. It is plausible that DreI is an ancestral gene, which would not be retained in modern species of higher vertebrate branches. The migration of ancient tetrapods from water to land is likely a transition point for the disappearance of DreI.

Taken together, our study have characterized DreI as an antiviral ISG which is regulated by the conserved RIG-I/MDA5 signaling pathway through transcription factor IRF3, and demonstrated that the ISRE motif is indispensable for its induction. We also illustrate the essential role of DreI in host innate immune response against both RNA and DNA viruses. Further studies should be required to specify the interaction between DreI and viral proteins to illuminate its antiviral mechanisms.

Materials and Methods

Cells viruses, and rIFN

Epithelioma papulosum cyprinid (EPC) cells were cultured at 25°C in medium 199 supplemented with 10% fetal calf serum (FCS) [41]. ZFL cells were purchased from the American type culture collection (ATCC) and grown at 28°C in Leibovitz’s L-15 medium with 2 mM L-glutamine, 50%; Dulbecco’s modified Eagle’s medium with 4.5 g/L glucose and 4 mM L-glutamine, 55%; Ham’s F12 with 1 mM L-glutamine, 15% and supplemented with: 0.15 g/L sodium bicarbonate, 15 mM HEPES, 50 ng/ml EGF, heat-inactivated fetal bovine serum, 5%. Spring viremia of carp virus (SVCV) [42], a negative ssRNA virus, and Rana grylio virus (RGV), a DNA virus, were maintained in our lab and propagated in EPC cells [43]. For viral infection,
EPC cells seeded in 24-well plates overnight were transfected with 0.5 μg DreI or empty vector. At 24 h posttransfection, the cells were infected with 10 TCID_{50} of SVCV or RGV per well. Five days later, the supernatant aliquots were harvested for measurement of virus titers by the standard TCID_{50} method. And then the cell monolayers were washed with PBS, fixed by 30% formaldehyde for 30 min, stained by 1% (w/v) crystal violet for 30 min, and observed for cytopathic effect (CPE). The results were the representative of three independent experiments. The source of hIFN was described previously [23].

Identification of the putative transcription start site

The putative transcription start site of DreI were identified by several softwares, including GENSCAN Web Server at MIT (http://genes.mit.edu/GENSCAN.html), Promoter 2.0 Predication Server (http://www.cbs.dtu.dk/services/Promoter/), Neural Network Promoter Predication (http://fruitfly.org/seq_tools/promoter.html), and Promoter Scan (http://thr.cit.nih.gov/molbio/proscan/), and no any putative transcription start site was found between the ISRE motif and the first intron. It was likely that DreI has a noncanonical promoter such that was unable to identify by current softwares. However, we have retrieved several EST sequences (like HQ269356, EH281212, EH278211) from NCBI database, and found that one EST sequence (HQ269356) is transcribed from the Adenosine (A) about 250 bp upstream of translation start codon. Moreover, a noncanonical TATA box (TAATA) probably recognized by RNA polymerase II was observed to locate at 26 nt ahead of the mentioned Adenosine (A). Taken together, we presumed that the Adenosine (A) might be the transcription start site and designated it as position +1.

Transfection and luciferase reporter assays

EPC cells seeded in 24-well plates overnight were cotransfected with the mixture containing 0.525 μg plasmid and 1 μl FuGENE HD Transfection Reagent (Roche) in 50 μl OPTI-MEM® I Reduced Serum Medium (Invitrogen). At 6 h after the poly I:C transfection or other stimulation, the cells were washed three times and cell medium was replaced by fresh 10%-FCS199 medium. The amount of expression vector, DreI-Luc and pRL-TK was at a ratio of 10:1. pRL-TK vector was used to normalize the expression level. At 48 h posttransfection, the cells were harvested and lysed according to the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were measured by Junior LB9509 Luminometer (Berthold) and normalized to the amount of Renilla luciferase activities. The results were representative of three independent experiments, each in triplicates.

Fusion protein expression, polyclonal anti-DreI antiserum preparation and Western blotting

The plasmid pET-32a(+) - DreI was transformed into DE3 (BL21) E. coli strain. The recombinant fusion protein DreI-His was induced by isopropyl-b-D-thiogalactopyranoside (IPTG) and purified by Ni^{2+}-NTA affinity chromatography (Novagen). The purified fusion protein was applied to immunized white rabbit to produce polyclonal anti-DreI antiserum according to a previous report [20].

For Western blotting, equal amounts of protein extracts from ZFL cells stimulated with poly I:C or not were separated by 12% SDS-PAGE gels and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane was blocked in freshly prepared TBST buffer containing 5% nonfat milk for 1 h at room temperature, incubated with primary antibody at 4°C overnight. After three times wash with TBST buffer, the membrane was incubated with secondary antibody for 1 h at room temperature. Finally, the membrane was stained with ECL system after another three washes with TBST buffer. For

RNA extraction and real-time PCR

Total RNA was extracted from ZFL cells by TRIZOL Reagent (Invitrogen). The first-strand cDNA was synthesized using random primers and M-MLV reverse system (Promega). The semi-quantitative RT-PCR was performed in a volume of 20 μl containing 1 μl cDNA, 0.2 μM each primer, 0.5 U of Taq polymerase (MBI, Ferments), 0.1 μM of dNTP and 1 × buffer for Taq polymerase (MBI, Ferments). PCR was conducted with the protocol: 94°C for 4 min; then 94°C for 30 s for 25–28 cycles; 72°C for 30 s, 54°C for 30 s, and 72°C for 30 s for 25–28 cycles; 72°C for 5 min. β-actin was introduced as an endogenous control gene. Real-time PCR was performed on ABI Step One real-time PCR system (Applied Biosystems, Foster City, CA, USA) with a dsDNA-binding dye, Fast SYBR green master mix (Applied Biosystems, Foster City, CA). All amplifications were performed using a two step temperature profile with annealing and extension at 60°C. The expression levels of mRNA were normalized by the median expression of β-actin. Each sample was analyzed in triplicates.

Gene clonning and plasmids

The open reading frame (ORF) of DreI was amplified using two pairs of primers by PCR from ZFL cells stimulated with poly I:C and cloned into the KpnI and XhoI sites of pcDNA3.1/myc-His(−)A vector (Invitrogen) for overexpression, and into pET-32a(+) vector (Novagen) for prokaryotic expression, respectively. For promoter activity analysis, genome DNA was extracted from the zebrafish tail fin by Wizard® Genomic DNA Purification Kit (Promega). The 5' flanking regulatory region (−200 to +24) of DreI was cloned and inserted into pGL3-Basic luciferase reporter vector (Promega). For mutation assay, the purine G/A (−59/−50) in the putative ISRE motif were mutated to pyrimidine C/T, respectively. CaRIG-I, CaMDA5, CaIRF3 and CaIRF3-DN were previously described [15]. All constructs were verified by sequencing the used primers are listed in Table 1.

Table 1. Primers used in this study.

| Primers       | Sequence (5’-3’) | Application               |
|---------------|-----------------|--------------------------|
| EE-DreI-R     | GAATTCCTGAGAAAATGCTCAACCATTT | overexpression           |
| PE-DreI-R     | TCTCGAGAAAATGCTCAACCATTT | Prokaryotic expression   |
| Drel-Pro-R    | TGGTGACCTGGTATAAAAGATTTAAACCA | Promoter activity analysis |
| Drel-mut-Pro-R| AACTCGAGGATCTGAAAGATTCTAGCA | mutation assay           |
| Drel-mut-Pro-R| TCTCACGATTGTTTATG   |                          |
| Drel-F        | GAAGACCAAGAGATGTTAG | Real-time PCR            |
| Drel-R        | AACCATTTTCCTCTTCG |                          |
| IFN-F         | ATGAAAATCTAAATGGAGCTA |                          |
| IFN-R         | GATAGTTTAAAAACCATTTCAATTAA |                          |
| β-Actin-F     | CACTTCTGTCGGAGGTATC | Real-time PCR, control   |
| β-Actin-R     | CCATCTCTGTCGGAGGTATC |                          |

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specificity detection, the membrane was incubated with anti-DreI antisemum that had been preabsorbed with excess antigen (purified DreI-His) at 4°C overnight.

**Immunofluorescent microscopy**

ZFL cells were seeded overnight on microscopic coverglass in 6-well plates, and transfected with poly I:C, or infected with SVCV or RGV. Forty-eight hours later, cell monolayers were fixed with 4% paraformaldehyde (PFA) for 15 min, treated with 0.2% Triton X-100 for 15 min, blocked with 10% normal goat serum for 1 hour and incubated with polyclonal anti-DreI rabbit serum overnight at 4°C. Then, the cells were incubated with fluorescein-labeled goat anti-rabbit IgG for 2 h in dark at room temperature. After three 10-min washes with PBS, the cells were stained with Hoest 33342 (Sigma) for 10 min in dark. Finally, the cells were examined under a Leica DM IRB fluorescence microscope.

**Author Contributions**

Conceived and designed the experiments: QYZ. Performed the experiments: SL. Analyzed the data: SL, FS YBZ. Contributed reagents/materials/analysis tools: QYZ, JFG. Wrote the paper: SL QYZ, JFG.