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# Fish viperin exerts a conserved antiviral function through RLR-triggered IFN signaling pathway

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ABSTRACT

Mammalian viperin is a typical interferon (IFN)-induced antiviral protein. Fish have viperin homologs; however, little is known about the expression regulation of fish viperins. In this study, we report the expression regulation and antiviral function of a fish viperin from crucian carp *Carassius auratus* during IFN response. Crucian carp viperin is induced at mRNA and protein levels by fish IFNs and IFN stimuli such as poly(I:C). Consistently, this gene promoter contains multiple transcription factor binding sites including IFN-stimulated response elements (ISRE) and IFN gamma activation sequences (GAS), and is activated by two types of fish IFNs and also by the intracellular and extracellular poly(I:C). Activation of crucian carp viperin promoter by the intracellular poly(I:C) is mediated by retinoic acid-inducing gene I (RIG-I)-like receptors (RLR)-triggered IFN signaling pathway, which is further verified by the findings that each signaling molecule of RLR pathway is able to induce the expression of crucian carp viperin at mRNA and protein levels. Finally, overexpression of crucian carp viperin in cultured fish cells confers significant protection against infection of grass carp reovirus (GCRV). These data suggest that similar to mammalian homologs, crucian carp viperin exerts a conserved function through RLR-triggered IFN signaling pathway.



## 1. Introduction

In mammals, double-stranded RNA (dsRNA), which accumulates in viral-infected cells, are recognized by pattern recognition receptors (PRRs) such as retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs) (Stark, 2007). During the recognition processes, RLR family members RIG-I and MDA5 (melanoma-differentiation-associated gene 5) interact with an adapter protein mitochondrial antiviral signaling protein (MAVS), while TLR3, one of TLR family member, recruits another adapter protein TIR-domain-containing adapter-inducing interferon (IFN)-β (Trif). Such interaction recruits a cytosolic protein kinase TBK1 (TANK-binding kinase 1) that activates transcription factors IRF3/7 (IFN regulatory factor 3/7) to initiate the expression of IFN-β (Baum and Garcia-Sastre, 2010). Recently, MITA (mediator of IRF3 activation) has been characterized as a novel adapter protein to mediate signaling transduction between MAVS and downstream protein kinase TBK1 (Zhong et al., 2008) and also to link cytosolic DNA sensing signal to evoke the expression of IFN-β through activation of TBK1 and IRF3 (Ishikawa and Barber, 2008). The produced IFNs in turn induce the expression of hundreds of ISGs to establish host antiviral state by Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway (Sadler and Williams, 2008; Stark, 2007).

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) is first identified as a viral-induced and IFN-induced EST from human cytomegalovirus (HCMV)-infected primary human foreskin cells (Zhu et al., 1997), and subsequently characterized as an IFN-inducible antiviral protein against human cytomegalovirus (HCMV) replication in fibroblasts (Chin and Cresswell, 2001). Viperin is capable to inhibit the release of influenza virus by perturbing lipid rafts (Wang et al., 2007) or to limit the replication of hepatitis C virus by interacting with nonstructural protein 5A (Helbig et al., 2011). Overexpression of viperin promotes TLR7- and TLR9-mediated production of type I IFN in mouse plasmacytoid dendritic cells (Saitoh et al., 2011). These results indicate that viperin is an important antiviral effector

*Abbreviations:* IFN, Interferon; Viperin, virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma-differentiation-associated gene 5; TBK1, TANK-binding kinase 1; IRF, interferon regulatory factor; ISRE, IFN-stimulated response elements; GAS, IFN gamma activation sequences.

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involved in IFN response, which accords with the findings that this gene is highly upregulated by type I, II and III IFNs (Boudinot et al., 1999; Chin and Cresswell, 2001; Grewal et al., 2000; Zhou et al., 2007; Zhu et al., 1997), and by pivotal molecules of TLR and RLR signaling cascades (Severa et al., 2006). In addition, the activation of viperin mRNA accumulation by HCMV does not require the action of IFN and expression of viral genes (Zhu et al., 1997), indicating that there is IFN-independent pathways for induction of viperin. Another report showed that IRF-1 is required for IFN-independent viperin induction by vesicular stomatitis virus (VSV) but not by Newcastle disease virus and not by a VSV mutant that is unable to block IFN expression and secretion (Stirnweiss et al., 2010). These studies suggest that the expression regulation of viperin gene seems to be complex.

Similar to mammals, virus infection elicits a conserved fish IFN antiviral response that is initiated by TLR pathway (Matsuo et al., 2008) or RLR pathway (Biacchesi et al., 2009; Chang et al., 2011; Sun et al., 2011). Two types of fish IFN genes have been identified in fish genomes, one most similar to mammalian type I IFNs and the other orthologous to mammalian type II IFN (Altmann et al., 2003; Hamming et al., 2011; Stolte et al., 2008; Zou et al., 2005). These fish IFNs can establish host antiviral state by inducing the expression of many fish IFN-stimulated genes (ISGs), such as PKR, Mx, ISG15 and some novel ISGs including PKZ, Gig1 and Gig2 (Langevin et al., 2013; Larsen et al., 2004; Li et al., 2012; Liu et al., 2011; Sun et al., 2014; Zhu et al., 2008). Fish viperin homologue is firstly identified in rainbow trout *Oncorhynchus mykiss* (Boudinot et al., 1999), and then in other fish species (Dang et al., 2010; Lee et al., 2013; Levraud et al., 2007; Padhi, 2013; Rise et al., 2008; Sun and Nie, 2004; Workenhe et al., 2009; Zhang et al., 2014; Zhang et al., 2007). Rainbow trout viperin is directly induced by VHSV without the requirement of ongoing protein synthesis and also by a rainbow trout IFN-like factor (Boudinot et al., 1999), indicating that there are IFN-dependent and IFN-independent pathways for rainbow trout viperin induction. Recent studies have shown that red drum *Sciaenops ocellatus* viperin mRNA is upregulated by different bacterial pathogens (Dang et al., 2010), and rock bream *Oplegnathus fasciatus* viperin induced by megalocytivirus infection (Zhang et al., 2014). Sequence analysis of Chinese perch *Siniperca chuatsi* viperin promoter reveals existence of ISRE-like motifs (Sun and Nie, 2004). However, the signaling cascades responsible for fish viperin expression remain unclear. In this study, we characterized the expression regulation of a fish viperin from crucian carp *Carassius auratus* and confirmed its ability to inhibit GCRV replication in cultured fish cells.

## 2. Materials and methods

### 2.1. Cell culture, virus and reagents

Crucian carp (*C. auratus*) blastulae embryonic cells (CAB) and grass carp (*ctenopharyngodon idellus*) ovary cells (CO) were grown at 28 °C in medium 199 (Gibco) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Zhang et al., 2007). Grass carp reovirus (GCRV), a dsRNA virus, was propagated in CAB cells according to a previous report (Zhang et al., 2003). Transfection reagents Lipofectamine 2000, Opti-MEM® Reduced-Serum Medium and poly(I:C) were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibody against human viperin (sc-102099) and mouse monoclonal antibody against human GAPDH (sc-166574) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against human β-tubulin (E021040-01) was purchased from EarthOx (San Francisco, CA). The purchased viperin antibody is recommended for detection of viperin of mouse, human, dog and zebrafish by Santa Cruz Biotechnology.

### 2.2. Plasmids

For overexpression, wild type gene plasmids crucian carp RIG-I, MDA5, LGP2, MITA, TBK1, IRF3, IRF7, IFN, IRF9, STAT2, dominant negative mutant plasmids IRF3-DN and IRF7-DN, wild type gene plasmids zebrafish IFN1, IFN2, IFN3, IFNγ1 (IFNrel) and IFNγ2 were previously described (Shi et al., 2013; Sun et al., 2010, 2011). Wild type viperin plasmid was generated by cloning the open reading frame (ORF) of crucian carp viperin (Genbank accession No. AY303809) into *EcoR* I/*Xho* I sites of pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). A 5′ flanking region (−1832/+23) of crucian carp viperin (GenBank accession No. KF442617) was obtained *via* genome walking PCR with genome walking kit (Takara) and the putative transcription factor binding sites were analyzed by TFsearch internet online software (http://www.cbrc.jp/research/db/TFSEARCH.html). This genomic DNA sequence was inserted into *Kpn* I/*Xho* I sites of pGL3-Basic luciferase reporter vector (Promega) to construct crucian carp viperin promoter-driven luciferase vector (Viperinpro-Luc, VP). All of these generated plasmids were verified by sequencing analysis. The primers used are listed in Table 1.

### 2.3. Transfection and luciferase assays

Transfection and luciferase assays were performed as described previously (Liu et al., 2011; Sun et al., 2010). In brief, CAB or CO cells seeded in 6-well or 24-well plates overnight were transfected with a mixture containing of 2 μg or 0.5 μg plasmids and 4 μl or 1 μl lipofectamine 2000 (Invitrogen) in 500 μl or 100 μl FCS-free opti-MEM (Invitrogen) per well. At 6 h post transfection, the mixture was replaced with 2 ml or 0.5 ml pre-warmed fresh medium (28 °C). At 24 h post transfection, the transfected cells were harvested and lysed according to the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were measured by a Junior LB9509 luminometer (Berthod, Pforzhiem, Germany) and normalized to the amounts of Renilla luciferase activities. All experiments were performed in triplicates and repeated at least three times.

**Table 1**
Primers used for all of the studies.

| Name | Sequence (5′–3′) | Usage |
|---|---|---|
| CaViperin -F | CGGGGTACCAGTGTCTACGCACCTTAACC | Eukaryotic expression |
| CaViperin -R | CCGCTCGAGATTGCCAGTCCTGTATGAC | |
| CaViperin -1F | CAGCGCGCAGCTGACCACTCC | Real-time PCR |
| CaViperin -1R | GCAGTACCGCACCAACTCTCCCAG | |
| CaMx1-F | ACAGAAGGAACTGGAGGCGTA | |
| CaMx1-R | CGCAGGTTCCTCCAACAGC | |
| CaViperin-SP1 | ACGACTCATCCGTCCAAGAGGTG | Genomic walking |
| CaViperin-SP2 | TCCATCTCAACAGGGCTGAAACG | |
| CaViperin-SP3 | GACCAACTTGGTTTGATGTCACCATG | |
| VP-F | CGGGGTACCCCACAATGATGGTGAACACGAAC | Promoter sequence verification |
| VP-R | CCGCTCGAGGGTTAAGGTGCGTAGACACTGATAC | |