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Identification and functional characterization of viperin of amphioxus *Branchiostoma japonicum*: Implications for ancient origin of viperin-mediated antiviral response

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ABSTRACT

Viperin, an antiviral protein, has been shown to be active against a wide range of DNA and RNA viruses, but no information is available regarding functional characterization of viperin in invertebrate species. In this study, we clearly demonstrate that amphioxus (*Branchiostoma japonicum*) viperin, BjVip, has features in common with those of vertebrate viperin, including the presence of the SAM superfamily domain with the characteristic CNYKCGFC motif, syntenic conservation, and predicted 3D structure. *Bjvip* exhibits a tissue-specific expression with abundant levels in the hepatic cecum, hind-gut, gill and muscle, and following challenge with the viral mimic poly I:C, its expression is significantly up-regulated, suggesting an involvement of BjVip in immune response of amphioxus against viral infection. Importantly, we show that the cells transfected with *Bjvip* is able to kill LCDV or inhibiting its propagation, and co-incubation of rBjVip with WSSV markedly attenuates its infectivity. Thus, we provide the first evidences that amphioxus viperin, like that of vertebrates, is capable of promoting resistance against viral infection *in vitro* and *in vivo*, indicating that viperin-mediated antiviral response already emerged in the primitive chordate. We also prove that amphioxus viperin has evolved under positive selection.

1. Introduction

Vertebrate interferon (IFNs) proteins, mainly comprising type I (IFN-α/β), type-II (IFN-γ) and type III (IFN-λ) IFNs, are rapidly induced following viral infection and subsequently activate thousands of interferon-regulated genes (IRGs) or interferon-stimulated genes (ISGs) (De Veer et al., 2001; Sen and Sarkar, 2007). ISGs encode proteins that generally display a range of antiviral activities against diverse pathogens (Sadler and Williams, 2008; Sen, 2001). Viperin (*v*irus *i*nhibitory *p*rotein, *e*ndoplasmic *r*eticulum-associated, *i*nterferon-inducible) is one of ISGs-encoding proteins, which could inhibit many DNA and RNA viruses such as CHIKV, HCMV, HCV, DENV, WNV, SINV, influenza, and HIV LAI strain etc. Initially identified as cig5 in HCMV-infected primary human foreskin cells (Zhu et al., 1997), viperin is subsequently found in rainbow trout as the vig1 gene inducible by viral haemorrhagic septicaemia virus and in mouse as the vig1/cig5 homologue inducible by vesicular stomatitis virus and pseudorabies virus (Boudinot et al., 1999, 2000). Viperin has now been identified in various species of vertebrates. All viperin homologues are highly conserved in the primary sequence, which includes a relatively less conserved N-terminal amphipathic α-helix required for endoplasmic reticulum (ER) localization (Hinson and Cresswell, 2009a,b), a central radical S-adenosylmethionine (SAM) domain which contains a CxxxCxxC motif important for the formation of a [4Fe–4S] cluster (Frey et al., 2008), and a highly conserved C-terminal region that is known to be critical for antiviral activity against a number of viruses (Helbig and Beard, 2014; Helbig et al., 2013). Positive selection has been reported to drive rapid evolution of certain amino acid residues in the N-terminal regions of the highly conserved viperin proteins of fishes (Padhi, 2013).

Viperin could be induced in a variety of cell types by different cellular factors, such as type I, II and III IFNs, DNA and RNA viral proteins, viral mimic polyriboinosinic polyribocytidylic acid (poly I:C) and polysaccharide (Boudinot et al., 1999; Chin and Cresswell,

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2001; Grewal et al., 2000; Zhou et al., 2007; Zhu et al., 1997). Interestingly, the activation of viperin mRNA accumulation by HCMV does not require the action of IFNs and expression of viral genes (Zhu et al., 1997), suggesting that there is IFN-independent pathway for induction of viperin. Therefore, vertebrate viperin could be induced in either IFN-dependent or IFN-independent pathway. Viperin appears to use a variety of different antiviral mechanisms from interacting directly with viral and host proteins essential for viral replication to interaction with host organelles such as the ER, lipid droplets and mitochondria (Blasius and Beutler, 2010; Gao et al., 2004; Jiang et al., 2008; Reizis et al., 2011; Saitoh et al., 2011; Wang et al., 2012, 2007).

Viperin homologues have also been predicted in a variety of invertebrate species, including sponge, daphnia, insect and amphioxus (Padhi, 2013). However, up to now, functional characterization of viperin has been exclusively restricted to vertebrate organisms; little is known about the function and evolution of invertebrate viperin, although it was shown that viperin gene expression was up-regulated in response to poly I:C in Pacific oyster (Green et al., 2014). In addition, as invertebrates in general and amphioxus in particular lack IFNs, whether viperin of invertebrates, like that of vertebrates, plays direct antiviral roles remains unknown. Amphioxus, the extant survivor of an ancient chordate lineage, is the best available stand-in for the proximate invertebrate ancestor of vertebrates and thus an ideal model for gaining insights into the origin and evolution of vertebrates (Stach, 2008; Zhang et al., 2009). The aims of this study were, therefore, to clone viperin homologue from the amphioxus *Branchiostoma japonicum*, to examine its evolution and expression, and to investigate its antiviral activity.

2. Materials and methods

2.1. Cloning and sequence analysis of amphioxus viperin cDNA

Adult amphioxus *B. Japonicum* were collected during the breeding season in the vicinity of Qingdao, China and cultured in aerated seawater for one week before experiments at room temperature. Total RNAs were extracted with Trizol (Invitrogen) from *B. japonicum* according to the manufacturer's instructions. After digestion with RNase-free DNase (TaKaRa) to eliminate the genomic contamination, the first-strand cDNA was synthesized with reverse transcription system (Promega) using oligo d(T) primer, and used as PCR template. The fragment of the viperin gene, named *Bjvip* thereafter, was amplified by PCR with the primer pairs P1−F and P1−R (Table 1) that were designed using Primer Premier 5.0 program on the basis of viperin sequences identified in *Branchiostoma floridae* genome database (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html) and *Branchiostoma belcheri* genome database (http://mosas.sysu.edu.cn/genome/index.php). After determination of the partial cDNA sequence, rapid amplification of cDNA ends (RACE) was employed to obtain the full-length cDNA. The gene-specific primer pairs P2−F and P3−F as well as P4−R and P5−R (Table 1) were used in RACE reactions for the cloning of 3′-end and 5′-end cDNAs, respectively. The 3′- and 5′- RACE-Ready cDNAs were synthesized from the total RNAs using the 3′ Full RACE Core Set and 5′ Full RACE Core Set (TaKaRa, Dalian, China) according to the manufacturer's instructions. The products of 3′- and 5′- RACE were gel-purified, sub-cloned, sequenced and assembled.

The cDNA sequence obtained was analyzed for coding probability with the EditSeq in DNASTAR software package (DNASTAR Inc., Madison, WI, USA). Sequence comparison against known viperin homologues of human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), mouse (*Mus musculus*), platypus (*Ornithorhynchus anatinus*), chicken (*Gallus gallus*), zebra finch (*Taeniopygia guttata*), anole lizard (*Anolis carolinensis*), frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), tilapia (*Oreochromis niloticus*), elephant shark (*Callorhinchus milii*) and amphioxus (*B. floridae*) were performed using the MegAlign program by CLUSTAL W method (Chenna et al., 2003) in DNASTAR software package. The SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional sites and domains and the SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) used to predict the signal peptide. The molecular mass (MW) and isoelectric point (pI) of the mature peptide were analyzed using ProtParam (http://www.expasy.ch/tools/protparam.html). A three-dimensional (3D) modeling of BjVip was predicted by fully-automated protein structure homology modeling (http://

Table 1
Sequences of the primers used in this study.

Primer	Primer sequence (5′−3′)	Primer amplification efficiencies (%)	Amplicon length (bp)
P1−F	TCAGGTTCTGTAAGKDSGAGTT		559
P1−R	CGAGATGGTTCCTTCCGT		
P2−F	TGGCGTTCAAGATAAACTCGG		395
P3−F	AGCGTCAGCATAGTCAGCAAC		
P4−R	GTTGCTGACTATGCTGACGCTC		71
P5−R	CCGAGTTTATCTTGAACGCCAC		
P6−F	ACCGCCAAGAAAGGGCTGAG	97.69	169
P6−R	GGCTGCCGTTGCTGACTATGC		
P7−F	TTCCAGCCTTCATTCCTCG	99.06	109
P7−R	CGGTGTTGGCGTACAGGTC		
P8−F	CGGAATTCATGCTGATTGTGACGTGT		1071
P8−R	CCGCTCGAGCCAGTCCAACTTTAAGTC		
P9−F	CGGGGTACCATGGTGAGCAAGGGCGAGGAG		723
P9−R	CGCGGATCCGATTACTTGTACAGCTCGTCCCATGC		
P10−F	CAGGTACAAACAGCACCTAAACATG	94.08	173
P10−R	CACCGTCAAAGATTACAGGAGAAG		
P11−F	CCCATCTACGAGGGCTACGC	98.15	127
P11−R	TCTCGGCTGTGGTGGTGAAG		
P12−F	GGAATTCCATATGATAATATTCCCGAAAGTCAAGAAGT		1002
P12−R	CGGAATTCTTACCAGTCCAACTTTAAGTCAGCC		
P13−F	AGCTCCAACACCTCCTCCTTCA	97.24	162
P13−R	TTACTCGGTCTCAGTGCCAGA		
P14−F	AGTAGCCGCCCTGGTTGTAGAC	94.08	240
P14−R	TTCTCCATGTCGTCCCAGT		