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Molecular and transcriptional insights into viperin protein from Big-belly seahorse (*Hippocampus abdominalis*), and its potential antiviral role

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ABSTRACT

Viperin is recognized as an antiviral protein that is stimulated by interferon, viral exposures, and other pathogenic molecules in vertebrate. In this study, a viperin homolog in the Big-belly seahorse (*Hippocampus abdominalis*; HaVip) was functionally characterized to determine its subcellular localization, expression pattern, and antiviral activity *in vitro*. The *HaVip* coding sequence encodes a 348 amino acid polypeptide with predicted molecular weight of 38.48 kDa. Sequence analysis revealed that HaVip comprises three main domains: the N-terminal amphipathic α-helix, a radical S-adenosyl-L-methionine (SAM) domain, and a conserved C-terminal domain. Transfected GFP-tagged HaVip protein was found to localize to the endoplasmic reticulum (ER). Overexpressed-HaVip in FHM cells was found to significantly reduce viral capsid gene expression in VHSV infection *in vitro*. Under normal physiological conditions, *HaVip* expression was ubiquitously detected in all 14 examined tissues of the seahorse, with the highest expression observed in the heart, followed by skin and blood. *In vivo* studies showed that *HaVip* was rapidly and predominantly upregulated in blood, kidney, and intestinal tissue upon poly (I:C) stimulus. LPS and *Streptococus iniae* challenges caused a significant increase in expression of *HaVip* in all the analyzed tissues. The obtained results suggest that HaVip is involved in the immune system of the seahorse, triggering antiviral and antibacterial responses, upon viral and bacterial pathogenic infections.

1. Introduction

Viperin is an antiviral protein, alternatively termed as Cig5 or RSAD2 in human [1,2]. Viperin (Interferon-Stimulated Gene - ISG) was initially recognized in humans as an induced protein in macrophages after exposure to interferon-γ (IFN-γ) and a protein in primary fibroblast infected by cytomegalovirus [3,4]. Viperin is involved in innate immunity to fight against pathogens, including DNA and RNA viruses and other infectious microbes [4]. Viperin has been characterized in different species such as mammals [4], reptiles [5], aves [6,7], and fish [8], including amphioxus [9].

Expression of viperin gene is regulated by either IFN-dependent or IFN-independent pathways [10,11]. Three types of interferons (IFNs) are rapidly produced by virus infected cells: type I (IFN-α, IFN-β), type II (IFN-γ), and type III (IFN-λ). The IFN-dependent pathway is initiated with the stimulation of Toll-like receptor 3 (TLR3) by virus-derived dsRNA. TLR3 mediates expression of important transcription factors such as IFN regulatory factors-3, -7 (IRF3, IRF7), and NF-κB. Type I IFNs subsequently trigger the activation of the JAK-STAT pathway, inducing different ISGs, including viperin, through signal cascades that limit the viral infections [1,2,10,12,13]. In the IFN-independent pathway, transcription factors such as IRF-1, IRF-3 or AP-1 have been directly involved in the regulation of viperin induction [1,14].

Vertebrate viperins distinctively comprise three conserved domains. The N-terminal domain substantially differs between species. Besides, it is comprised of an amphipathic α-helix motif and a leucine zipper motif. The central domain includes a CxxxCxxC motif that is a feature of the radical S-adenosylmethionine (SAM) family. The C-terminal domain of the viperin proteins are highly conserved across species [15,16].

Detection of viperin was reported in various animal species [5–7,9]. Fish viperin was initially identified as *Vig-1* in rainbow trout, and its expression was highly upregulated in lymphoid tissues in viral hemorrhagic septicemia virus (VHSV)-infected fish [17]. Additionally, expression analysis of viperin was carried out in red drum (*Sciaenops ocellatus*) and tilapia *(Oreochromis niloticus)* upon bacterial pathogen challenges [8,18]. Furthermore, viperin expression was analyzed in mandarin fish (*Siniperca chuatsi*) [19], Atlantic salmon (*Salmo salar*) [20], crucian carp (*Carassius auratus*) [21], and rock bream

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(*Oplegnathus fasciatus*) [22] with virus stimulations. However, until date, antiviral properties of viperin enzyme has been comprehensively studied only in a few fish species [21,22]. Additionally, molecular characterization, antiviral activity, and expression modulation by pathogenic stimulants of viperin in the Big-belly seahorse (*Hippocampus abdominalis*) has not yet been demonstrated.

The Big-belly seahorse species is reputed for its economical, ecological and biological significance and this teleost fish is used in traditional medical applications in China, Japan and Korea [23,24]. Mariculture industry and natural habitats of seahorses face serious pathogenic infections and environmental changes, resulting in severe economic loss as well as extinction of the seahorse [25,26].

In this study, the viperin paralog of *Hippocampus abdominalis* was molecularly characterized along with its mRNA transcriptional profile under immune challenges with lipopolysaccharide (LPS), polyinosinic:polycytidylic acid [poly (I:C)], and *Streptococcus iniae*. The coding sequence (CDS) of viperin was cloned to analyze the sub-cellular localization in the cell and for determining its antiviral capacities against VHSV infection *in vitro*.

2. Materials and methods

2.1. Experimental seahorse

Seahorses (body length ~12 cm) were obtained from the Korean Marine Fish Breeding Center (Jeju, South Korea), and adapted for one week at a constant temperature (18 ± 2 °C) and salinity (34 ± 0.6 °C) in laboratory aquarium tanks (300 L). Frozen Mysis shrimps were provided twice daily in this acclimatization period. Challenge experiments were revised and approved by Animal Care and Use Committee of Jeju National University.

2.2. Sequence identification and characterization of viperin from the Big-belly seahorse

National Center for Biotechnology Information (NCBI) - BLAST online program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the viperin sequence from laboratory generated transcriptome library of big-belly seahorse [27]. Seahorse viperin was designated as HaVip; complete CDS was obtained with the full amino acid sequence. Conserved domain prediction, and signal peptide sequence detection were carried out by NCBI CDD search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SignalP server (http://www.cbs.dtu.dk/services/SignalP), respectively. The sequence of amphipathic α–helix at N-terminal was predicted by helical wheel projections (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi) and the physico-chemical characteristics of amphipathic α-helix was determined using HELIQUEST [28]. Multiple sequence alignment, pairwise sequence alignment and 3D structure prediction was obtained by Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), EMBOSS needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and SWISS-MODEL (https://swissmodel.expasy.org/) online software, respectively. Phylogenetic analysis was carried out using MEGA 6.0 software in which the neighbor-joining method was used with 5000 bootstrap replicates.

2.3. Tissue isolation and challenge experiment

Six healthy seahorses (three male and three female, with an average body weight of 8 g) were selected for tissue-specific expression analysis. Collected blood was centrifuged at 3000 × *g* at 4 °C for 10 min to obtain peripheral blood cells (PBC). Brain, heart, gill, intestine, stomach, spleen, liver, kidney, muscle, pouch, skin, testis, and ovary were sampled and snap-frozen in liquid nitrogen. All tissues were stored at −80 °C.

Seahorses (average body weight ~3 g) were separated into three immune challenge groups and one control group. Thirty seahorses were included in each group and were not fed during the challenge experiment period. LPS (1.25 μg/μL), *S. iniae* (10^5 CFU/μL), and poly (I:C) (1.5 μg/μL) injections were prepared in PBS in a final volume of 100 μL and the fish were injected intraperitoneally. The control group was injected with 100 μL PBS. Kidneys, intestines, and PBCs were collected from five seahorses at 0, 3, 6, 12, 24, 48, 72 h post-injection (p.i.) time points.

2.4. RNA purification and cDNA synthesis

Tissues were collected and equal amounts of tissues from six unchallenged seahorses and five challenged seahorses were pooled for each time point. RNAiso plus (TaKaRa, japan) was used for total RNA extraction of tissue samples and then purified with an RNeasy spin column (Qiagen, USA). RNA quality was examined using agarose gel (1.5%) electrophoresis. RNA concentration was measured spectrophotometrically using μDrop Plate (Thermo Scientific, USA) at 260 nm. RNA (2.5 μg) was taken from each sample for cDNA synthesis. PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) was used in a 20 μL final reaction mixture. Prepared cDNA was diluted in nuclease-free H_2O up to 40-fold and stored at – 80 °C.

2.5. Quantitative real-time PCR

mRNA expression of *HaVip* was measured in the un-challenged and challenged seahorse tissues using qPCR technique. The 40S ribosomal protein S7 (ShRPS7, Accession no: KP780177) was selected as the seahorse internal control gene. The internal control gene and gene-specific primers for *HaVip* were designed according to the minimum information for publication of qPCR experiments (MIQE) guidelines [29]. The qPCR reactions were carried out in a Thermal Cycler Dice™ system III TP950 (TaKaRa), using 3 μL of diluted cDNA as template in a 10 μL final volume with 0.5 μL of each gene specific primer (10 pmol/μL), 5 μL of 2 × TaKaRa Ex Taq™ SYBR premix, and 1 μL of nuclease free water. The qPCR program contained one cycle of 95 °C for 30 s, followed by 45 amplification cycles of 95 °C for 5s, 58 °C for 10 s, 72 °C for 20 s and final dissociation cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. All qPCR experiments were conducted in triplicate, and relative expression of HaVip mRNA was analyzed according to the Livak $2^{-\Delta\Delta Ct}$ method [30]. qPCR results were further normalized with corresponding PBS controls at each time point.

2.6. Plasmid construction

The coding sequence of *HaVip* was cloned into *Nde*I/*Eco*RI sites of pcDNA™ 3.1(+) expression vector (Thermofisher, USA) for over-expression. Similarly, same sequence was cloned into *Hin*dIII/*Eco*RI sites of the pEGFP-N1 vector (Clontech, USA). Cloning primers were designed including relevant restriction sites (Table 1). Cloned insertion sequences were confirmed after sequence analysis (Macrogen, Korea). Constructed plasmids were purified using QIAfilter Plasmid Midi Kit (QIAGEN, Germany).

2.7. Cell culture, virus, and transfection

The fat head minnow (FHM) cell line was cultured at 20 °C in L-15 medium, supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Constructed *HaVip*-gene containing pcDNA 3.1(+) or empty pcDNA 3.1(+) plasmid vectors (1 μg from each vector) were transfected into cultured cells using X-tremeGENE™ 9 reagent (Roche, Germany) according to the manufacturer's protocols. For transfection, the FHM cells (10^5 cells/well) were distributed into 6-well cell culture plates. Lab stock of Korean isolate of VHSV (FWando05) was used in this experiment which was isolated from VHSV infected Olive flounder (*Paralichthys olivaceus*) [31]. VHSV titers were measured through 50% tissue culture infective dose ($TCID_{50}$) according to the

Table 1
Nucleotide sequences of PCR and qPCR primers used in this study.

Primer name	Application	Sequence of primer (5′-3′)
HaVip_F	ORF amplification (For pcDNA3.1 +)	GAGAAAGCTTGCTATGAATGCATCCAACGTCCTTAC
HaVip_R		GAGAGAGAATTCTCACCACTCCAGTTTCATATCAGCTTT
HaVip_F	ORF amplification (For pEGEFP-N1)	GAGAGAAAGCTTATGAATGCATCCAACGTCCTTACGG
HaVip_R		GAGAGAGAATTCCCACTCCAGTTTCATATCAGCTTTACTC
HaVip_qF	qPCR amplification	AGGAAGAGCCACCTGGACAATCT TCAATCAGCAGACACTGGAACACCT
HaVip_qR		
VHSV_qF		TCGGATGCTGGGAGAGTCCTACTA
VHSV_qR		TCGCGGAACTTCTCTGTCACCTT
40S ribosomal protein S7_qF 40S ribosomal protein S7_qR	qPCR internal reference of seahorse	GCGGGAAGCATGTGGTCTTCATT
		ACTCCTGGGTCGCTTCTGCTTATT
EF1α_qF	qPCR internal reference of FHM cells	GGCTGACTGTGCTGTGCTGAT
EF1α_qR		GTGAAAGCCAGGAGGGCATGT
FHM Vip_qF	qPCR Viperin amplification of FHM cells	AAGACTTCCTGGACCGCCATAAGA
FHM Vip_qR		GCCTCTTCCACACCAACATCCA

Reed-Muench method [32].

2.8. Subcellular localization of HaVip

The FHM cell line was used to study the subcellular localization of HaVip protein. Constructed pEGFP-N1/HaVip and pEGFP-N1 empty plasmid were transfected into FHM cells and incubated at 25 °C for 24 h for protein expression. Cells were stained with DAPI (Invitrogen, USA) and ER-Tracker™ Red dye (Invitrogen, USA) according to manufacturer's protocol. Briefly, transfected cells were fixed with 4% formaldehyde and washed twice with 1 × PBS. Thereafter staining solutions were added and incubated for 20 min at 37 °C before wash with 1 × PBS. Subcellular localization was observed by fluorescence microscope under 400X magnification (Leica Microsystems, Germany). Images were processed using Leica Application Suite X version 3.3.

2.9. Antiviral activity assay

The FHM cells were transfected with pcDNA3.1 + /HaVip and pcDNA3.1 + empty expression vectors. Twenty four hours post transfection, pcDNA3.1 + /HaVip and pcDNA3.1 + transfected cells were infected with VHSV at multiplicity of infection (MOI) of 0.01, and another pcDNA3.1 + and pcDNA3.1 + /HaVip transfected cell series were kept uninfected with virus. Samples were incubated for 24 h and 48 h post infection. RNA extraction, cDNA synthesis and qPCR were performed as described above. For the FHM cells, elongation factor 1 alpha gene (Accession no: AY643400) was used as the internal control gene and the endogenous viperin (Accession no: KM099177) expression was determined. Viral gene expression was measured using nucleocapsid protein (Accession no: NP_049545) of VHSV.

2.10. Statistical analysis

All experiments were conducted in triplicate. Final results are indicated as mean ± standard deviation (SD). Student's t-test was applied to the collected data for the evaluation of significance between groups. P− values less than 0.05 were considered to indicate statistically significant differences ($P < 0.05$).

3. Results

3.1. Sequence characterization of HaVip, phylogenetic relationship analysis, and tertiary structure

The complete open reading frame (ORF) of *HaVip* is 1047 bp in length (Accession No: MH229987), which encodes a protein sequence of 348 amino acids with a predicted molecular weight of 38.48 kDa. The theoretical isoelectric point (pI) is 6.40. A signal peptide was not detected in the HaVip protein sequence according to the SignalP 4.1 software. According to NCBI CDD tool, HaVip sequence comprises non-conserved N-terminal amphipathic α–helix domain, and radical SAM domain (Tyr^{64} – Asn^{196}) containing conserved motifs and a C-terminal conserved domain (Fig. 1). The HaVip amino acid sequence and vertebrate viperin orthologs were aligned to determine sequence identities; also the alignment results indicated that HaVip shares the highest amino acid identity and similarity with Nile tilapia viperin sequence at 77.7% and 84.2% respectively, and the lowest identity and similarity with mouse, at 62.5% and 75.5% respectively. However, HaVip showed sequence identity with human at 65.5% and similarity, at 77.7% (Fig. 1). The N-terminal amphipathic α–helix was detected at Gly^{9} – Gly^{42} in the protein sequence (Fig. 1). The helical wheel projection views of amphipathic α-helix (residues from 9 to 42) of viperin from human, Nile tilapia and big-belly seahorse are illustrated with their compared physicochemical properties (Fig. S1). The predicted hydrophobic face of viperin α-helix in human, Nile tilapia and seahorse consisted of 13 (LLLLFLALLFLFW), 11 (IGFWPAIALLF) and 4 (PFVA) residues, respectively (Fig. S1B). To determine the evolutionary relations among HaVip and viperin orthologs from different animals, phylogenetic analysis was carried out; and in the phylogeny tree, HaVip clustered with the fish species (Fig. 2). A tertiary structure model of HaVip protein was predicted, based on the crystal structure of mouse viperin (PDB: 5vsl.1), which showed 75.0% identity with each other (Fig. 3). The complete 3D structure consisted of 9 β-strands and 9 α-helices, from the N-terminal to the C-terminal end (Fig. 3A). The deduced 3D structure exposed the active site design with complete SAM domain (Fig. 3B).

3.2. HaVip localizes to endoplasmic reticulum (ER)

To examine the subcellular localization of viperin, FHM cells were transfected with pEGFP-N1/HaVip, which expresses green fluorescent protein (GFP)-tagged seahorse viperin protein (HaVip/GFP). Fluorescent stains indicated the ER in red color and the nucleus in blue color. HaVip/GFP expression was concentrated around the ER and dispersion was observed throughout the cells (Fig. 4).

3.3. HaVip reduces viral gene expression in FHM cells

To observe the possible effect of HaVip on viral infection, cultured FHM cells were transfected with pcDNA3.1 + /HaVip or empty pcDNA3.1 + vector. The transfected cells were infected with VHSV and the viral load in the FHM cells was determined at 24 h and at 48 h p.i. Compared to control, FHM cells transfected with pcDNA3.1 + /HaVip resulted in significantly lower amounts of virus nucleocapsid transcript expression at 24 h and 48 h p.i (Fig. 5). There was no significant effect on the expression of exogenous HaVip by expression of the endogenous

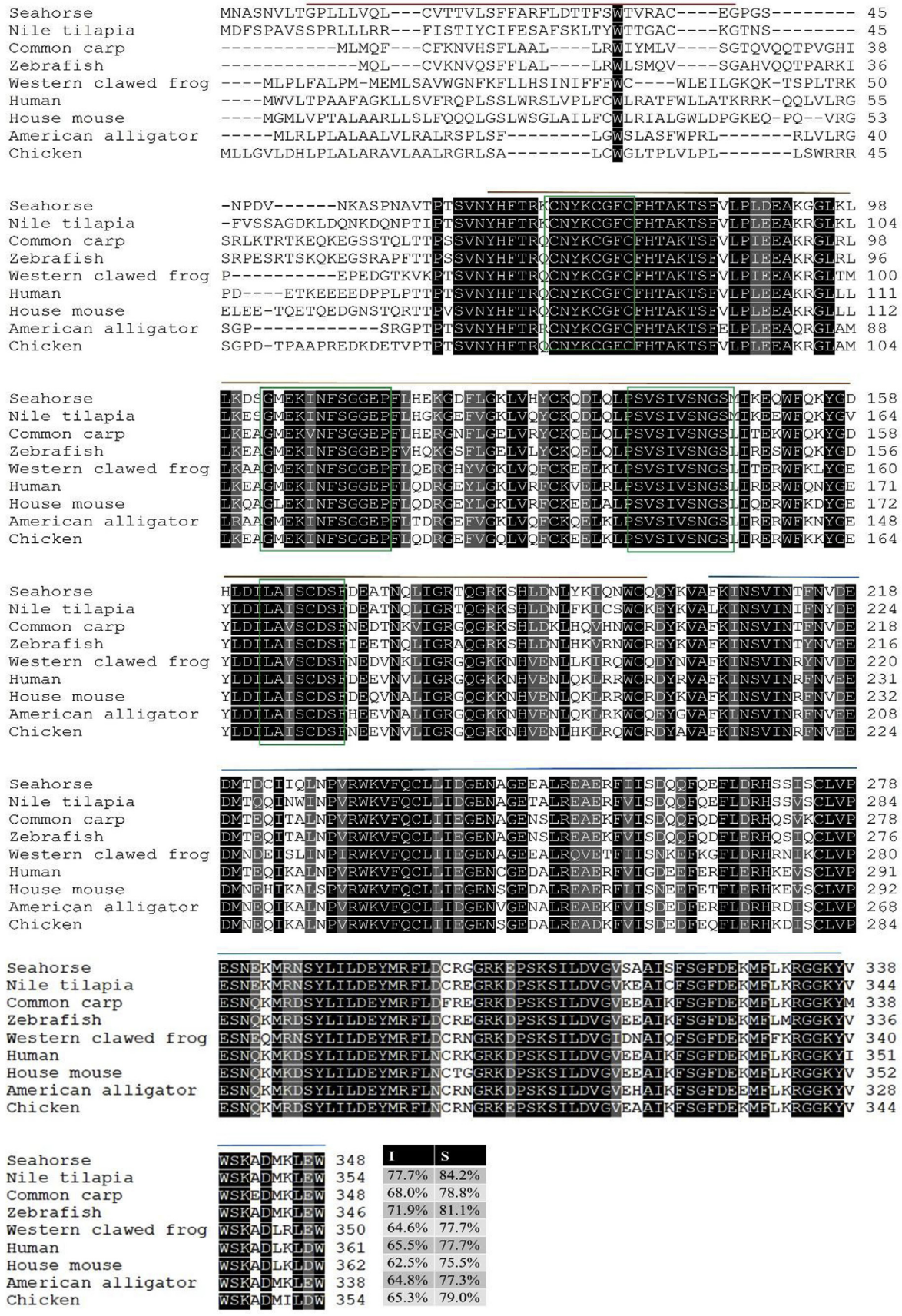

Fig. 1. Multiple sequence alignment of amino acid sequences of HaVip with ortholog sequences from different organisms. Fully conserved amino acids are shown in black. Strongly conserved and weakly conserved residues are shown in dark gray and light gray, respectively. The N-terminal amphipathic α–helix sequence is indicated by redline. Radical SAM domain is indicated by brown line and conserved motifs are shown in green boxes. The blue line indicates C-terminal conserved domain. At the end of each sequence, the identities (I) and the similarities (S) of each ortholog with HaVip are shown as percentages (%). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

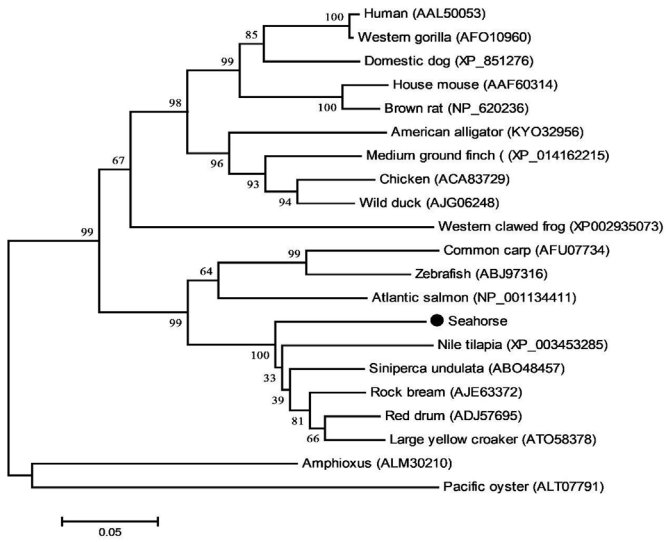

Fig. 2. Phylogenetic analysis of HaVip with selected full-length viperin amino acid sequences of other species. The tree was constructed using neighbor-joining methods with 5000 replicates. For each protein, corresponding bootstrap values are indicated on the branches and the NCBI accession numbers are indicated with the common names.

viperin (Data not shown).

3.4. Quantitative analysis of tissue specific mRNA expression of HaVip

In order to quantify the tissue distribution of HaVip mRNA expression under normal physiological conditions, qPCR was performed with gene specific primers. Single dissociation curve was observed in 40S ribosomal protein S7 and *HaVip*, ensuring their specific amplification. *HaVip* revealed a diverse range of gene expression patterns among all the analyzed tissues. The highest mRNA expression level was obtained in the heart (27-fold) tissue than that the lowest expression value in spleen, followed by skin (16.5-fold) and blood (10-fold) (Fig. 6). The lowest expressions were detected in the spleen and the liver (2.1-fold) tissues.

3.5. Comparative analysis of mRNA expression of HaVip upon immune stimulation

In the blood (Fig. 7A), *HaVip* expression was significantly upregulated upon poly (I:C) challenge at 3 h p.i. indicating maximum fold changes at 24 h (82.7 fold), continuing its expression to 50 fold at 72 h p.i. Upon LPS stimulation, the highest mRNA expression was observed at 12 h p.i. (4.5 fold), after which, from 48 h p.i, *HaVip* expression was significantly downregulated. The transcript of viperin was observed to be the highest at 6 h p.i (4 fold) and continue to be upregulated until 12 h p.i. with *S. iniae* infection. In the kidney (Fig. 7B), upon poly (I:C) stimulation, levels of viperin mRNA transcripts were gradually and significantly elevated from 3 h p.i (25 fold) to peak at 12 h (192 fold) and then the elevation decreased until at 72 h (19.5 fold). Both LPS and *S. iniae* injections resulted in increased mRNA expression of viperin until at 12 h. p.i. LPS stimulation significantly downregulated the transcription at 48 h (0.6) and at 72 h (0.57). Similarly, decreased transcript levels of HaVip were observed in animals infected by *S. iniae* at 48 h (0.8 fold) and at 72 h p.i. (0.2 fold). In the intestine (Fig. 7C), mRNA expression of HaVip was significantly increased at all the time points, with the highest levels at 48 h p.i. (61 fold) and 12 h p.i. (37 fold) with poly (I:C). LPS stimulus elicited the mRNA expression at 12 h p.i. (~3 fold) and 48 h p.i. (2.5 fold). However, *S. iniae* infection was found to be elevate the expression at 3 h p.i (~1.7 fold), 24 h p.i. (~5 fold), and 48 h p.i. (~4 fold) and later at 72 h p.i. (~4 fold).

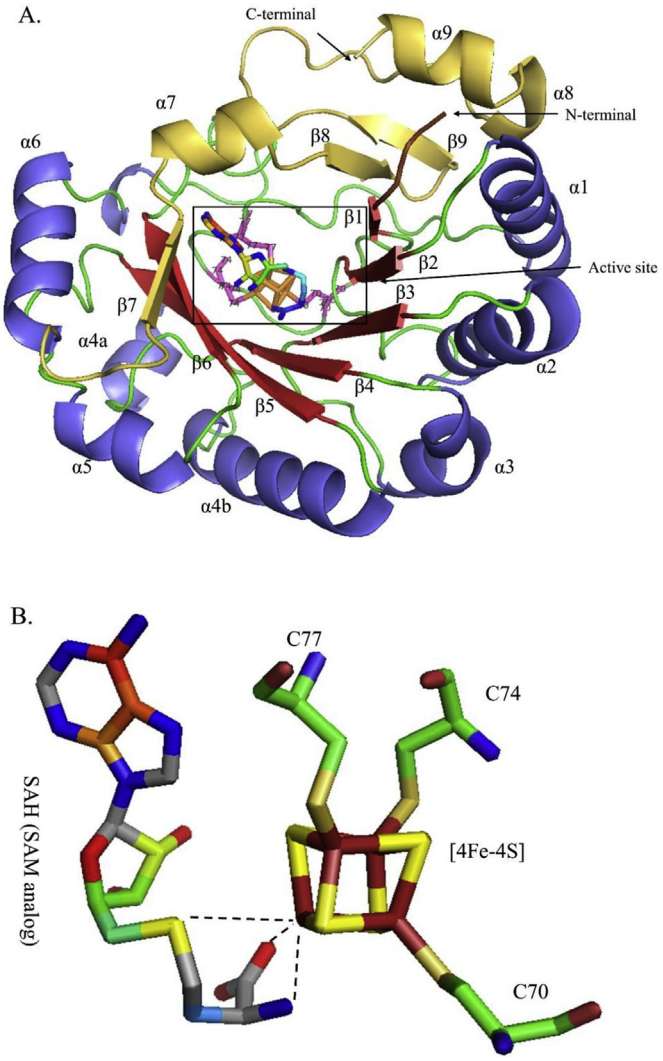

Fig. 3. Tertiary structures of seahorse viperin and structure of a SAM enzyme according to the homology based modelling (A) The domain architecture of the seahorse viperin (B) The structure of the [4Fe−4S] cluster in complex with conserved motif ($C_{70}NYKC_{74}GFC_{77}$) and the ligand SAM. Strands, β-sheets, and α-helixes are represented using different colors to highlight the contrasts between the domains. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Recent experiments on viperin have shown its broad antiviral properties against various viral pathogens in mammals [33–35]. The immune response to viral and bacterial pathogens has not been extensively studied in many fish species [21,22]. Hence, HaVip was characterized by detecting its antiviral activity *in vitro*. As all other known viperin counterparts, HaVip was found to contain an N-terminal amphipathic α-helix (residues from 9 to 42), a typical SAM domain with the highly conserved CxxxCxxC ($C_{70}NYKC_{74}GFC_{77}$) motif positioned after β1 strand and a conserved C-terminal domain. In the phylogenetic tree, seahorse viperin was grouped with fish, showing its evolutionary conservation. Additionally, the predicted 3D structure of HaVip is greatly comparable to that of mouse viperin. Based on nuclear magnetic resonance (NMR) and circular dichroism (CD) studies, mouse viperin was categorized as a radical SAM enzyme and its residues (45–362) constitute the [4Fe−4S] cluster [16]. It has been reported that the conserved three cysteine residues at the active site in SAM domain is required for [4Fe−4S] formation in human, mouse as well as in fish

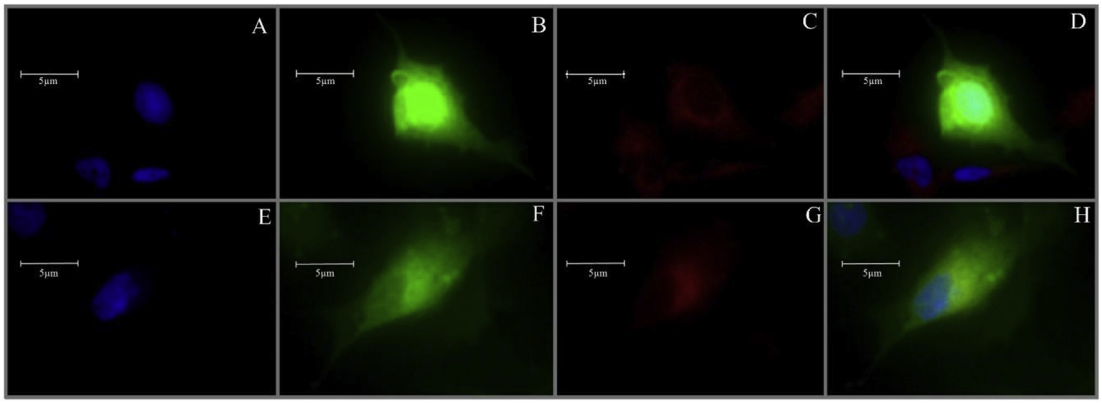

Fig. 4. ER-associated **s**ubcellular localization of HaVip. FHM cells were transfected with empty pEGFP-N1 vector (A–D) and pEGFP-N1/HaVip (E–H). GFP and HaVip tagged GFP expression were visualized in green color (B and F). The transfectants were stained with DAPI (blue) (A and E) and ER-Tracker Red (red) (C and G) that detected the nucleus and endoplasmic reticulum (ER), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

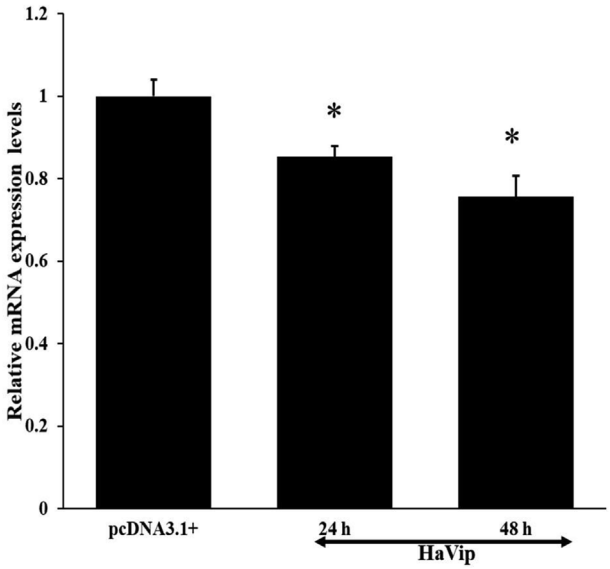

Fig. 5. *In vitro* effect of HaVip on VHSV infection. FHM cells were transfected with pcDNA3.1 + /HaVip or empty pcDNA3.1 + and infected with VHSV 24 h post transfection. Viral protein content was determined at 24 h and 48 h p.i. using qPCR analysis. Results are indicated as mean ± SD of three replicates (n = 3). Statistically significant values ($P < 0.05$) are represented with asterisk (*).

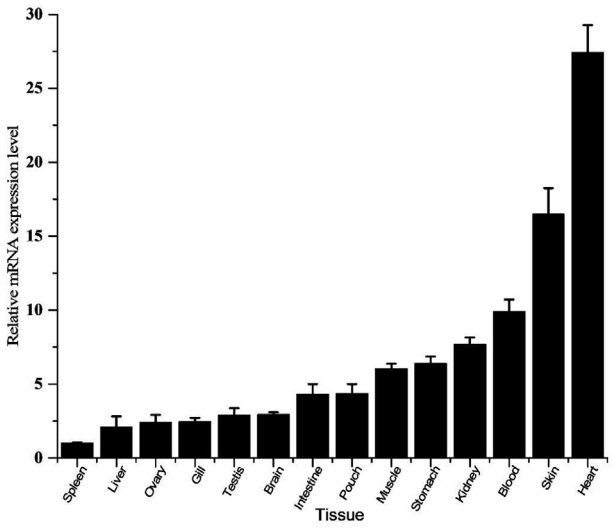

Fig. 6. Tissue-specific expression profiles of HaVip (presented relative to the expression of mRNA in spleen) mRNA in unchallenged *H. abdominalis*. Results are indicated as mean ± standard deviation (SD) of three replicates (n = 3).

[36,37]. Collectively, the amino acid sequence analysis results revealed that HaVip remains highly homologous to other teleost and vertebrate viperin counterparts [5,6,8,19,38].

Previous studies have shown that the mammalian viperin is associated with the cytosolic face of the ER, using its N-terminal amphipathic α-helix region [39]. The study of rock bream and crucian carp viperin showed that fish viperin is also associated with ER [22,40]. Moreover, it was shown that the N-terminal amphipathic α-helix of crucian carp viperin is not only essential, but also acts as a guide for ER-localization [40]. Experimental evidence indicating that the antiviral activity of viperin is linked to the ER was also confirmed [41]. Thus, HaVip-ER-localization could due to the amphipathic α-helix region, in order to assist the antiviral activity.

Studies of mammalian viperin have indicated its involvement in antiviral responses. West Nile virus (WNV) infected, viperin$^{-/-}$ mice displayed an increased lethality and enhanced viral titer over the wild type mice *in vivo* [42]. Besides, the viperin-deficient mice experiment showed that viperin mediates optimal Th2 immune response via NF-κB and AP-1 activities [43]. In contrast, overexpression of mice viperin confirmed that its enhanced ability to restrict viral replication of Hepatitis C virus (HMV), alphavirus, human cytomegalovirus (HCMV), and influenza A virus [4,14,44,45]. In fish, the rock bream viperin reduced the viral copy number of megalocytivirus RBIV-C1 in Grunt fin cells [22]. In this study, the antiviral activity of HaVip was analyzed against VHSV, as VHSV infection is one of the world's most severe fish diseases [46]. HaVip-transfected-FHM cells showed significant suppression of VHSV nucleocapsid transcript expression 24 h and 48 h post VHSV infection, compared to their respective controls. In previous studies, nucleocapsid proteins were used to detect the viral content of VHSV that infected the fish species [47–49]. Cytopathic effect on FHM cells was not observed until 48 h p.i at 0.01 MOD (data not shown). Hence, the experimental results suggested that overexpression of *HaVip* is capable of inhibiting or killing the VHSV propagation *in vitro*. It has been shown that viral budding from the cell plasma membrane can be inhibited by either human or murine viperin by disturbing lipid rafts and enhancing membrane fluidity [35,50]. Therefore, it may be proposed that the seahorse viperin exhibits antiviral properties. However, at present, the antiviral mechanism of viperin has not been completely elucidated.

Currently, tissue-specific expression of viperin has been observed

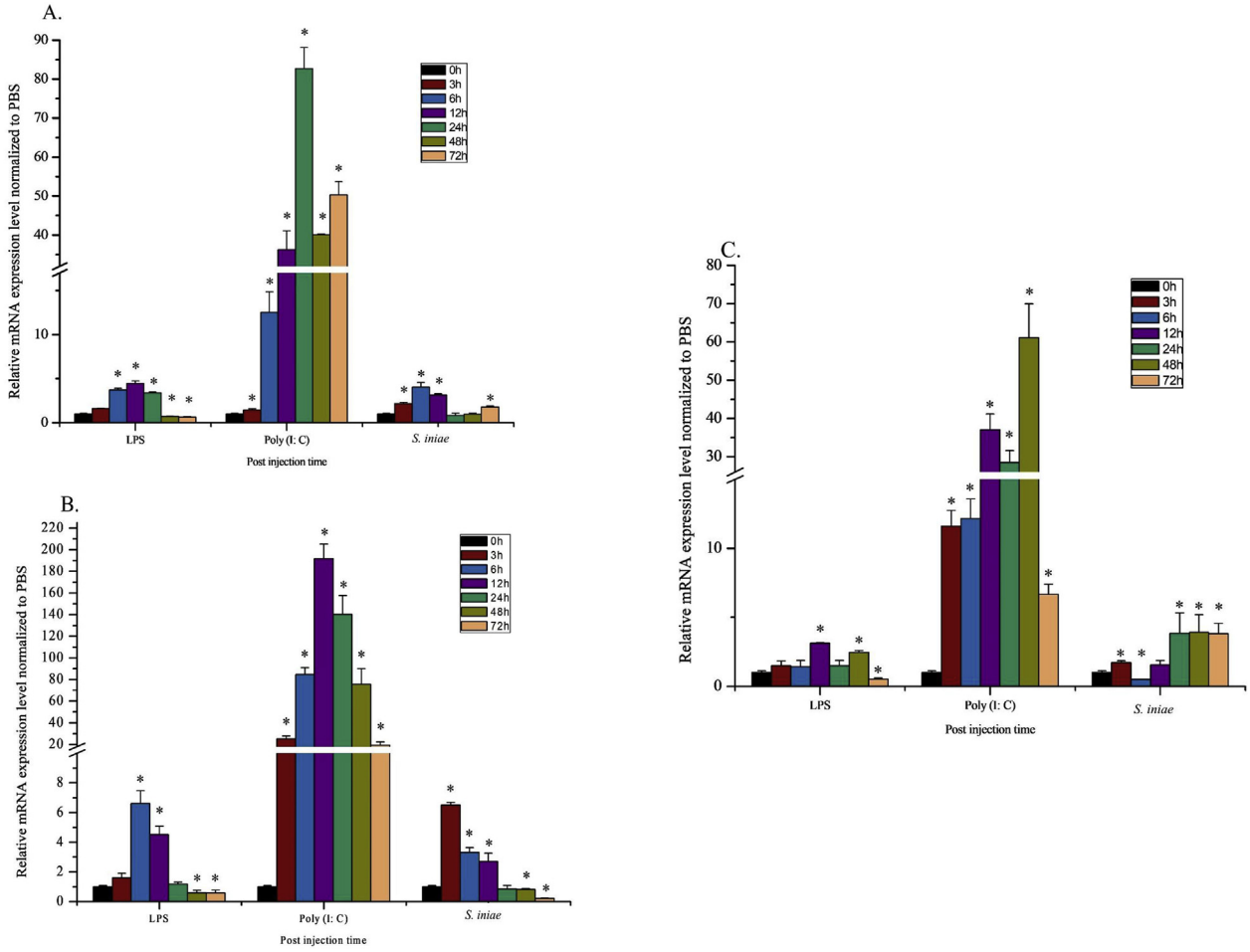

Fig. 7. Relative expression analysis after immune challenge with LPS, poly (I:C), and *S. iniae* by qPCR. HaVip expression analysis in (A) blood tissue (B) Kidney (C) intestine. Results are indicated as mean ± SD of three replicates (n = 3). Statistically significant values ($P < 0.05$) are represented with asterisk (*).

only in four fish species (mandarin fish, red drum, rock bream, and large yellow croaker) [18,19,22,38]. Tissue-specific expression of *HaVip* was obtained by qPCR in all examined tissues with different magnitude levels. Though, the heart tissue showed the highest viperin expression in seahorse, in large yellow croaker and rock bream, heart tissue ranked second and third respectively, in terms of magnitude of viperin expression [22,38]. It was observed that viperin expression was the highest in blood of large yellow croaker as well as in red drum [18,22]. Moreover, viperin expression in mandarin fish was reported to be higher in the head kidney, trunk kidney and spleen [19]. Hence, spatial expression pattern of viperin varied from one organ to another organ as well as fluctuated among fish species. Blood mainly consists of cells such as natural killer cells and macrophages etc. which are directly involved in innate immunity [51]. The mucosal tissues such as skin and gill serve as initial barrier against the entry of pathogen. Once the initial barriers are breached, and an invading pathogen comes into contact with the circulation, then the kidney and spleen play vital roles in originating immune responses by trapping these antigens [52]. Seahorse kidney was observed to exhibit higher expression of HaVip transcripts compared to its expression in the spleen. Hence, the blood and kidney were selected for challenge experiments. The intestine was selected as it is directly exposed to pathogenic substances, in which viperin expression has not been studied with pathogenic challenges [53]. Gram-positive *S. iniae*, poly (I:C) and LPS were selected as immune stimulants. *S. iniae* is considered as a pathogen causing severe infections in aquaculture [54]. Poly (I:C) (synthetic dsRNA) has been used as a viral mimic immune stimulant of fish [55]. LPS triggered immune responses, are similar to Gram-negative bacterial endotoxins [56].

The expression of the *HaVip* gene was significantly and quickly upregulated after challenge by poly (I:C) in blood, kidney and intestine. Besides, in the kidney, expression reached a peak level at 12 h compared to blood (peak at 24 h) and intestine (peak at 48 h). But in the large yellow croaker, LcViperin expression peaked at 24 h in blood with poly (I:C) stimulus [38]. In kidneys of rock bream and red drum, viperin was significantly expressed 12 h p.i with megalocytivirus and expressed 4 h p.i with poly (I:C) challenge, respectively [18,22]. These results suggested that the kidney of seahorse seems to respond to poly (I:C) stimulation much quicker than its blood and intestine tissues. Viperin expression was found to be induced by poly (I:C) in mandarin fish and Atlantic cod tissues [19,57]. Viperin expression was upregulated in macrophage/dendritic-like cell line of Atlantic salmon after treatment with infectious salmon anemia virus (ISAV) and in erythrocytes after treatment with piscine orthoreovirus (PRV) [20,58]. Similarly, VHSV and interferon-like factor induced viperin expression in rainbow trout leukocytes [17]. Crucian carp blastulate embryonic (CAB) cells overexpressed viperin and IFN system genes after being induced by grass carp hemorrhagic virus (GCHV) and poly (I:C) [59]. It was reported that the poly (I:C) induced activation of the crucian carp viperin promoter and its subsequent expression was facilitated by RLR-triggered IFN signaling pathway [21]. Additionally, a study of large yellow croaker confirmed that poly (I:C) stimulation greatly induced the RIG-I-like receptor signaling pathway genes such as MDA5, LGP2 and MAVS [60]. These interferon signaling pathways are involved in antiviral action as a part of the innate immunity [61–63]. In mice, it has been reported that viperin is highly produced in macrophages and neutrophils during

Lymphocytic choriomeningitis virus (LCMV) infection. However, viperin was produced with delayed kinetics in dendritic cells in order to successfully activate the adaptive immune system through efficient T cell priming [64]. Macrophages and neutrophils are mainly involved in the innate immune responses upon pathogenic invasions [65]. Interestingly, at later time points, *HaVip* expression was found to drop gradually once maximum expression was achieved in the three examined tissues. The pattern of drop in expression resembled the LcViperin expression patterns in poly (I:C) challenged tissues of large yellow croaker [38]. Hence, in fish species, late reduction of viperin expression may facilitate subsequent direction of the adaptive immune system. Accordingly, it can be proposed that significant viperin expression observed in seahorse kidney, blood and intestine may be due to antiviral reactions resulting from the activation of innate immune responses, to poly (I:C) stimulus.

Upon LPS and *S. iniae* challenges, *HaVip* was significantly expressed in blood, kidney and intestine. Earlier viperin responses appeared to be initiated in these tissues (kidney, blood and intestine) at 3 h p.i by *S. iniae* compared to LPS responses. In red drum kidney, *S. iniae* significantly down regulated the *SoVip* expression at every time point until 72 h, while *Listonella anguillarum* upregulated the expression [18]. However, upregulated *HaVip* expression was seen to decrease only after 48 h p.i by *S. iniae*. In tilapia, viperin expression was considerably induced upon LPS stimulation in many tissues, including kidney, liver, gill, fin and spleen [8]. Moreover, transfection of tilapia viperin-expressing plasmid into zebrafish muscle reduced bacterial counts and upregulated the expression of inflammatory genes such as NF-κB and IFN-1 after bacterial infection [8]. *In silico* studies confirmed that HaVip showed the highest similarity and the highest identity with tilapia viperin. Thus, LPS and *S. iniae* challenged seahorse tissues reflected the innate immune response via HaVip expression as shown by tilapia viperin. In all challenged tissues, both expression patterns were almost parallel to each other upon LPS and *S. iniae* stimulation. Likewise, post poly (I:C) challenge, viperin expression in blood, kidney and intestine also appeared as a bell shaped pattern. In conclusion, higher and rapid expression of seahorse viperin may trigger the innate immune response upon poly (I:C), *S. iniae* and LPS challenges. Later, expression of viperin decreases in order to facilitate the adaptive immunity in seahorse. Therefore, further experiments must be performed in the future to obtain a comprehensible explanation for the involvement of seahorse viperin in the innate and adaptive immune responses.

In conclusion, full length of viperin CDS was identified and cloned into pcDNA3.1 (+) and pEGFP-N1 vectors. Recombinant vectors were transfected into FHM cells and analyzed for antiviral activity against VHSV; the subcellular localization of HaVip was detected in the FHM cells. *In silico* studies revealed that HaVip belongs to the radical SAM domain containing enzyme family and it shows high similarity to the other viperin proteins found in vertebrate species. Expression of *HaVip* was distributed in unchallenged seahorse tissues, in a species-specific manner. Upon challenge with poly (I:C), LPS and *S. iniae*, expression of *HaVip* was significantly elevated in blood, kidney and intestine. Finally, based on our observed results, we propose that HaVip not only contributes to the antiviral activity through ER localization, but also modulates other immune stress responses and may be associated with the host immune defense mechanism.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.006.

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