Oyster viperin retains direct antiviral activity and its transcription occurs via a signalling pathway involving a heat-stable haemolymph protein

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Little is known about the response of non-model invertebrates, such as oysters, to virus infection. The vertebrate innate immune system detects virus-derived nucleic acids to trigger the type I IFN pathway, leading to the transcription of hundreds of IFN-stimulated genes (ISGs) that exert antiviral functions. Invertebrates were thought to lack the IFN pathway based on the absence of IFN or ISGs encoded in model invertebrate genomes. However, the oyster genome encodes many ISGs, including the well-described antiviral protein viperin. In this study, we characterized oyster viperin and showed that it localizes to caveolin-1 and inhibits dengue virus replication in a heterologous model. In a second set of experiments, we have provided evidence that the haemolymph from poly(I:C)-injected oysters contains a heat-stable, protease-susceptible factor that induces haemocyte transcription of viperin mRNA in conjunction with upregulation of IFN regulatory factor. Collectively, these results support the concept that oysters have antiviral systems that are homologous to the vertebrate IFN pathway.

INTRODUCTION

IFNs are a class of cytokines that induce vertebrate cells into an antiviral state (Randall & Goodbourn, 2008). Typically, virus-infected cells secrete IFNs to alert other cells in the body to the presence of a virus (Robertsen, 2006). IFNs induce an antiviral state by binding to IFN receptors, which are present on all nucleated cells (Biron & Sen, 2001). Receptor engagement activates signal transduction via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, leading to the transcription of hundreds of IFN-stimulated genes (ISGs) (Schoggins & Rice, 2011). The products of these ISGs exert numerous antiviral effector functions, many of which are still not fully described (Schoggins & Rice, 2011). Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN inducible) is one of a few ISGs that has been shown to have direct antiviral activity against a range of RNA and DNA viruses (Mattijssen & Pruijn, 2012), and is one of the earliest and most significantly upregulated genes in response to viral infection in humans (reviewed by Helbig & Beard, 2014). Viperin was first isolated from fibroblast cells and was shown to be an inducible cytoplasmic antiviral protein that is induced by IFNs and human cytomegalovirus (HCMV) (Chin & Cresswell, 2001). Subsequently, viperin has been characterized in a variety of vertebrate species and shown to be a highly conserved evolutionary host protein (Goossens et al., 2015; Helbig et al., 2011; Milic et al., 2015; Wang et al., 2007; Zhang et al., 2014). Viperin localizes to the endoplasmic reticulum (ER) and lipid droplets (reviewed by Mattijssen & Pruijn, 2012) and inhibits the release of influenza virus and human immunodeficiency virus by altering the formation of lipid rafts, which are the known sites of virus budding (Nasr et al., 2012; Wang et al., 2014b). Viperin also inhibits the replication of both hepatitis C virus and dengue virus by interacting with viral non-structural proteins (Helbig et al., 2011, 2013).

The evolutionary origins and divergence of major immune response pathways have generally been inferred from comparisons between vertebrates and model invertebrate species, such as Drosophila melanogaster, Anopheles gambiae, Caenorhabditis elegans and Ciona intestinalis (Robalino et al., 2004). The absence of IFN or its major effectors from the genomes of these model invertebrates has been used to imply that the IFN pathway is a vertebrate innovation (Loker et al., 2004; Robalino et al., 2005). However, non-model invertebrate species might have
antiviral systems that are homologous to the vertebrate type I IFN response (Green & Montagnani, 2013; He et al., 2015). In particular, transcriptome sequencing of the Pacific oyster (Crassostrea gigas) infected with ostreid herpesvirus type 1 (OsHV-1) has revealed an ancient IFN pathway in the oyster genome with key components [Toll-like receptor (TLR), Rig-like Receptor (RLR), IFN regulatory factors (IRFs), JAK/STAT and ISGs] conserved and highly upregulated in response to OsHV-1 infection (He et al., 2015; Renault et al., 2011; Rosani et al., 2014; Segarra et al., 2014b). Many of these antiviral genes are upregulated in C. gigas tissues following injection with poly(I:C) to mimic a virus infection (Green et al., 2014a, b), and this inducible immune response can inhibit OsHV-1 infection (Green & Montagnani, 2013). Viperin is also reported to be one of the earliest and most upregulated of the C. gigas genes in response to OsHV-1 (Rosani et al., 2014 and poly(I:C) (Green et al., 2014b), but it is unknown whether oyster viperin has direct antiviral activity.

The concept that non-model invertebrates, such as oysters, have a type 1 IFN response is still contrary to established views in innate immunology. Many comparative immunologists are sceptical that oysters have an IFN response because bioinformatics analysis of all fully sequenced invertebrate genomes (including the oyster) have failed to identify an IFN cytokine (He et al., 2015; Loker et al., 2004). In addition, it is unknown whether invertebrate genes that share sequence homology to vertebrate ISGs have also retained their antiviral functions over a long evolutionary time period. Therefore, the first objective of this study was to characterize oyster viperin and determine whether it had direct antiviral activity. The second objective was to confirm that expression of oyster viperin is induced via a cytokine.

RESULTS

Sequence analysis of oyster viperin

Utilizing the oyster genome database (http://www.oysterdb.com), primers were designed to amplify the complete coding sequence of C. gigas viperin. The full-length coding sequence of oyster viperin was amplified and sequenced from four individual oysters and confirmed to be 1050 bp, encoding 350 aa. Comparison of these four nucleotide sequences revealed a single 3 bp insertion in the N terminus and 13 single-nucleotide polymorphisms (SNPs) in the C terminus of C. gigas viperin. Only two of these SNPs were associated with amino acid substitutions at aa 250 and 273. In the vertebrate phylum, viperin amino acid sequences are highly conserved. A comparison of C. gigas viperin with human (Homo sapiens; GenBank accession no. NP_542388), fish (Danio rerio; GenBank accession no. NP_001020727), chicken (Gallus gallus; GenBank accession no. ACA83729) and lancelet (Branchiostoma floridae; GenBank accession no. EEN65148) viperin revealed 62–64% amino acid identity. The C-terminal region (essential for some of the antiviral activity of this protein) of viperin was highly conserved between vertebrates and C. gigas (Fig. 1). The amphipathic helix in the N-terminal region of human viperin allows it to tether to the ER and lipid droplets. However, an amphipathic helix could not be predicted in C. gigas viperin (Amphipaseek, https://prabi.ibcp.fr/htmv/pbil_ibcp_Amphipaseek.html), although it does retain the radical S-adenosyl methionine (SAM) domain (Conserved Domains search, http://www.ncbi.nlm.nih.gov).

dsRNA induces oyster viperin expression

Mammalian viperin is rapidly induced (within 2 h) in response to viruses, IFN and bacterial by-products, such as dsRNA and lipopolysaccharide (LPS) (reviewed by Helbig & Beard, 2014). We chose to investigate oyster viperin expression in response to poly(I:C), which is a synthetic dsRNA molecule. The use of synthetic dsRNA in place of a replicating virus allowed a full analysis of a cellular response to dsRNA in the absence of any interference that may occur via specific viral proteins. In contrast to vertebrates, injection of poly(I:C) in the oyster’s adductor muscle resulted in the delayed expression of viperin (Fig. 2a). Haemocyte expression of oyster viperin remained stable at 3 and 9 h p.i. (P>0.05, Fig. 2a) and then increased rapidly to peak at 27 h.p.i. (Fig. 2a, P<0.05). At 27 h p.i., viperin mRNA was also elevated in adductor muscle, gill and mantle tissues (P<0.05) but not in digestive gland or gonad tissues (Fig. 2b, P>0.05).

Stimulation of primary haemocyte cell cultures with different concentrations of poly(I:C) or LPS revealed a dose threshold for C. gigas viperin expression (Fig. 3). Expression of haemocyte viperin was induced by poly(I:C) at a concentration of 24.0 μg ml⁻¹ (P<0.05) but not at 2.4 and 0.24 μg ml⁻¹ (P>0.05). Stimulation of haemocytes with three different concentrations of LPS failed to induce the expression of C. gigas viperin (Fig. 3, P>0.05).

Haemolymph protein/peptide induces haemocyte viperin expression

Our results demonstrated that injection of poly(I:C) into the oyster adductor muscle resulted in elevated viperin expression in the majority of tissue compartments. Three possibilities exist for the systemic expression of oyster viperin: (i) cells within the adductor muscle secrete a cytokine; (ii) poly(I:C) diffuses from the site of injection to other tissue compartments; or (iii) stimulated haemocytes are migrating from the adductor muscle to other tissue compartments. We therefore undertook a series of experiments to show that cells within the adductor muscle are secreting a cytokine. First, adult C. gigas were induced into an antiviral state by intramuscular injection with poly(I:C) or seawater (control). The circulating haemoocytes from oysters injected with poly(I:C) had elevated expression levels of many genes in the IFN pathway, including TLR, retinoic acid inducible gene I-like helicase...
Oyster viperin has direct antiviral activity

Viperin has remarkably high evolutionary conservation in animals. C. gigas viperin (GenBank accession no. KT334231) has 68% amino acid identity to viperin sequences isolated from human (Homo sapiens; GenBank accession no. NP_542388), fish (Danio rerio; GenBank accession no. NP_001020727), chicken (Gallus gallus; GenBank accession no. ACB3729) and lancelet (Branchiostoma floridae; GenBank accession no. EEN65148). The viperin amino acid sequences were aligned by CLUSTAL W using the phylogenetic software package MEGA v.6.06. The radical SAM domain is underlined. Asterisks indicate conserved amino acids.

![Fig. 1.](http://jgv.microbiologyresearch.org)
stimulated CFH activated expression of IRF, JAK, STAT6 and viperin (Fig. 4b, P<0.05). The upregulation of RLH in Fig. 4(b) is an anomaly: RLH was only upregulated in one experiment, whereas IRF, JAK, STAT6 and viperin were consistently upregulated in three independent experiments. Fig. 4(c) demonstrates that the haemolymph compound that induces viperin expression must be potent because diluting stimulated CFH (20%, v/v) did not reduce viperin expression in primary haemocytes (P>0.05). Furthermore, this haemolymph component appeared to be a heat-stable, protease-susceptible factor because the ability of the stimulated CFH to induce viperin expression was retained after digestion with RNase A or heat inactivation (Fig. 4c, P>0.05), but was eliminated by proteinase K digestion (Fig. 4c, P>0.05).

Oyster viperin localizes to caveolin-1 but not to lipid droplets

Many viperin molecules to date have been shown to localize to lipid droplets and/or the ER, including murine, human, crocodile and fish viperin (Helbig et al., 2011; Hinson & Cresswell, 2009b; Milic et al., 2015; Wang et al., 2014a), and in most cases this has been attributed to the amphipathic helix of the N terminus of viperin (reviewed by Helbig & Beard, 2014). Oyster viperin has a considerably shorter N terminus than human viperin, and our analysis of oyster viperin suggested that it is unlikely to form an amphipathic helix in its N terminus (see above).

To determine the localization of oyster viperin, we performed a number of expression studies using the Huh-7 cell line, which is known to have prominent lipid droplet formation, making viperin’s potential localization to this organelle easier to observe. To assess the ability of both oyster and human viperin to localize to lipid droplets, we co-transfected FLAG-tagged oyster and human viperin into Huh-7 cells in conjunction with MCherry conjugated to adipocyte differentiation-related protein (MCherry–ADRP), which is a resident lipid droplet marker. As can be seen in Fig. 5(a), human viperin co-localized extensively with the lipid droplet; however, oyster viperin expression appeared more cytoplasmically punctate in nature and did not co-localize with ADRP. Due to the punctate nature of oyster viperin, we analysed its potential co-localization with a number of organelle markers displaying a similar localization pattern, including those for lysosome (LAMP1), early...
and late endosomal compartments (Rab5 and -7), mitochondria (Cox IV) and the peroxisome (pex19); however, no co-localization was observed (data not shown). Interestingly, oyster viperin was observed to co-localize with caveolin-1 in Huh-7 cells (Fig. 5b). Caveolin-1 is a marker of caveolae, a specialized form of lipid raft (Parton & Simons, 2007).

To examine the divergent localization of human and oyster viperin in vitro, we co-expressed human viperin–GFP in conjunction with FLAG-tagged oyster viperin in Huh-7 cells. Fig. 5(c) shows that the two viperin molecules displayed extensive co-localization to putative lipid droplets, as well as to punctate cytoplasmic loci. Human viperin has been shown to dimerize previously (Hinson & Cresswell, 2009b), independent of its N terminus, and, given the inability of oyster viperin to localize to lipid droplets in the absence of human viperin expression (Fig. 5a), we can presume that oyster viperin maintains the ability to dimerize and is able to do so with human viperin, relocating to lipid droplets in vitro.

**Oyster viperin inhibits dengue virus replication**

Cell lines for marine bivalves do not exist (Yoshino et al., 2013), and the methodology to culture OsHV-1 in primary cells isolated from C. gigas has not yet been developed. Therefore, we utilized a heterologous model to investigate whether oyster viperin directs antiviral activity. Human viperin restricts the replication of a number of human viral pathogens, including dengue virus (DENV-2; Helbig et al., 2013). We compared the ability of oyster and human viperin to restrict DENV-2 replication in Huh-7 cells. Cells were transiently transfected with either FLAG expression control vector, FLAG-tagged oyster viperin or a FLAG-tagged human viperin expression plasmid, and then infected with DENV-2 at 24 h post-transfection.

Both oyster and human viperin were able to restrict DENV-2 replication at 24 h post-infection (p.i.) by 60 and 54 % respectively (Fig. 6, P<0.05). No significant difference was observed between the ability of oyster and human viperin to restrict DENV-2 replication in vitro. The ability of oyster viperin to restrict DENV-2 replication demonstrated that the lack of an amphipathic helix in the N terminus of this protein did not inhibit its anti-DENV activity.

**DISCUSSION**

A growing body of evidence supports the concept that some invertebrates may have an ancient antiviral pathway that is homologous to the vertebrate type I IFN response (reviewed by Wang et al., 2015). Despite numerous studies describing C. gigas genes that are homologous to vertebrate ISGs (Green & Montagnani, 2013; He et al., 2015; Renault et al., 2011; Rosani et al., 2014), there are outstanding questions regarding whether these invertebrate genes have a similar biological function and whether a C. gigas type I IFN cytokine exists. In the current study, we showed oyster viperin has a direct antiviral activity (Fig. 6) and provided evidence that intramuscular poly(I:C) injection induces a haemolymph protein/peptide (cytokine) that induces haemocyte expression of viperin in conjunction with upregulation of IRF and JAK/STAT (Fig. 4b). As herpesviruses pose the biggest threat to the global production of Pacific oysters (Renault et al., 2014; Segarra et al., 2010) and other marine molluscs (reviewed by Green et al., 2015a), these results are of considerable interest in progressing novel therapeutics for the aquaculture industry.
Viperin has been characterized from many different animals within the subphylums of Vertebrata (Goossens et al., 2015; Helbig et al., 2013; Milic et al., 2015; Wang et al., 2014b; Zhong et al., 2015) and Cephalochordata (Lei et al., 2015). To the best of our knowledge, this is the first study to characterize viperin isolated from an animal without a notochord (non-chordates). The amino acid sequence of C. gigas viperin has high homology to human and teleost viperin (Fig. 1) and has a similar domain arrangement with the conserved motif of CxxxxCxxC that exists in radical SAM enzymes and a conserved C-terminal domain. However, we were unable to predict an amphipathic helix in the N-terminal region. Vertebrate viperin requires the amphipathic helix for its association with the ER and its ability to localize to lipid droplets (Hinson & Cresswell, 2009a, b). The amphipathic helix of human viperin is important for its direct antiviral activity against hepatitis C virus (Helbig et al., 2013).

**Fig. 5.** C. gigas viperin co-localizes to caveolin-1 but not to lipid droplets. (a) Huh-7 cells were transfected with either FLAG-tagged oyster viperin (O. viperin) or FLAG-tagged human viperin (H. viperin) in conjunction with MCherry–ADRP. ADRP is a resident lipid droplet marker. No co-localization was observed between oyster viperin and ADRP. (b) FLAG-tagged oyster viperin has considerable co-localization to caveolin-1-GFP. (c) Co-expression of FLAG-tagged oyster viperin and GFP-tagged human viperin in Huh-7 cells revealing that human viperin molecules dimerize with oyster viperin localizing to lipid droplets.
mediated within the C-terminal 17 aa. The C-terminal region of human viperin was shown to restrict early DENV-2 RNA production/accumulation by interacting with DENV-2 capsid, non-structural protein (NS3) and viral RNA (Helbig et al., 2013). Phylogenetic analysis revealed that human and C. gigas viperin share 88 % amino acid identity within the C-terminal region.

Herpesviruses are renowned for their ability to modulate the host’s immune response and co-opt host antiviral proteins to facilitate the infectious process (Aresté & Blackbourn, 2009; White et al., 2012). Both HCMV (human herpesvirus-5) and human herpesvirus-1 [herpes simplex virus type 1 (HSV-1)] have been shown to counteract viperin’s antiviral activities (See et al., 2011; Shen et al., 2014). The HCMV vMIA protein has been demonstrated to interact with viperin, resulting in the relocalization of viperin from the ER to the mitochondria, where it reduces cellular ATP generation, resulting in actin cytoskeleton disruption and enhancement of HCMV infection (See et al., 2011). HSV-1 does not co-opt viperin; rather, the endoribonuclease activity of its UL41 protein has been shown to restrict viperin mRNA accumulation and to abolish its ability to limit HSV-1 infection (Shen et al., 2014). It is presumed that OsHV-1 can also modulate the immune response of C. gigas, as the viral genome encodes four inhibitors of apoptosis that are highly expressed during the early stages of infection (Green et al., 2015b; Segarra et al., 2014a, b). There are fewer data available regarding the ability of OsHV-1 to modulate the other evolutionarily conserved antiviral proteins, such as viperin. Interestingly, younger developmental stages of C. gigas induce viperin to higher expression levels when infected with OsHV-1 (unpublished data) and these earlier developmental stages also happen to be more susceptible to OsHV-1 infection (Paul-Pont et al., 2014; Peeler et al., 2012). Further research is therefore warranted to determine whether OsHV-1 diverts viperin from its antiviral role and co-opts it to facilitate the infection process.

In vertebrates, viperin is a highly inducible gene and its expression rapidly increases following viral infections and treatment with poly(I : C) and LPS (reviewed by Helbig & Beard, 2014; Mattijssen & Pruijn, 2012). We conducted several \textit{in vitro} experiments to determine which pathogen-associated ligands and sensors are responsible for inducing C. gigas viperin. In contrast to vertebrates, C. gigas viperin was induced by poly(I : C) but not by LPS (Fig. 3). These results suggest that viral replication products, such as dsRNA, are responsible for inducing viperin expression in C. gigas haemocytes via a TLR or retinoic acid inducible gene 1-like helicase (RLH) sensor. \textit{In vivo} experiments revealed viperin expression is delayed in C. gigas haemoocytes following poly(I : C)-injection when compared with other animals from the vertebrate phylum. Vertebrate cells usually express viperin within 2 h following a stimulus, and its expression typically peaks between 4 and 6 h following stimulation with poly(I : C) (Goossens et al., 2015; Zhang et al., 2014), whereas haemocyte expression of C. gigas viperin remained stable for the

![Fig. 6. C. gigas viperin has direct antiviral activity against DENV-2 in a heterologous model. Huh-7 cells were transfected with either oyster viperin (O. viperin), human viperin (H. viperin) or an empty control vector, 24 h prior to infection with DENV-2 (m.o.i.=1). Cells were harvested for RNA at 24 h p.i. and reverse transcriptase PCR was performed to detect viral RNA levels in comparison with the controls. C. gigas viperin had the same level of antiviral activity as human viperin against DENV-2. Asterisks denote a significant difference compared with control (*P<0.05).](http://jgv.microbiologyresearch.org)
first 9 h after poly(I : C)-injection and did not peak until 27 h post-injection (Fig. 2a). Poly(I : C)-injection in the adductor muscle also resulted in the upregulation of C. gigas viperin in the majority of tissue compartments (Fig. 2b). Several plausible explanations for the delayed but systemic expression of C. gigas viperin are that: (i) stimulated haemocytes migrate from the site of poly(I : C) injection to other tissue compartments; (ii) poly(I : C) diffuses from the site of injection to other tissue compartments; or (iii) poly(I : C) induces the cells within the adductor muscle to secrete a type I IFN cytokine. We therefore devised several experiments to indirectly test whether C. gigas has a type I IFN response. Genes with clear homology to type I IFNs have been identified in tetrapods (amphibians, reptiles, birds and mammals) and fishes but not in non-vertebrate chordates (tunicates or lancelets). This has previously been taken to suggest that IFNs first evolved in early vertebrates (Langevin et al., 2013). However, another interpretation is that functionally active IFNs from other animal groups simply lack sufficient sequence conservation with their vertebrate counterparts to be identified by homology searches. Comparisons of mammalian and teleost IFNs reveal low overall amino acid similarity (≤ 25 %) and a dissimilar gene domain architecture of type I IFNs even within the vertebrate phylum (Robertsen, 2006). Our results showed that C. gigas injected with poly(I : C) produced a haemolymph compound that activated viperin expression in primary haemocyte cell cultures in conjunction with upregulation of IRF and JAK/STAT (Fig. 4b). Furthermore, this compound(s) is likely to be a heat-stable protein/peptide (cytokine) because its activity was retained after digestion with RNase A and heat inactivation (Fig. 4c) but was eliminated by proteinase K digestion. Previous research has shown that all type I IFNs from vertebrates are heat stable (Oritani et al., 2003).

Conclusion

In summary, our results provide further support to the concept of an ancient type I IFN response existing in the common metazoan ancestor. We demonstrated that C. gigas viperin has direct antiviral activity and provided evidence that viperin expression is induced by non-specific dsRNA via a haemolymph protein/peptide (cytokine). Research is currently underway to purify this haemolymph protein/peptide(s) that activates viperin expression. The existence of a type I IFN response in the oyster creates exciting new possibilities for future research into novel therapeutic treatments for viral diseases that are threatening global aquaculture production.

METHODS

Oyster challenge experiments. Juvenile oysters (C. gigas) had a notch filed in their shell adjacent to their adductor muscle to allow delivery of poly(I : C) (3 mg ml⁻¹ in seawater; Sigma) according to previous published procedures (Green & Barnes, 2009; Green et al., 2014b). At 0 h, oysters were injected with 50 μl poly(I : C) or seawater (control) and placed in replicated aquariums (salinity 35 p.p.t., 19 °C, aerated). Six oysters per treatment were sampled at 0, 3, 9, 27 and 54 h post-injection. Sampling consisted of excising oyster tissues, snap freezing in liquid nitrogen and storage at −80 °C for RNA extraction. The oysters were not fed for the duration of the experiment.

Primary cell culture and pathogen-associated molecular patterns (PAMP) stimulation. Experiments investigating the effects of PAMPs on naïve haemocytes were carried out using primary haemocyte cell cultures that were established from individual oysters. Six primary cell cultures were established from individual C. gigas according to previously published procedures (Morga et al., 2011; Renault et al., 2011). Briefly, haemolymph was withdrawn from the pericardial cavity using a sterile 21-gauge needle and syringe. Haemolymph from individual oysters was kept separate and divided into seven replicate wells of a 24-well tissue culture plate (0.4 ml per well). Haemocytes were allowed to adhere to the tissue culture wells for 30 min at 22 °C before the acellular fraction of the haemolymph was removed from each well, filtered (0.2 μm) and retained on ice. Adhered haemocytes were washed three times with sterile seawater before 0.4 ml acellular haemolymph with 2 % (v/v) penicillin/streptomycin was replaced as the culture medium. Three concentrations of the PAMPs poly(I : C) and LPS (5.0, 0.5 and 0.05 mg.ml⁻¹) were prepared in seawater and 20 μl PAMP suspension (control=seawater) was added to each well. Haemocytes from each individual oyster were therefore exposed to three concentrations of poly(I : C) and LPS. Haemocytes were incubated for 6 h at 22 °C in a humid incubator and then used for RNA extraction. This set of experiments was repeated on two separate occasions.

Stimulation of naïve haemocytes with haemolymph collected from oysters injected with poly(I : C). Six adult oysters were injected with either 100 μl poly(I : C) or with seawater as above. Haemolymph from poly(I : C)-injected and control oysters was collected at 27 h post-injection using a 21-gauge needle and syringe, pooled and filtered (0.2 μm), and the acellular fraction of the haemolymph was retained on ice.

Primary haemocyte cultures were established from individual oysters (n=6) as described above. Pooled haemolymph from poly(I : C)- and seawater-injected adult oysters was used as the culture medium to determine whether a haemolymph component induced viperin expression (Fig. 7). Additional treatments included digestion of the haemolymph from poly(I : C)-injected oysters with RNase A (3.3 μg ml⁻¹, 37 °C, 1.5 h), proteinase K (0.1 mg ml⁻¹, 37 °C, 1.5 h) and heat inactivation (85 °C for 15 min) before using as culture medium. Haemolymph from poly(I : C)-injected oysters was also diluted with haemolymph from control oysters (20 %, v/v) before being used as a culture medium. Haemocytes were incubated for 6 h at 22 °C in a humid incubator and then used for RNA extraction. This set of experiments was repeated on two separate occasions.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR). Total RNA was purified from oyster samples using TriSure (Bioline) and reverse transcribed using a Tetro cDNA synthesis kit (Bioline). Quantitative real-time PCR was performed in a Viia7 thermocycler (Applied Biosystems), as described previously, using the primers in Table 1, which included the internal reference gene eEF1x (Green et al., 2014b).

Oyster viperin coding sequence and synthesis. The complete coding sequence of oyster viperin was amplified from cDNA samples of oysters injected with poly(I : C) (n=4, 27 h p.i.), using the primers 5′-ACATGGCTATTACGCSGTAC-3′ and 5′-CCAGGATTACAAATCC-3′. PCR amplicons were DNA sequenced at the Australian Genome Research Facility. The N-terminal FLAG-tagged oyster viperin was generated in two steps. The consensus nucleotide sequence of oyster viperin was directly synthesized (GenScript USA)
Oyster viperin has direct antiviral activity

Table 1. Primer pairs used in RT-qPCR expression analysis

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<th>Gene name</th>
<th>Accession no.</th>
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<th>Antisense primer (5'→3')</th>
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The GenBank accession number is provided for each gene. STING, Stimulator of IFN genes; viperin, virus inhibitory protein, ER-associated, IFN inducible.

Immunostaining and co-localization studies. The human hepatocellular carcinoma cell line Huh-7 were cultured on gelatin-coated glass coverslips and transiently transfected with plasmids expressing human or oyster viperin that were FLAG tagged at their N terminus in the vector pCI-neo. Co-transfection was performed with vectors expressing either mCherry–ADRP or a caveolin-1–GFP. Cells were fixed in 4% paraformaldehyde at 24 h post-transfection (Eyre et al., 2018).
DENV assay. HuH-7 cells were seeded in a 12-well dish and infected 24 h after seeding at an m.o.i. of 1 for 90 min at 37 °C with DENV-2 in a volume of 300 μl per well as described previously (Milic et al., 2015). Cells were then washed with PBS three times before being reincubated in culture medium. At 24 h p.i., the cells were harvested for RNA purification as described above, and real-time PCR was performed utilizing the DENV-2-specific primers 5’-ATCCTCCTATGGTGACGCACAAA-3’ and 5’-CTCCAGTATATTGGAAGCTGCTATCC-3’ in combination with primers for the internal RPLPO (large ribosomal protein) reference gene: 5’-AGATGCAGCAGATCCGTGCAT-3’ and 5’-GGATGGCCCTTGGCGCA-3’.

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Oyster viperin has direct antiviral activity


