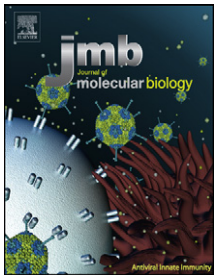
Review



The Role of Viperin in the Innate Antiviral Response

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Abstract

Viral infection of the cell is able to initiate a signaling cascade of events that ultimately attempts to limit viral replication and prevent escalating infection through expression of host antiviral proteins. Recent work has highlighted the importance of the host antiviral protein viperin in this process, with its ability to limit a large variety of viral infections as well as play a role in the production of type I interferon and the modulation of a number of transcription factor binding sites. Viperin appears to have the ability to modulate varying conditions within the cell and to interfere with proviral host proteins in its attempts to create an unfavorable environment for viral replication. The study of the mechanistic actions of viperin has come a long way in recent years, describing important functional domains of the protein for its antiviral and immune modulator actions as well as demonstrating its role as a member of the radical SAM enzyme family. However, despite the rapid expansion of knowledge regarding the functions of this highly conserved and ancient antiviral protein, there still remains large gaps in our understanding of the precise mechanisms at play for viperin to exert such a wide variety of roles within the cell.

Introduction

Viral infection of mammalian cells results in activation of a number of viral recognition pathways triggered by replication intermediates and or viral proteins, which ultimately induce innate defenses to limit viral replication [1]. Pivotal to this antiviral response is the induction of interferon (IFN). There are three types of IFN, type I IFNs (IFN-α and IFN-β), type II IFN (IFN-γ) and type III IFNs (IFN-λ1, IFN-λ2 and IFN-λ3). The type I IFNs are essential for immune defenses against viruses and, following binding to the type I IFN receptor, induce the expression of hundreds of interferon-stimulated genes (ISGs), many of which act to limit viral replication. The importance of this early IFN response to viral infection is highlighted by the fact that most viruses have evolved strategies to combat either IFN production or IFN action [2]. Despite our increasing knowledge of the host's response to viral infection, the role of most ISGs remains elusive, and only a handful of antiviral host effectors have been described in the literature. Viperin is one of the few ISGs shown to have direct antiviral activity and has received increasing attention due to its ability to limit a broad range of viruses and play an emerging role in modulating innate immune signaling. Here we review the literature surrounding the spectrum of viruses for which viperin has antiviral activity.

Structure and Characterization

Viperin (also known as cig5 and RSAD2) is a highly species conserved, 361-amino-acid protein with a predicted molecular mass of 42 kDa. It was first identified as a human cytomegalovirus (HCMV)-inducible gene in fibroblasts, where two cDNA fragments termed cig5 and cig33 were later found to form the one transcript that was renamed viperin [for virus inhibitory protein, endoplasmic reticulum (ER)

associated, interferon inducible] [3,4]. Viperin is expressed by a wide variety of mammals and fish and has been more recently demonstrated in reptiles. It is composed of three distinct domains: an N-terminal domain that varies considerably between species and contains both an amphipathic helix and a leucine zipper domain, a highly conserved central domain containing a "radical SAM domain" and a C-terminal domain that shows striking similarity across species and has recently been shown to be critical for viperin's antiviral properties against a number of viruses (Fig. 1). The amphipathic helix is located from residue

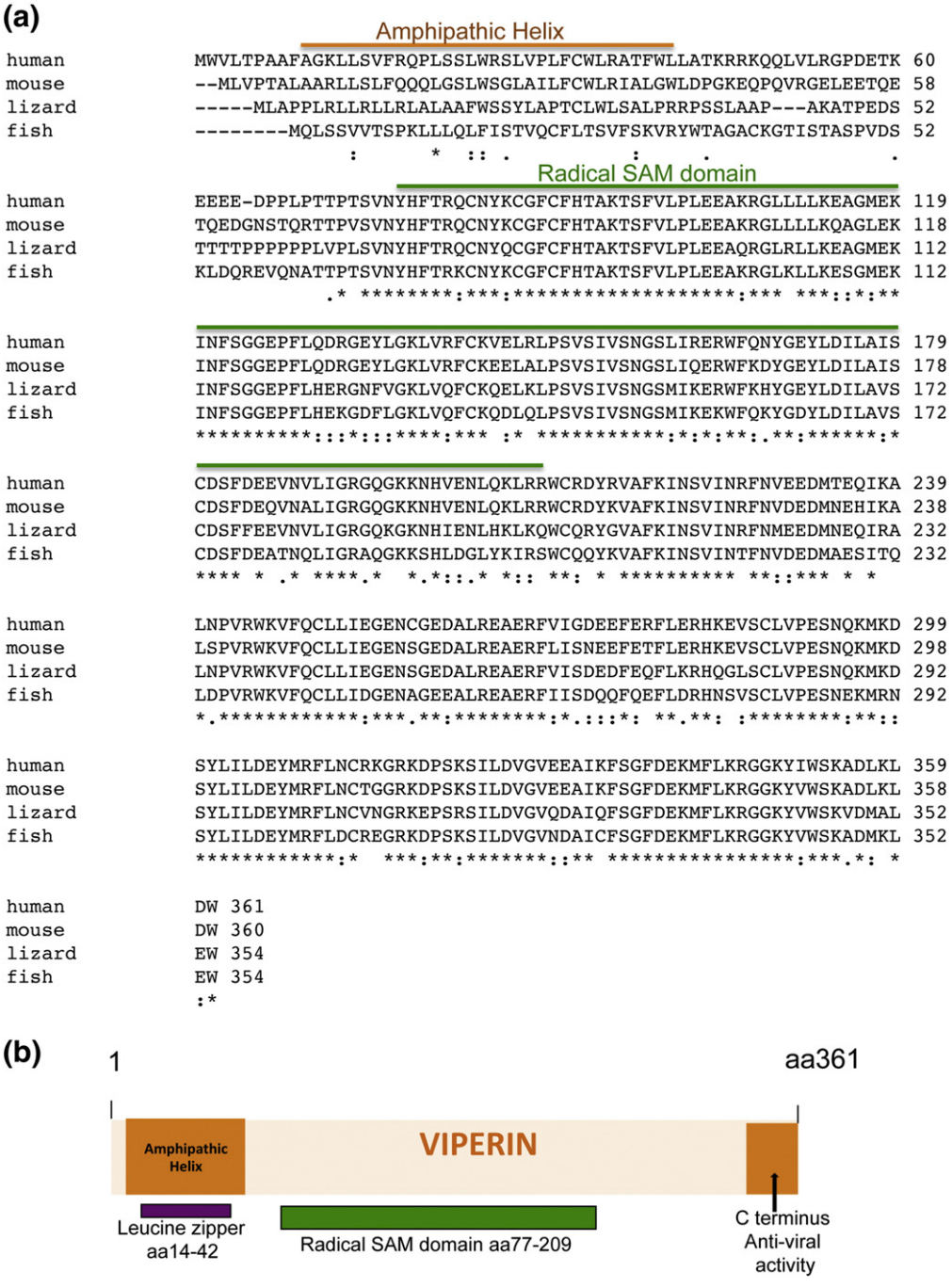

Fig. 1. Viperin is highly conserved. (a) Viperin homology across multiple species. (b) Viperin contains a number of conserved domains including the radical *S*-adenosyl methionine (SAM), a leucine zipper domain and an amphipathic helix. The C-terminal region of viperin is also highly conserved and has been demonstrated to be critical for its ability to limit replication of a number of viruses.

9 to residue 42 in the proteins' N-terminus and mediates viperin's ability to bind to the cytosolic face of the ER, as well as its association with lipid droplets [5,6]. The central domain of the protein (residues 71–182) carries 4 motifs characteristic of members of the radical SAM (*S*-adenosyl-L-methionine) enzymes, with motif 1 containing a CxxxCxxC sequence responsible for binding iron sulfur clusters. Recent work has been able to demonstrate that viperin can bind FeS clusters and reduce *S*-adenosyl-L-methionine to 5′deoxyadenosine, a characteristic function of radical SAM enzymes [7,8]; however, no enzymatic function has been assigned to viperin as yet, although this domain has been shown to be important in viperins ability to limit both human immunodeficiency virus (HIV) and Bunyamwera virus [9,10] and in the ability of HCMV to co-opt viperin as a proviral host protein (discussed below) [11]. The C-terminal domain of viperin is highly conserved, and its presence is critical for its antiviral function against the Flaviviridae members hepatitis C virus (HCV) and dengue virus (DENV); however, its exact role still remains unknown [5,12].

Viperin Regulation

Viperin is expressed in most cell types at very low basal levels and has been demonstrated to be induced by type I IFN, type II IFN, type III IFN, double-stranded DNA, double-stranded RNA (dsRNA) analogues, LPS and multiple viruses. Viperin induction through classical IFN pathways has been demonstrated via stimulation with LPS, double-stranded DNA, poly I:C and multiple viruses, including Pseudorabies, Sendai virus, Sindbis virus and lymphocytic choriomeningitis virus [11,13–16]; all of which bind to and activate pattern recognition receptors such as Toll-like receptors TLR3 and TLR4, the cytosolic retinoic acid-inducible gene I-like receptors (RLRs) and cytosolic DNA sensors. These in turn are able to activate interferon regulatory factors IRF3 and IRF7 to produce IFN-β, which is able to act in both a paracrine manner and an autocrine manner to bind the cell surface type I IFN receptor and initiate a signaling cascade that culminates in the formation of the ISG factor 3 (ISGF3), which is able to bind the viperin promoter and induce expression. Viperin has been previously demonstrated to be very tightly regulated by ISGF3, with counter-regulation exerted by the PRDI-binding factor-1 (BLIMP-1) [15]; however, more recent work by Xu *et al*. has indicated that the transcription factor promyelocytic leukemia zinc finger protein (PLZF) is essential for IFN-regulated viperin expression [17]. IFN stimulates an association of PLZF with promyelocytic leukemia protein and histone deacetylase 1 to induce a specific subset of ISGs, including viperin. Mice deficient for PLZF were unable to efficiently upregulate viperin and other key antiviral effectors upon IFN-α stimulation and, consequently, were more susceptible to viral infection [17].

Viperin can also be upregulated independently of IFN, through an IRF1 or IRF3 mechanism [18–20], and a number of viruses, including HCMV, vesicular stomatitis virus, Japanese encephalitis virus (JEV) and Chikungunya virus (CHIKV), can induce viperin independently of IFN production [3,13,16,21]. Following viral dsRNA stimulation of RLRs, they interact with the adaptor protein MAVS (mitochondria-associated adaptor molecule, also termed Cardif, IPS-1 or VISA) to promote the expression of IFN-β and, consequently, ISGs. MAVS is now known to reside on both the peroxisome and the mitochondrial membrane, and it is the initial activation of peroxisomal MAVS by viral stimulated RLRs that results in rapid and transient induction of some ISGs, including viperin through an IRF1- and IRF3-dependent gene induction [22]. In contrast, mitochondrial MAVS activates an IFN-dependent induction of viperin and other ISGs to set up a stable antiviral environment within the cell. Therefore, viperin IFN-dependent expression is mediated by ISGF3 and PLZF, whereas its IFN-independent expression is controlled via IRF1 and IRF3.

Antiviral Functions of Viperin *In Vitro*

Viperin was first shown to be antiviral against HCMV, where overexpression of viperin in human fibroblasts prior to HCMV infection was found to significantly decrease expression of the late viral proteins gB, pp28 and pp65 [3]. Viperin has since been demonstrated *in vitro* to have antiviral activity against a broad range of viruses from both DNA and RNA viral families; however, the full elucidation of antiviral mechanisms at play still remains somewhat elusive (Table 1).

Viperin expression has been shown to inhibit both influenza A and HIV-1 through blocking viral particle release [9,26,27]. HeLa cells overexpressing either murine or human viperin have been shown to inhibit viral budding from the plasma membrane by disrupting lipid rafts and increasing membrane fluidity [26,27] (see Fig. 2). In this instance, viperin was found to bind farnesyl diphosphate synthase (FPPS), a critical enzyme in the mevalonate pathway, which is involved mainly in cholesterol and sterol biosynthesis, protein farnesylation and geranylgeranylation. The effect of viperin on influenza budding from the plasma membranes was reversed via overexpression of FPPS, substantiating its importance in the viral budding of influenza from the plasma membrane; however, the precise mechanism by which viperin affects membrane fluidity and disrupts lipid rafts was unable to be determined [27]. In parallel, viperin overexpression in HEK293 cells is also able to inhibit HIV-1 egress from the cell, and this was found to be dependent on the enzymatic domain (SAM domain) of viperin [9]. HIV-1 infection of primary monocyte-derived macrophages (MDMs) was found to induce viperin and redistribute it

Table 1. Critical antiviral domains of viperin

Virus	Mechanism	Critical domains	Ref.
RSV	nd	nd	[39]
DENV	Inhibits replication via interaction with DENV NS3	C′-terminus	[12,32]
HCV	Inhibits replication via localization in RC with HCV NS5A and VAP-A	C′-terminus and amphipathic helix	[28,33]
CHIKV	nd	Amphipathic helix	[36]
Influenza A	Inhibits viral budding from the plasma membrane, possibly via interacting with FPPS	nd	[26,27]
HIV	Inhibits viral egress	SAM domain	[9]
Bunyamwera virus	Inhibits replication	SAM domain	[10]
WNV	nd	nd	[31]
Sindbis virus	nd	nd	[23]
HCMV	nd	nd	[3]
Rhinovirus	nd	nd	[24]
HCMV	nd	nd	[3]
Vesicular stomatitis virus	nd	nd	[25]

(in comparison to the ER-localized viperin in IFN-α-treated MDMs) into foci containing p24 and CD81, a marker for the site of HIV-1 viral assembly and accumulation. Interestingly, the addition of exogenous farnesol was also able to increase HIV-1 cDNA levels, indicating that, in this infection system, viperin's interaction with FPPS may also be inhibiting HIV-1 viral egress, although this still needs to be validated [9].

Viperin also inhibits members of the Flaviviridae family of viruses, with HCV, DENV and West Nile virus (WNV) all restricted by viperin *in vitro* [5,12,28–32]. With the use of a HEK293 HCV replicon-based system, with viperin under the transcriptional control of tetracycline, the protein was shown to impede HCV replication. In this model system, the radical SAM domain and the C-terminal residue were found to be

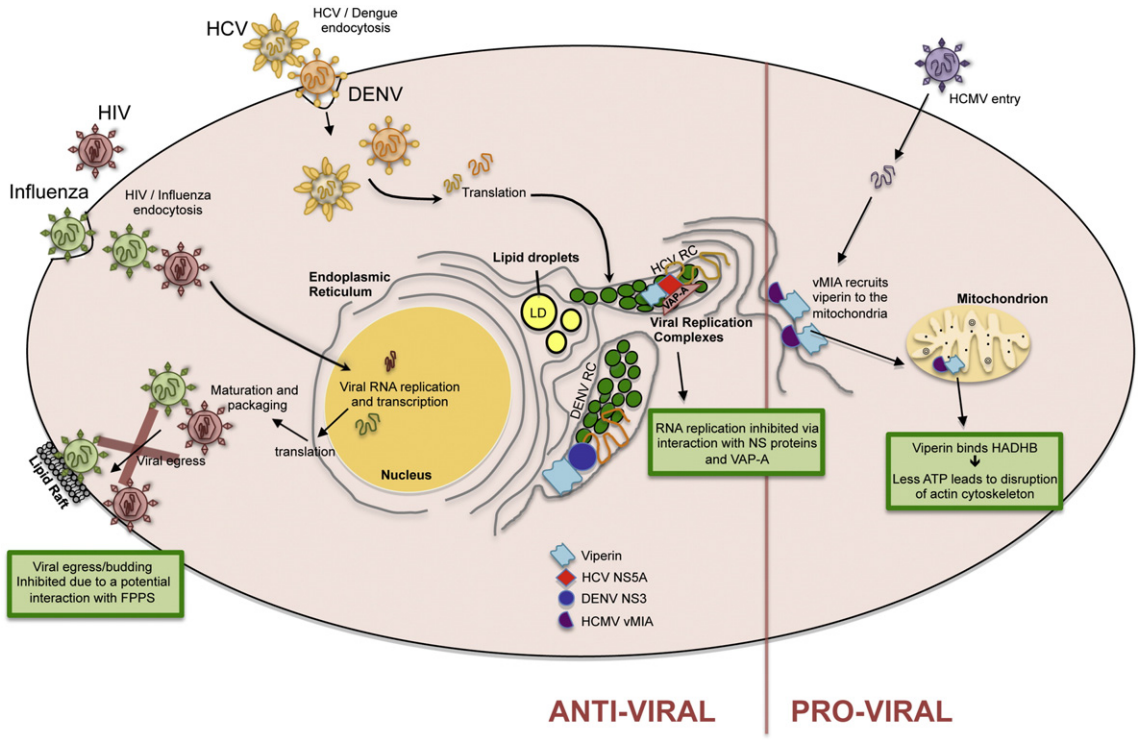

Fig. 2. Viperin acts as both an antiviral and a proviral host protein. Viperin is able to act at multiple stages of the viral life cycle; it has been demonstrated to associate with the RCs of both HCV and DENV, by interacting with the non-structural protein 5A (NS5A) and NS3, respectively. In the case of HCV NS5A, viperin also associates with the proviral host factor VAP-A. Viperin is also able to limit viral egress of both HIV and influenza through the plasma membrane and lipid rafts, respectively, although the mechanism surrounding this remains elusive. HCMV has also been demonstrated to co-opt viperin as a proviral host factor, by trafficking it to the mitochondria attached to the viral mitochondria-localized inhibitor of apoptosis protein (vMIA) where it is able to bind HADHB and cause disruption of the cytoskeleton, thereby increasing viral entry.

critical domains of the viperin against HCV; however, the N-terminal region containing the amphipathic helix was found to be dispensable [29]. Further work by two groups looking at the ability of viperin to limit HCV in the more physiological setting of the complete HCV life cycle in hepatocyte cell lines was able to demonstrate that the C-terminal domain was essential for viperin to limit HCV *in vitro*; however, the radical SAM domain was not required [5,33]. The N-terminal amphipathic helix was also shown to be important for viperin's ability to bind and interact with the HCV NS5A protein on the lipid droplet surface and within the replication complex (RC) of HCV, both of which were required for viperin to impart its antiviral activities [5]. Viperin was also shown to interact with the proviral host factor, vesicle-associated membrane protein-associated protein-A (VAP-A) in RCs, and impair its association with NS5A, which is critical for HCV replication [5,33].

Utilizing the same HEK293-inducible cell lines as mentioned above for HCV, we also showed viperin expression to limit both DENV-1 and WNV using a virus-like particle/replicon system, and once again, the radical SAM domain was shown to be critical [30]. More recently, with the use of an infectious cell culture model, viperin was shown to be antiviral against DENV-2 in Huh-7, A549 and primary human MDMs [12]. Viperin was shown to impede early RNA replication and co-localized and co-precipitated with dsRNA in DENV-2-infected cells, suggesting a possible interaction of viperin at sites of DENV-2 replication. Viperin was also shown to interact with DENV-2 NS3 (protease/helicase) protein, which is essential for replication, and this interaction was crucial for its antiviral activities and mediated by the C-terminus of the protein. In contrast to previous reports, in using the DENV-2 infectious system, neither the N-terminal amphipathic helix nor the radical SAM domain of viperin was found to be important in its ability to limit DENV-2 replication, which either highlights a difference between viperin's ability to restrict DENV-1 and DENV-2 or perhaps displays the importance of performing these studies in physiologically relevant cell culture systems that harbor the complete virus life cycle.

Viperin has also been shown to interact with DENV-2 capsid protein and HCV core on the surface of lipid droplets, a cellular organelle that is hypothesized to be a scaffold for viral assembly [5,12]. In the case of DENV-2, the loss of the N-terminal amphipathic helix of viperin, which mediates its association with both the ER and lipid droplets, abrogated its anti-DENV activity, indicating that its association with the capsid protein is not its main mechanism for limiting DENV viral replication. In parallel, the amphipathic helix of viperin was required for its ability to restrict HCV replication, as well as its ability to interact with HCV core and NS5A on the surface of the lipid droplet and with NS5A within putative RCs. As mentioned above, viperin's association with NS5A and VAP-A in the HCV RC was demonstrated to mediate its anti-HCV effects, and one plausible explanation is that the association seen between HCV core and viperin on the surface of the lipid droplet is in fact due to the ability of NS5A and HCV core to still co-localize at this site while NS5A is associated with viperin [34].

The amphipathic helix of viperin is required for its association with the ER and its ability to localize to lipid droplets [5,6,35]. Its presence is essential for its anti-HCV activities and its ability to bind and localize in the RC with HCV NS5A; however, it is dispensable for its anti-DENV activities and its association with DENV NS3, perhaps due to the varied distribution of DENV NS3 during infection. Viperin's amphipathic helix is also essential for its ability to limit CHIKV [36]. CHIKV was found to co-localize with viperin in the ER of HEK293 cells, and CHIKV nsP2 (an RNA helicase/protease that mediates replication) was found to also localize to the periphery of the ER with significantly reduced levels observed during viperin overexpression. CHIKV nsP2 is known to interact with many host proteins in the ER to facilitate replication; however, it is unknown whether viperin's inhibitory effect on nsP2 is direct or indirect [36,37].

In Vivo Antiviral Functions of Viperin

Studies translating the antiviral activity of viperin in cell culture to its function in an *in vivo* model have been somewhat lacking, most likely reflecting redundancy in the innate antiviral response in which the absence of viperin can be compensated by other ISGs resulting in mice that can still respond to a viral infection. This is certainly the case with influenza virus (IFV) in which ectopic viperin expression in HeLa cells can inhibit IFV release from the plasma membrane by affecting the formation of lipid rafts and direct interaction with the FPPS, an enzyme essential for isoprenoid biosynthesis (as discussed previously). However, this *in vitro* observation did not translate to a mouse model of IFV infection using viperin knockout mice in which no difference was observed in susceptibility, pulmonary damage or IFV titers between wild type (WT) and viperin$^{-/-}$ mice [26]. This discrepancy may be due to a number of factors. As suggested above, redundancy in the ISG innate response may contribute and, together with the acute nature of infection and its ability to block the host innate immune signaling, may suggest that IFV can overcome the antiviral nature of viperin. Interestingly, viperin expression peaked at 5 days post infection, after which IFV infection is well established.

In contrast to the experience with IFV, viperin has recently been shown to restrict the replication of both WNV and CHIKV in mouse models [31,36]. In contrast to WT mice, mice deficient for viperin expression succumbed to subcutaneous injection of WNV and, in the case of CHIKV, had higher viremia and more

severe joint inflammation. Interestingly for WNV, this antiviral effect was noted predominantly in the CNS and direct intracranial injection of WNV resulted in regional differences in WNV titers between WT and viperin$^{-/-}$ mice [31]. Notably, increased WNV titers were seen in the cortex, spinal cord and white matter in viperin$^{-/-}$ mice and it was suggested that the antiviral function of viperin may restrict the spread and infection of neuronal subsets. This was confirmed in a follow-up study revealing that different neuronal subtypes have distinct antiviral IFN responses [38]. Notably, granule cell neurons were more resistant to WNV infection compared to cortical neurons a function that was attributed to expression of a subset of ISGs, one of which was viperin [38]. In fact, cortical neurons could be made resistant to WNV infection after ectopic expression of viperin, while in the mouse brain, granule cell neurons were less vulnerable to WNV infection compared to cortical neurons. Importantly, in human brain tissue infected with WNV, granule cell neurons were relatively spared from WNV infection compared to other neuronal populations. Clearly different cell types even within the same tissue can express different ISG programs that may confer resistance to viral infection.

Infection with CHIKV virus causes a very different pathology from that of WNV in that there is generally no CNS involvement with patients developing an incapacitating arthralgia during the acute phase with symptoms usually resolved within 7–10 days; however, in some patients, symptoms can last for many months to years. Patients infected with CHIKV show a strong IFN response that often correlates with the level of viremia and significant corresponding ISG expression, including viperin in PBMCs [36]. While viperin was shown to restrict CHIKV replication *in vitro*, its importance as a host antiviral ISG was confirmed by *in vivo* CHIKV infection of viperin$^{-/-}$ knockout mice. Loss of viperin expression resulted in a significant increase in CHIKV viremia, and while loss of viperin had no impact on mortality of the mice, it resulted in increased disease susceptibility and joint inflammation [36]. Combining the observations from human studies and the ability of viperin to restrict CHIKV replication *in vitro* and *in vivo*, it was proposed that viperin expression induced early as a result of activation of the innate arm of the immune response can limit CHIKV replication and the accompanying inflammation. It will be interesting to determine in humans the spectrum of viperin expression in individuals that have mild compared to those that have protracted disease and the existence of viperin polymorphisms that may impact disease severity.

The only study to date to analyze the ectopic expression of viperin *in vivo* was performed in the context of respiratory syncytial virus (RSV) change of the chinchilla airway [39]. Viperin expression was found to be significantly upregulated in the nasopharynx of the chinchilla up to 7 days post RSV challenge, and the pre-infection delivery of a recombinant adenoviral associated vector was able to further increase viperin expression in the nasopharynx by up to 51%. This increased viperin expression was able to significantly reduce viral titers of RSV in nasopharyngeal lavage fluid by approximately 1 log at 7 days post infection [39]. This study highlights the importance of viperin as an antiviral at the mucosal barrier and potentially underpins the possibility for the use of a viperin mimic in therapeutic situations in the distant future.

Viperin and Modulation of Immune Signaling

While there is clear evidence that viperin has antiviral activity against a diverse group of pathogens, it is emerging that viperin may also contribute to immune signaling. This was first identified in mice deficient for viperin (viperin$^{-/-}$) expression that exhibited no significant phenotypic changes; however, challenge of these mice with ovalbumin (OVA) that induces a mixed Th1/Th2 response resulted in a significant reduction in OVA-specific IgG1 suggesting that viperin may be involved in regulating the Th2 response [40]. Consistent with this observation, stimulation of T cells from viperin$^{-/-}$ mice with anti-CD3/CD28 resulted in a significant decrease in the expression of Th2 cytokines IL-4, IL-5 and IL-13 compared to WT T cells. This decrease was attributed to a defect in the induction of GATA-3 (a well-characterized regulator of Th1/Th2 differentiation) and decreased DNA binding activities of NF-κB/p50 and AP-1. The authors concluded that viperin was crucial for T cell activation and differentiation; however, no mechanism was provided and it will be interesting to determine if similar findings will be seen in *in vivo* Th2-associated disease models.

Not only does viperin posses direct acting antiviral activity but recent data suggest that it may impact innate antiviral signaling. Viperin can modulate TLR7 and TLR9 recognition of viral nucleic acids in pDCs to promote type I IFN production [41] (see Fig. 3). Type I IFN production is crucial in the host response and control of viral infections through inducing the expression of antiviral effector genes and modulation of the adaptive immune system. Saitoh and colleagues showed that pDCs deficient for viperin were also deficient in type I IFN production in response to TLR7 and TLR9 ligands. This effect was dependent on the N-terminal amphipathic helix of viperin that anchors it to the lipid droplet and mediates signaling from TLR7 and TLR9. Specifically, viperin was found to recruit the adaptor molecules IRAK1 and TRAF6 to the lipid droplet to facilitate the ubiquitination of IRAK1 by TRAF6 that, in turn, results in nuclear translocation of IRF7 to the nucleus and stimulation of type I IFN expression. Consistent with the abovementioned

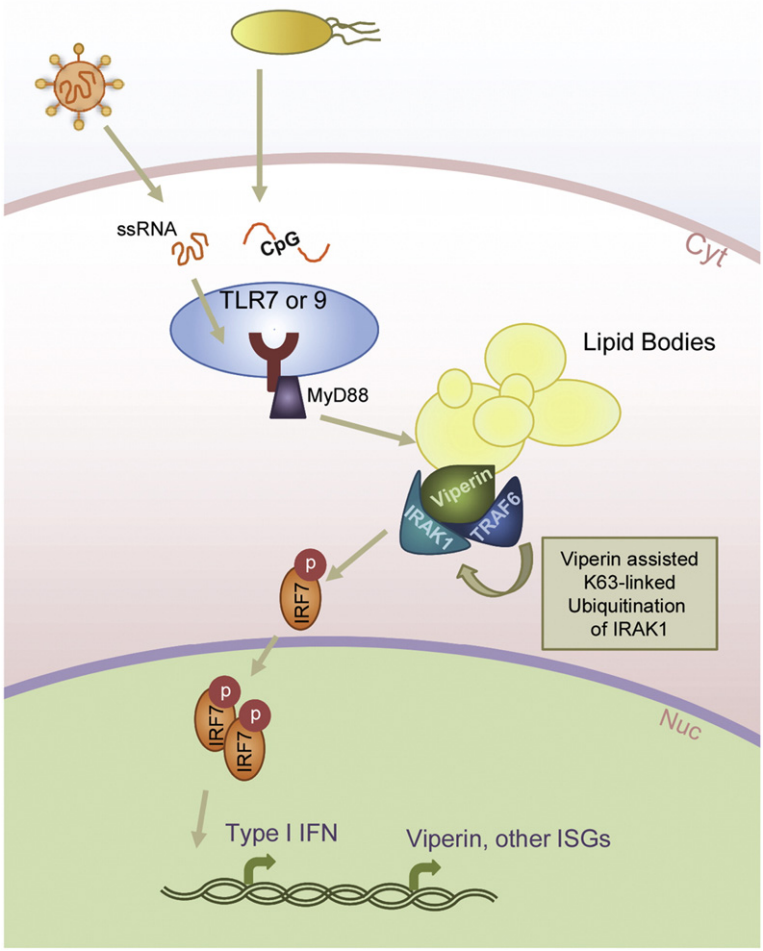

Fig. 3. Viperin is required for TLR7/TLR9 activation in pDCs. Viperin is essential for activation of TLR7 and TLR9 and the subsequent downstream upregulation of type I ISGs. Following activation of TLR7/TLR9 by single-stranded RNA (ssRNA) or CpG, myeloid differentiation primary response gene 88 (MyD88) is able to mediate the TLR7/TLR9 signals to lipid bodies. Viperin then interacts with the signal mediators, interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) to recruit them to the lipid bodies and facilitate K63-linked ubiquitination of IRAK1 to induce the nuclear translocation of transcription factor IRF7 and subsequent upregulation IFN-regulated gene transcription.

study linking viperin and IRF7 nuclear translocation, it was recently demonstrated that knockdown of viperin reduces CD40-ligand-mediated activation of NF-κB in carcinoma cells and downstream recruitment of IRF7 to the nucleus resulting in a decrease in IFN-β gene expression [42]. Thus, it is becoming clear that not only does viperin play a direct role in attenuating viral infection but it also augments innate immune signaling and thus it may contribute to the host antiviral response on multiple fronts.

Escape and Utilization of Viperin by Viruses

Viruses have evolved multiple mechanisms to combat varying arms of the host innate immune system, including evasion of antiviral host proteins, with PKR being an excellent example [43,44]. To date, two viruses, JEV and HCMV, have been reported to evade the antiviral properties of viperin, with the later actually co-opting viperin to its advantage [11,13]. In contrast to other plus-strand viruses such as HCV and WNV, overexpression of viperin was not found to have an impact on JEV replication and, moreover, JEV does not replicate to increased titers in cells depleted of viperin; however, JEV is able to directly induce viperin in an IFN-independent manner [13]. With the use of A549 cells, induction of viperin by JEV can only be seen in the presence of the proteasome inhibitor MG132, suggesting that JEV is negatively controlling viperin protein expression to circumvent its antiviral effects; however, no mechanism was determined for this degradation [13].

HCMV has been shown to be sensitive to ectopic viperin expression *in vitro* when it is expressed in the cell prior to infection [3]. However, when human fibroblasts cells were infected with HCMV, the virus induced viperin expression that localized to the ER after 24 h, but at day 3 post infection, viperin relocalized to the Golgi and to unidentified cytoplasmic vesicles where it co-stained with the viral envelope protein, gB [3]. This was originally thought to be a viral evasion mechanism, but it is now known that the virus transports viperin to the mitochondria before it relocalizes it to the assembly compartment as a strategy to enhance viral infection through actin cytoskeleton disruption [11]. The HCMV viral protein, vMIA (*v*iral *m*itochondrial *i*nhibitor of *a*poptosis) was found to interact with viperin and relocate it to the mitochondria, where it was able to interact with the β subunit of the mitochondrial trifunctional protein (TFP), a protein that mediates β-oxidation of fatty acids to generate adenosine triphosphate; this interaction causes inhibition of TFP and, subsequently, a decrease in cellular adenosine triphosphate levels [11]. The main consequence of viperins' interaction with TFP was the disruption of the actin cytoskeleton that served to increase viral infection, and this was found to be mediated by the radical SAM domain of viperin (Fig. 2). Interestingly, a similar effect was also observed when the N-terminal amphipathic helix of viperin was replaced with a mitochondrial targeting sequence, indicating that these effects were mediated directly by viperin itself and that the involvement of HCMV vMIA is simply to transport viperin to the mitochondria [11].

Biological Mechanisms of Viperin Action

Viperin has been shown to play a role in innate immune signaling, to limit varying viruses through both direct inhibition of replication and interference with viral budding/release and to disrupt the actin cytoskeleton to increase infectivity of HCMV in an example of evolutionary escape of HCMV from the antiviral properties of viperin. Viperin has been shown to bind the five host proteins FPPS, TFP, IRAK1, VAP-A and TRAF6 and the three viral proteins DENV NS3, HCV NS5A and HCMV vMIA in order to accomplish this list of biological functions. It is unusual for viperin to be able to interact with such a divergent range of other proteins and to potentially mediate quite distinct cellular functions. The extreme C-terminal region of the protein has been demonstrated to be important for protein dimerization and in binding HCV NS5A, VAP-A and DENV NS3 [5,6,12,33]; however, its function in binding the other mentioned interacting partners has not been assessed to date and may shed further light on the broad biological functions of this protein. Interestingly, recent studies on the evolution of primate viperin over some 60 million years show that it has evolved under positive selection pressure similar to other antiviral proteins such as PKR [45,46] and that the C-terminus of viperin displays significant positive selection. The structural regions of viperin under the highest positive selection were found to be the middle region (immediately following the N-terminal amphipathic helix), the radical SAM domain and the C-terminus, in particular, the C-terminal amino acid. This pattern of dispersion of residues is similar to that seen for PKR and demonstrates escape from viral antagonism over the course of primate evolution, highlighting regions of importance within the protein.

The ability of viperin to bind to the cytosolic face of the ER has also been associated with its capacity to inhibit secretion of soluble proteins and reduce the rate of ER-to-Golgi traffic, as well as induce heightened membrane curvature of the ER [6]. It is plausible that this property of viperin may inhibit the trafficking of viral and host proteins required for replication, budding and egress or potentially interfere with the formation of ER membranous complexes required for the life cycle of some viruses. However, in the case of HCV, this does not appear to be the case. HCV RCs are derived from the ER and form the platform for viral replication within the cell; viperin was found to localize to these RCs in conjunction with NS5A and the proviral host factor VAP-A but did not limit the number or spatial distribution of RCs in a cell (K.J.H. and M.R.B., unpublished results); however, further work remains to be done to ascertain the functionality of these RCs [5,47].

The ER in conjunction with the Golgi is also an important site for the synthesis of bulk lipids and cholesterol, both of which are important components for viral entry, assembly and budding, not only in the context of lipid raft formation but also in the context of viral replication for some members of the Flaviviridae family of viruses. Cholesterol and lipids must be transported from the ER to other organelles, such as endosomes, mitochondria and plasma membranes; the ER resident protein, VAP-A, is an important protein implicated in these processes along with oxysterol-binding protein (OSBP) [48]. Viperin is able to interact with VAP-A, and it is possible that this association may underpin its ability to limit multiple viruses [5,33]. Recent work has shown that the ISG IFITM3 (*i*nter*f*eron-*i*nducible *t*rans*m*embrane protein-*3*) is able to also bind VAP-A and, in doing so, inhibits its ability to interact with OSBP and consequently alters the balance of cholesterol in late endosomes, inhibiting viral egress into the cytosol [49]. IFITM3 is a transmembrane protein, and its ability to bind VAP-A will have localized effects; however, viperin has been demonstrated to reside in the ER, as well as piggyback with viral proteins, such as HCMV vMIA to the mitochondria and HCV NS5A and DENV NS3 to sites of replication [5,11,12]. It is possible that viperin may indeed contribute to an alteration in cholesterol or lipid

homeostasis in the cell, contributing to its ability to limit multiple viruses; however, further work will need to be performed to investigate this in multiple viral systems and to characterize its ability to bind VAP-A and the downstream consequences of this interaction.

Concluding Remarks

Since its discovery as an interferon-induced gene and the identification of its role as a potent host antiviral protein against HCMV in 2001, there has been significant advances in our understanding of this unique host protein regarding its regulation, its antiviral properties and its ability to modulate innate immune signaling. However, the majority of studies have investigated its antiviral function and it is now clear that viperin is a genuine host antiviral protein. Even more intriguing about this protein is its promiscuous nature by which it seems to exert its antiviral effect. 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. In an interesting twist from its described antiviral activities, and perhaps further reiterating its ancient origin, it has also recently been shown to play a positive role in HCMV replication and furthermore can modulate type I IFN production in pDCs via modulating signaling from TLR7 and TLR9. Thus, it seems that viperin may impact viral replication via both direct and indirect mechanisms, which raises the question of how one protein can have such a diverse array of functions and possess antiviral activity to a vast array of viruses using a variety of antiviral mechanisms. Clearly, more investigation into this mysterious protein is warranted to fully elucidate its functional characteristics not only in its role as an antiviral but also in its emerging role in innate immune control of viral infection.

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Abbreviations used:
ISG, interferon-stimulated gene; HCMV, human cytomegalovirus; ER, endoplasmic reticulum; HIV, human immunodeficiency virus; HCV, hepatitis C virus; DENV, dengue virus; dsRNA, double-stranded RNA; ISGF3, ISG factor 3; JEV, Japanese encephalitis virus; CHIKV, Chikungunya virus; MDM, monocyte-derived macrophage; WNV, West Nile virus; RC, replication complex; VAP-A, vesicle-associated membrane protein-associated protein-A; IFV, influenza virus; WT, wild type; RSV, respiratory syncytial virus.

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