Regulation of IRAK1 Ubiquitination by the Antiviral Radical SAM Enzyme Viperin

Arti B. Dumbrepatil[1], Soumi Ghosh[1], Ayesha M. Patel[1], Paige A. Malec[1], Kelcie Zegalia[1], J. Damon Hoff[2], Robert T. Kennedy[1] and E. Neil. G Marsh[1,3,*]

Departments of Chemistry[1], Biophysics[2] and Biological Chemistry[3], University of Michigan, Ann Arbor, Michigan 48109-1055

[*]Corresponding author:

Dr. Neil Marsh, Department of Chemistry, 930 N. University Ave. Ann Arbor MI 48109-1055; nmarsh@umich.edu; T: 734 763-6096

Key words

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Abstract

Viperin is a radical S-adenosylmethionine (SAM) enzyme that plays a multifaceted role in the cellular antiviral response; however, the mechanism by which viperin mediates its antiviral effects remains unknown. Viperin is implicated regulating K63-linked poly-ubiquitination of interleukin-1 receptor-associated kinase-1 (IRAK1) by the E3 ubiquitin ligase TNF Receptor-Associated Factor 6 (TRAF6) as part of the Toll-like receptor-7 and 9 (TLR7/9) innate immune signaling pathways. We show that poly-ubiquitination of IRAK1 requires the association of viperin with IRAK1 and TRAF6 and is SAM-dependent. Furthermore, IRAK1 and TRAF6 activate viperin to reductively cleave SAM in the presence of either ATP or CTP. Based on these and other observations, we propose a mechanism in which SAM cleavage switches the viperin-IRAK1-TRAF6 complex between poly-ubiquitination-active and inactive states, thereby regulating TLR7/9 signaling. This finding represents a new regulatory mechanism for protein post-translational modifications by radical SAM enzymes that may also explain viperin's seemingly unconnected antiviral effects.

Introduction

The innate immune system is involved in the initial detection of pathogens and coordinates the host's first line of defense against infection.[1] Viperin (**V**irus **i**nhibitory **p**rotein, **e**ndoplasmic **r**eticulum-associated, **in**terferon-inducible (also denoted as RSAD2 or Cig5 in humans) is an interferon-inducible protein that is up-regulated in response to viral infection (Fig. 1a) [2, 3, 4]. An N-terminal membrane-associated domain localizes viperin to the endoplasmic reticulum and lipid droplets, which are the assembly and replication sites for various viruses [5, 6, 7, 8]. Numerous studies have demonstrated viperin's role in restricting the infectivity of a range of viruses including HIV-1, influenza A, human cytomegalovirus (HCV), Bunyamwera virus and tick-borne encephalitis virus (TBEV), [3, 4, 9, 10, 11, 12] but no unified biochemical mechanism of action has immerged so far. Viperin has been reported to interact with a variety of host and viral proteins, among which are farnesyl pyrophosphate synthase[13] , the mitochondrial trifunctional protein[14], the viral nonstructural protein 5A (NS5A)[11] and the pro-viral host factor, vesicle-associated protein-A[15]. Most likely, viperin causes its targets to be degraded by the cell, thereby inhibiting metabolic pathways important for viral replication.

Viperin is unusual in being one of only eight radical SAM enzymes found in animals[16]. The vast majority of radical SAM enzymes catalyze reactions unique to microbial metabolism, which are initiated by the reductive cleavage of SAM to generate a 5′-deoxyadenosyl radical [16, 17, 18, 19, 20, 21]. The recently determined crystal structure of mouse viperin[5] shows that it adopts the canonical $(\beta\alpha)_6$-partial barrel fold observed for radical SAM enzymes[20], in which the catalytically essential [4Fe-4S] cluster is ligated by the three cysteinyl residues of the hallmark CxxxCxxC motif (Fig. 1b) [5]. The structural similarity of viperin to MoaA, a radical SAM enzyme that catalyzes the first step in molybdoterin biosynthesis using GTP as a substrate[22], has led to studies that

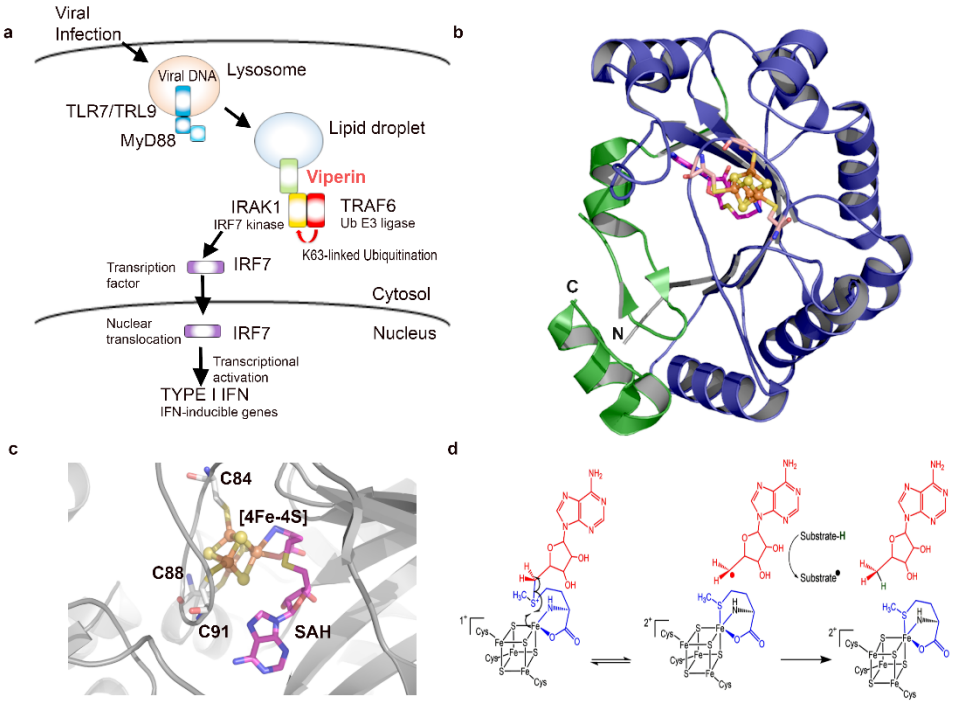

Fig.1: Role of radical SAM enzyme viperin in TLR7/9 signaling pathways. **a,** Viperin facilitates the activation of IRAK1 for phosphorylation of IRF7 in TLR7/9 signaling pathway. **b,** Structure of viperin (PDB code 5VSL); the canonical radical SAM domain shown in blue and the viperin-specific C-terminal domain in shown green; the [4Fe-4S] cluster is shown as spheres and the structure of SAH as sticks. **c**, Details of the active site showing the [4Fe-4S] cluster (spheres), the ligating cysteine residues of the CxxxCxxC motif and SAH bound (sticks). **d,** Canonical reaction mechanism for the reductive cleavage of SAM catalyzed by radical SAM enzymes to generate substrate-based radicals.

suggest that viperin catalyzes reactions on small molecule(s) rather than on proteins [23].

However, it is unclear how viperin's anti-viral properties depend upon its radical SAM activity, or what viperin's real substrate(s) are [12]. Furthermore, it appears that the radical SAM activity of viperin may be dispensable in some cases, such as restricting influenza A infection, [24]. Viperin, or viperin-like sequences have been identified in all kingdoms of life, which suggests a conserved biochemical function for the enzyme that may extend beyond the antiviral properties associated with its expression in animals [3, 4, 25, 26].

Recently, a connection between viperin and innate antiviral signaling has emerged. Toll-like receptor 7 (TLR7) and TLR9 are among various pattern recognition receptors that sense viral nucleic acids and induce production of type I interferons by plasmacytoid dendritic cells (pDCs) to protect the host from viral infection. Viperin has been implicated as a component of the signaling pathway that leads to type I interferon production [27, 28]. Viperin stimulates the K63-linked poly-ubiquitination of the IL-1R-associated kinase (IRAK1) by the E3 ubiquitin ligase, TNFR-associated factor 6 (TRAF6) [28, 29]. K63-linked poly-ubiquitination, in turn, activates IRAK1 to phosphorylate interferon regulatory factor 7 (IRF7), causing IRF7 to migrate to the nucleus where it activates transcription of type-1 interferons (Fig. 1a)[30, 31].

To examine the biochemical mechanism underling viperin's seemingly unconnected modes of action, we have reconstituted the interactions between viperin, IRAK1 and TRAF6 that lead to poly-ubiquitination of IRAK1 in HEK 293T cells. Based on our experiments, we propose that viperin regulates IRAK1 poly-ubiquitination in a manner that is controlled by the reductive cleavage of SAM and linked to cellular nucleotide pool. This mechanism reconciles many seemingly contradictory findings and may explain the mechanism of action of viperin on other cellular and viral protein targets.

Results

Viperin was initially implicated as a component the TLR7 and TLR9 signaling pathways leading to type-1interferon production in experiments using pDCs derived from transgenic mice [27, 28]. These studies focused on defining the signaling pathway, however they did not address the enzymatic function of viperin. To examine this question, we sought to reconstitute the interactions between viperin, TRAF6 and IRAK1 by transient expression of these proteins in HEK 293T cells. This allowed us to examine a specific biochemical function of viperin –

activation of IRAK1 by poly-ubiquitination – in a system that maintains the necessary cellular context for the enzyme to be functional. In particular, expression in eukaryotic cells allows viperin to localize to the ER membrane and lipid droplets and provides the additional components necessary for protein ubiquitination.

Viperin forms a complex with IRAK1 and TRAF6 at the ER membrane

We first sought to establish that viperin directly interacts with IRAK1 and TRAF6 to stimulate poly-ubiquitination of IRAK1, rather than through other intermediary protein(s). The interaction between viperin, IRAK1 and TRAF6 was examined by co-immunoprecipitation using FLAG-tagged viperin as the bait protein. Extracts of HEK 293T cells transfected with viperin, IRAK1 or TRAF6 were prepared, combined in 1:1:1 ratio and incubated for 16 h at 4º C; anti-FLAG M2 affinity gel was then used to immunoprecipitate viperin. IRAK1 co-precipitated with viperinin dependently of TRAF6 (Fig. 2a, Supplementary Fig. 4); however, TRAF6 was only co-precipitated in presence of both viperin and IRAK1. This observation implies that viperin binds to IRAK1 but suggests TRAF6 primarily binds to IRAK1. Similar results were obtained when a viperin mutant, viperin-Δ3C, in which the cysteine ligands binding the iron-sulfur cluster were mutated to alanine, was used as the bait protein. This suggests that the iron-sulfur cluster is unnecessary for viperin's physical association with IRAK1 and TRAF6.

The N-terminal domain of viperin serves to localize the enzyme to the cytoplasmic face of lipid droplets and the endoplasmic reticulum (ER) [8], whereas TRAF6 and IRAK1 are cytosolic enzymes [32]. Therefore, we examined whether co-expression of viperin with TRAF6 and IRAK1 caused these enzymes to re-localize to the ER membrane. Confocal immunofluorescence microscopy of HEK 293T cells co-transfected with IRAK1 and TRAF6 confirmed that in the absence of viperin these proteins are, as expected, distributed diffusely in

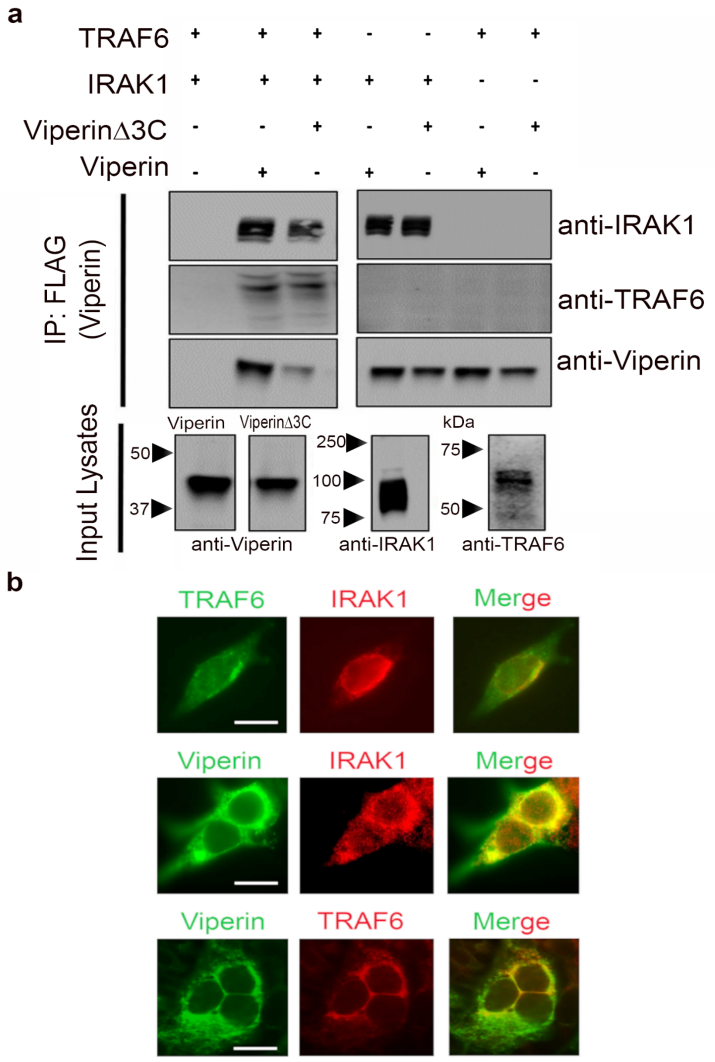

Fig. 2: Viperin co-localizes with IRAK1 and TRAF6 at the ER membrane. a, Co-immunoprecipitation analysis with viperin as bait protein demonstrates that viperin co-precipitates IRAK1 but only co-precipitates TRAF6 if IRAK1 is present. **b,** Confocal immunofluorescence images of HEK 293T cells transfected with viperin, TRAF6, and IRAK1. Scale bar = 5 μm. *Top panels*: cells co-transfected with IRAK1 and TRAF6 show diffuse expression throughout the cell. *Middle panels*: cells co-transfected with IRAK1, TRAF6 and viperin; staining for viperin (green) and IRAK1 (red) demonstrates co-localization (yellow) of viperin and IRAK1. *Bottom panels*: cells co-transfected with IRAK1, TRAF6 and viperin; staining for viperin (green) and TRAF6 (red) demonstrates co-localization (yellow). Co-expression of IRAK with viperin appears to result in IRAK1 also forming punctate structures that do not co-localize (middle panels). The reason for this is currently unclear.

the cytoplasm (Fig. 2b). In contrast, co-expression with viperin resulted in TRAF6 and IRAK1 localization to the ER membrane. Co-expression of IRAK1 alone with viperin resulted in IRAK1 partially localizing to the ER membrane. However, no localization of TRAF6 to the ER membrane was observed when viperin and TRAF6 were co-expressed (Supplementary Fig. 5). These observations are consistent with the immunoprecipitation results. Interestingly, when cells expressing viperin-Δ3C were examined, this mutant did not appear to localize to the ER membrane but remained cytosolic despite the presence of the N-terminal ER-localization domain (Supplementary Fig. 3). This precluded using viperin-Δ3C to conduct co-localization studies with IRAK1 and TRAF6.

Viperin is required for poly-ubiquitination of IRAK1

Viperin was initially implicated in the K63-linked poly-ubiquitination of IRAK1 by the E3 ubiquitin ligase TRAF6 based on studies that used mouse-derived viperin$^{+/+}$ and viperin$^{-/-}$ pDC cells. The TLR7/9 signaling pathways were stimulated in these cells with either dsRNA or lipopolysaccharides to induce viperin expression. [27, 28] We sought more direct evidence of viperin's involvement in the poly-ubiquitination of IRAK1 by recapitulating the system in HEK 293T cells. Cells were co-transfected with viperin and IRAK1 or TRAF6, or both, and harvested after 36 h. Cell extracts were analyzed by immunoblotting and probed with antibodies against IRAK1 or ubiquitin.

Viperin significantly stimulated poly-ubiquitination of IRAK1, as evidenced by the appearance of high molecular weight bands in immunoblots probed with polyclonal anti-ubiquitin antibodies (Fig. 3a). This finding indicates that viperin (when co-expressed with IRAK1) is able to either recruit endogenous TRAF6 and/or other E3 ubiquitin ligases to poly-ubiquitinate IRAK1[28, 33]. In contrast, the inactive viperinΔ3C mutant failed to stimulate poly-

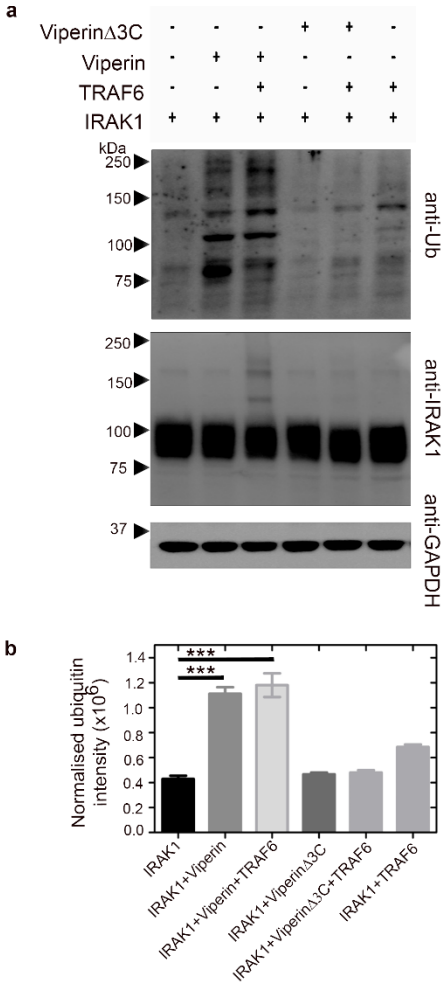

Fig.3: Viperin stimulates poly-ubiquitination of IRAK1 a, Immunoblot analysis of cells transfected with viperin or viperinΔ3C, IRAK1 and/or TRAF6 with staining for ubiquitin (*top panel*). Viperin significantly increases the level of IRAK1 ubiquitination (*bottom panel*) GAPDH staining was used as loading control for IRAK1staining. High M_r poly-ubiquitinated forms of IRAK1 are evident when both viperin and, IRAK1 and TRAF6 are co-expressed. **b,** Quantification of IRAK ubiquitination levels obtained by integrating intensities of bands for blot shown in the top panel of **a**. *** indicates statistical significance, $p < 0.001$

ubiquitination of IRAK1. This observation suggested that intact holo-enzyme is required for poly-ubiquitination of IRAK1 (Fig. 3a). Co-expression of TRAF6 with IRAK1 and viperin did not significantly alter the overall level of ubiquitination (Fig. 3b), but rather resulted in a shift to

higher molecular weight isoforms of poly-ubiquitinated IRAK1. We note that it is these long-chain ubiquitinated forms that are implicated in the activation of IRAK1 towards phosphorylation of IRF7, which is the penultimate step in TLR7/9 signaling [30, 31]. Interestingly, very little poly-ubiquitination of IRAK1 by TRAF6 was evident in the absence of viperin, even though these proteins were expressed at very high (non-physiological) cellular concentrations. This finding underscores the necessity of the interaction between viperin, IRAK1 and TRAF6 for poly-ubiquitination of IRAK1 to occur.

SAM is required for viperin to stimulate poly-ubiquitination of IRAK1

To investigate whether SAM was required for viperin to stimulate poly-ubiquitination of IRAK1, we examined the effect of depleting cellular SAM levels using cycloleucine, which is an inhibitor of SAM synthase [34]. Cycloleucine (50 mM) was added to the cell culture medium 12 h post-transfection and then the levels of cellular viperin were monitored for a period of 7 h. Surprising, we found that treatment with cycloleucine significantly reduced the half-life of viperin in the cells (Fig. 4a). This observation suggests that SAM binding to viperin stabilizes its structure, with viperin being subject to degradation in the absence of SAM. In the presence of cycloleucine, no poly-ubiquitination of IRAK1 was observed in cells expressing viperin, IRAK1 and TRAF6 (Fig. 4b). The lack of poly-ubiquitination could be a consequence either of the low levels of viperin expression under these conditions, or the low levels of SAM. To clarify this observation, examined the stability of the viperinΔ3C mutant, which is unable to cleave SAM.

When we expressed viperinΔ3C in HEK293T cells, we found that the mutant enzyme had a much shorter half-life than wild-type viperin, even in the absence of cycloleucine, (Fig. 4a). This result suggested that removing the iron-sulfur cluster significantly destabilizes the protein's structure, so that SAM may not bind to the mutant enzyme. We therefore examined the effect of

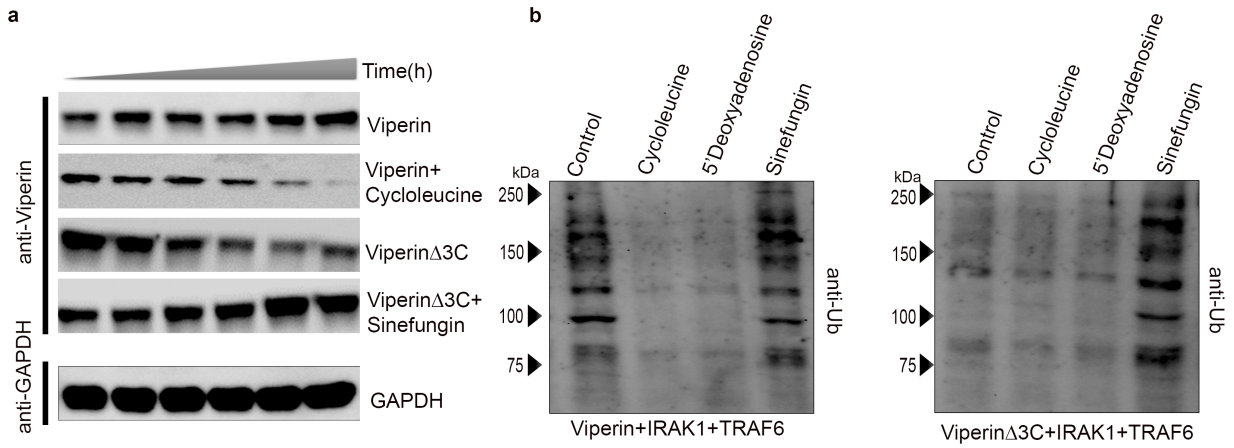

Fig.4: The effect of SAM, 5'-dA and sinefungin on viperin stability and poly-ubiquitination-stimulating activity. a, Stability of viperin and viperinΔ3C transiently expressed in HEK 293T cells over a period of 6 hours after addition of either cycloleucine (SAM synthase inhibitor) or sinefungin (non-hydrolysable SAM analogue) examined by immunoblotting. Depletion of cellular SAM levels by cycloleucine destabilizes viperin whereas sinefungin stabilizes viperin-Δ3C. **b,** Immunoblots showing that cycloleucine or 5'-dA inhibit the ability of viperin to stimulate poly-ubiquitination of IRAK1 in cells transiently expressing viperin, IRAK1 and TRAF6; in contrast, sinefungin rescues the ability of viperinΔ3C to stimulate poly-ubiquitination of IRAK1.

sinefungin, a tight-binding competitive inhibitor of many SAM-dependent enzymes [35], on the stability of viperinΔ3C. Sinefungin (20 nM) was added to the cell culture medium 4 h post-transfection and then the cellular levels of viperinΔ3C were followed for a further 12 h. Sinefungin effectively stabilized viperinΔ3C, thereby extending its cellular half-life to that of wild-type viperin (Fig. 4a). This observation suggested that sinefungin might stabilize the enzyme's structure.

Therefore, we examined whether, by stabilizing its structure, sinefungin could rescue the ability of the viperinΔ3C mutant to stimulate poly-ubiquitination of IRAK1 by TRAF6. HEK293T cells were co-transfected with viperinΔ3C, IRAK1 and TRAF6 and 8 h post-transfection treated with sinefungin (20 nM, final concentration). The cells were cultured for a

further 12 h , harvested and analyzed by immunoblotting for the presence of poly-ubiquitinated forms of IRAK1. We found that sinefungin does indeed rescue the ability of viperinΔ3C to stimulate poly-ubiquitination of IRAK1 (Fig. 4b). Lower levels of poly-ubiquitinated IRAK1 were seen in sinefungin-treated cells expressing only viperinΔ3C and IRAK1, suggesting that sinefungin-bound viperinΔ3C may recruit endogenous E3 ubiquitin ligases (Fig 4b). These observations imply that SAM (or a SAM analog) must be bound to viperin for it to form an active complex with IRAK1 and TRAF6.

Based on this observation, we hypothesized that 5'-dA, as the product of SAM cleavage, might inhibit viperin's ability to stimulate poly-ubiquitination of IRAK1. HEK 293T cells were co-transfected with wild-type viperin, TRAF6 and IRAK1 and treated with 5'-dA (5 μM final concentration). The treated cells were harvested after 12 h and analyzed by immunoblotting for the presence of poly-ubiquitinated IRAK1. In this case, we observed that 5'-dA significantly reduced the level of poly-ubiquitinated IRAK1 (Fig. 4b), suggesting that 5'-dA inhibits wild-type viperin's ability to stimulate poly-ubiquitination of IRAK1.

IRAK1 and TRAF6 activate viperin towards reductive cleavage of SAM

The contrasting effects of 5'-dA and sinefungin on the abilities of wild-type viperin and viperinΔ3C to stimulate the poly-ubiquitination of IRAK1, suggested that the radical SAM activity of viperin might serve as a regulatory mechanism to control the poly-ubiquitination of IRAK1. Therefore, we examined whether co-expression of IRAK1 and TRAF6 had any effect on the reductive cleavage of SAM catalyzed by viperin. Extracts of HEK 293T cells transfected with viperin, IRAK1 and TRAF6 were prepared and assayed under anaerobic conditions for viperin activity, which was quantified by measuring the formation of 5'-dA formed through the reductive cleavage of SAM (Supplementary Fig. 6). To normalize the data, the amount of

viperin present in the cell extracts was quantified by immunoblotting, as described in the Methods section (Supplementary Fig. 7).

In cell extracts expressing only viperin, only 0.94 ± 0.05 molecules of 5'-dA per molecule of viperin were generated in 1 h (Fig. 5; Table 1). However, when viperin was co-expressed with both IRAK1 and TRAF6, a 4-fold increase in the amount of 5'-dA produced was observed (k_{obs} = 4.30 ± 0.21 h^{-1}). Co-expression of viperin with IRAK1 alone resulted in a smaller, 2-fold, increase in 5'-dA formation, whereas co-expression of viperin with TRAF6 alone had no effect on 5'-dA formation, which is consistent with the lack of interaction between these two enzymes. Negligible amounts of 5'-dA were formed in cell extracts either lacking viperin or in cells expressing viperinΔ3C.

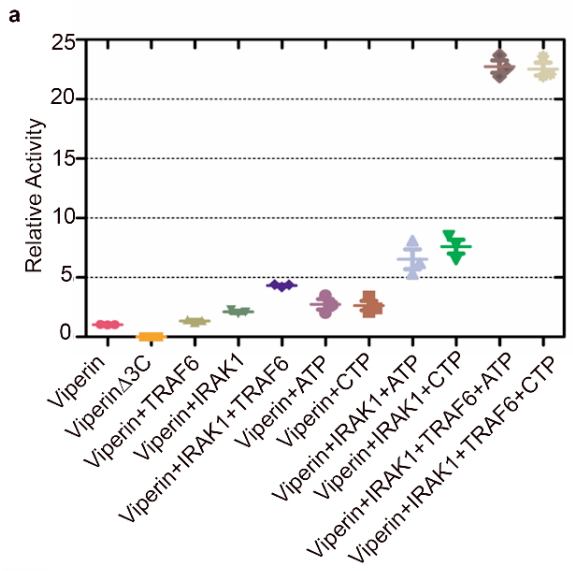

Fig. 5: Radical SAM activity of viperin is stimulated 23-fold by IRAK1 and TRAF6 and either ATP or CTP. Relative activity refers to the amount of 5'-dA formed in 1 h, normalized for the amount of viperin present in the cell extracts, and relative to the viperin-only sample which is set to 1.0 on the scale. The data represent the mean and standard deviation of three independent biological replicates with three technical replicates of each measurement.

Table 1: Effects of IRAK1, TRAF6, ATP and CTP on the radical SAM activity of viperin

Condition	Viperin (pmol)	5'-dA (pmol)	Turnovers (h^{-1})
Viperin	1.38 ± 0.07	1.30 ± 0.01	0.94 ± 0.05
Viperin +TRAF6	1.10 ± 0.06	1.27 ± 0.01	1.15 ± 0.06
Viperin + IRAK1	0.70 ± 0.04	1.49 ± 0.02	2.13 ± 0.11
Viperin +IRAK1+ TRAF6	0.40 ± 0.02	1.72 ± 0.03	4.30 ± 0.21
Viperin +ATP	1.38 ± 0.07	3.48 ± 0.18	2.67 ± 0.06
Viperin +GTP	1.38 ± 0.07	1.58 ± 0.11	1.21 ± 0.03
Viperin +CTP	1.38 ± 0.07	3.10 ± 0.15	2.38 ± 0.05
Viperin +UTP	1.38 ± 0.07	1.57 ± 0.12	1.20 ± 0.03
Viperin + IRAK1+ATP	0.70 ± 0.04	4.47 ± 0.11	6.83 ± 0.04
Viperin + IRAK1+CTP	0.70 ± 0.04	5.09 ± 0.17	7.78 ± 0.06
Viperin +IRAK1+ TRAF6+ATP	0.40 ± 0.02	9.17 ± 1.25	23.65 ± 0.42
Viperin +IRAK1+ TRAF6+CTP	0.40 ± 0.02	8.55 ± 0.48	22.05 ± 0.13

ATP and CTP activate viperin towards reductive cleavage of SAM

The recent crystal structure for mouse viperin enigmatically shows that the active site architecture closely resembles to that of the radical SAM enzyme MoaA [5, 36], which catalyzes the first step molybdopterin biosynthesis using GTP as the substrate [22]. The active site of viperin contains conserved amino acid residues that could also conceivably bind a nucleotide substrate [5, 23, 37]. Moreover, recent reports have demonstrated that, *in vitro*, a fungal viperin catalyzes the reaction of SAM with UDP-glucose[23] and that farnesyl- and geranyl-pyrophosphate stimulate 5'-dA formation by human viperin[38]. Although the physiological relevance of these reactions remains to be established, these observations suggest that, in addition to interacting with other proteins, viperin may require a small molecule co-substrate to perform its physiological function.

Noting the conservation of active site biding residues between MoaA and viperin, we examined the ability of nucleotides to activate viperin towards SAM cleavage. Interestingly, we found that inclusion of either 300 μM ATP (k_{obs} = 2.67 ± 0.06 h^{-1}) or CTP (k_{obs} = 2.38 ± 0.05 h^{-1}) in the assay resulted in a 2.4 – 2.7-fold increase in the amount of 5'-dA formed in cell extracts expressing viperin (Fig. 5; Table 1), indicating that ATP and CTP may also be substrates for viperin. In contrast, inclusion of 300 μM UTP or GTP provided no increase in 5'-dA, demonstrating that viperin has specificity for certain nucleotides. As typical cellular nucleotide triphosphate concentrations range from 1 – 10 mM for ATP and ~ 400 μM for CTP [39], this observation suggests that these nucleotides are likely to be physiological substrates for viperin.

IRAK1 and TRAF6 and either ATP or CTP synergistically activate viperin

Significantly, a large increase in viperin's radical SAM activity was observed when either ATP or CTP were added to lysates prepared from cells co-expressing viperin, IRAK1 and TRAF6. As shown in Fig. 5, addition of ATP or CTP (300 μM) to cell extracts that contained

viperin, IRAK1 and TRAF6 resulted in a 23-fold increase in specific activity over cell extracts expressing only viperin in the absence of nucleotides. This dramatic increase in activity implies that viperin undergoes multiple turnovers during the course of the assay, with the turnover time shortening from 1 h to about 2.5 min.

Discussion

The mechanism underpinning viperin's diverse effects on cellular and viral proteins has remained poorly understood. Prior studies have either tended to focus on indirect measures of viperin's activity, such as the reduction of viral titer or RNA levels or examined viperin's reactions with putative substrates *in vitro* in the absence of any cellular context. By reconstituting the interactions between viperin, IRAK1 and TRAF6 in HEK 293T cells we have been able to examine a specific, biochemically-defined function of viperin: the activation of IRAK1 by poly-ubiquitination.

Our experiments allow us to posit the following mechanism for viperin, depicted in Fig. 6, in which the reductive cleavage of SAM serves as a switch to regulate the TLR7/9 signaling pathway in a manner analogous to signal regulation by hetero-trimeric G proteins[40, 41]. In the absence of SAM, viperin is in an inactive conformation (apo-form) that is unable to stimulate ubiquitination of IRAK1. Upon binding SAM, viperin undergoes a conformational change to an active state (holo-form) that stimulates K63-linked poly-ubiquitination of IRAK1 by TRAF6 (or other E3 ligases). This step of the mechanism is supported by the fact that viperinΔ3C, which cannot catalyze reductive cleavage of SAM, is rescued by the SAM analogue, sinefungin. Once poly-ubiquitinated, IRAK phosphorylates IRF7, eventually inducing transcription of type I interferons [28]

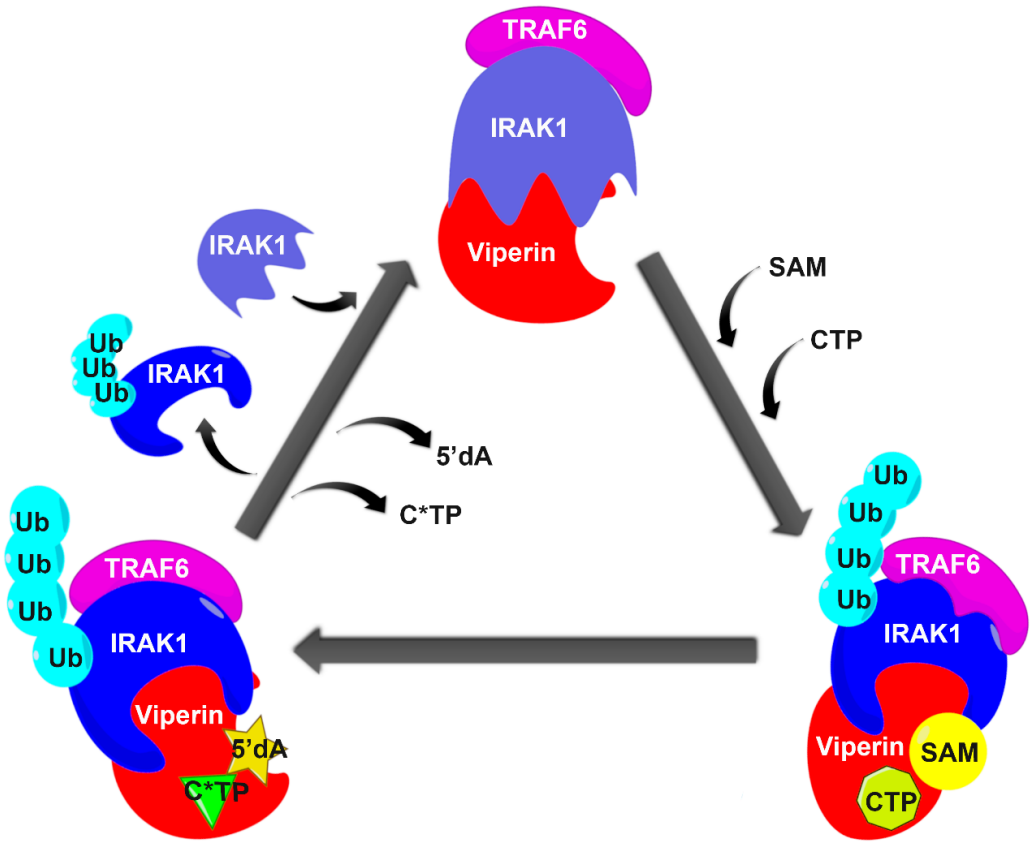

Fig.6: Mechanistic hypothesis for viperin's role in regulating ubiquitination of IRAK1. Reaction of SAM with CTP (or ATP; not shown for simplicity) generates 5'-dA and a modified nucleotide (C*TP). This switches the viperin-TRAF6-IRAK1 complex between active and inactive states. For details see the text.

When complexed with IRAK1 and TRAF6, viperin is activated to cleave SAM. The large increase in radical SAM activity elicited by ATP or CTP indicates that these nucleotides are co-substrates for viperin. We postulate that cleavage of SAM, leading to the formation of 5'-dA returns the viperin to its inactive state, preventing further poly-ubiquitination of IRAK1. This aspect of the mechanism is supported by inhibitory effect of 5'-dA on poly-ubiquitination of IRAK1. The slow rate, for an enzymatic reaction, at which viperin cleaves SAM makes this reaction well suited to serve as a "radical clock" to regulate a signaling pathway. Based on the

rate of 5'-dA formation, we estimate that viperin remains in the active state for an average of 2.5 min before cleavage of SAM reverts it to its inactive state.

The activation of viperin by ATP and CTP intriguingly hints at a mechanism by which viperin and by extension TLR7/9 signaling could be modulated by the cellular nucleotide pool. It is currently unclear how viperin modifies these nucleotides, assuming, as seems very likely, that they are true substrates for viperin, as opposed to simply activators of the enzyme. Unfortunately, the extremely small amounts of material produced in these experiments precluded us from characterizing the modified nucleotides. An interesting possibility is that the modified nucleotides might serve as novel second messengers that could regulate other cellular pathways involved in the innate immune response. It is also intriguing that, *in vitro,* viperin is reported to be activated to cleave SAM by geranyl- and farnesyl-pyrophosphate [38], which are key intermediates in isoprenoid biosynthesis. Other than their phosphate groups, these molecules have no structural similarity to CTP or ATP. This observation suggests that viperin might bind, and by extension be regulated by, a range of phosphorylated cellular metabolites.

In conclusion, our experiments establish a unifying mechanistic hypothesis for viperin that rationalizes many of the seemingly unconnected observations regarding the function of this intriguing enzyme. We note that protein ubiquitination plays multiple roles in cellular proteostasis and signal transduction [42, 43]. We suggest that the wide-ranging roles that viperin plays in the antiviral response could result from the ability of the enzyme to recruit and activate a subset of the many E3 ubiquitin ligases present in the cell to ubiquitinate target proteins, thereby regulating their cellular levels in response to viral infection. Lastly, we note that the utilization of radical SAM chemistry as a “radical clock” to regulate the activity of other cellular proteins constitutes new role for this ancient and diverse class of enzymes.

Methods

Materials

Cell lines

The HEK293T cell line was obtained from ATCC.

Antibodies

Rabbit polyclonal RSAD2/ Viperin antibody (11833-1-AP) was obtained from Protein Tech. Rabbit polyclonal IRAK1 antibody (PA5-17490) was obtained from Thermo Scientific. Rabbit polyclonal Ubiquitin antibody (sc-9133) and mouse monoclonal TRAF6 antibody (sc-8409) were purchased from Santa Cruz. Goat anti-rabbit (170-6515) and anti-mouse (626520) Ig secondary Abs were purchased from BioRad and Life Technologies respectively. Rabbit polyclonal GAPDH (TAB1001) was purchased from Thermo Scientific and Mouse monoclonal GAPDH antibody (6C5) was obtained from EMD Millipore.

Plasmids

Synthetic genes encoding human viperin, IRAK1 and TRAF6 (GenBank accession numbers AAL50053.1, NM145803, NM001569 respectively) were purchased from GenScript. IRAK1 (NM_001569) was cloned between EcoRI and XhoI with an N-terminal Myc tag. TRAF6 (NM_145803) was cloned between EcoRI and XhoI with an N-terminal HA tag.

Reagents The sources of other reagents were as described previously [44]

Transfection

HEK 293T cells, cultured as described above, were transiently transfected using FuGENE® HD (Promega) following the manufacturer's instructions.

Immunofluorescence analyses

Cells were grown on poly-L-lysine-coated coverslips to 30–40% confluence, transfected with plasmids expressing Viperin, IRAK1 and/or TRAF6 and incubated for 30 h. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100 dissolved in PBS, and washed three times with PBS containing 0.1% Tween20. The fixed cells were stained with the appropriate antibodies. Primary antibodies were diluted in PBS containing, 1% FBS and 0.1% Tween 20. Viperin was detected by using mouse monoclonal anti-viperin (Abcam) diluted 1:250, and calnexin was detected with rabbit polyclonal antibody (Abcam) diluted 1:250. IRAK1 was detected by using rabbit polyclonal IRAK1 antibody (Thermo Scientific) diluted 1:250 and mouse monoclonal c-Myc Tag antibody (Thermo Scientific) diluted 1:100. TRAF6 was detected by using mouse monoclonal TRAF6 antibody (Santa Cruz) diluted 1:100. After incubation at room temperature for 1 h or overnight at 4°C, the coverslips were washed with PBS containing 0.1% Tween20 and treated with Alexa Fluor 647-conjugated goat anti-mouse (Life Technologies) and Alexa Fluor 488-conjugated goat anti-rabbit (Abcam) secondary antibodies at a dilution of 1:400 at room temperature for 2 h. The coverslips were washed three times with PBS containing 0.1% Tween20 and mounted in ProLong™ Gold Antifade Mountant (Molecular Probes). Images were acquired with an Olympus IX81 microscope with a 60× 1.49NA objective on an Andor iXON Ultra EMCCD camera. 488 nm (Coherent Cube 488-50) and 640 nm (Coherent Cube 640–100) laser excitation was aligned in HILO imaging mode for axial sectioning using an Olympus cell^TIRF module.

Immunoblotting

Cells were lysed in TBST buffer (20 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween-20) containing protease inhibitors (SIGMAFAST™ Protease Inhibitor Tablets, S8830; Sigma).

Supernatants of lysates were collected and mixed with reducing sample buffer. The supernatants were separated on 10% SDS-PAGE gels. The bicinchoninic acid (BCA) assay (Thermo Scientific) was used to determine the total amount of protein in the lysates. Immunoblotting was performed as describes previously [44]. Primary antibodies used were directed against viperin (rabbit polyclonal diluted 1:2500), GAPDH (rabbit polyclonal diluted 1:5000), IRAK1 (rabbit polyclonal diluted 1:4000), TRAF6 (mouse monoclonal diluted 1:500) and GAPDH (mouse monoclonal diluted 1:5000). Rabbit polyclonal ubiquitin antibody was used at a dilution of 1:1000. Blots were visualized, and band intensities quantified using a Bio-Rad ChemiDoc Touch imaging system. Integrated density measurements were done using ImageJ software. Quantitative measurements of protein expression levels reported here represent the average of at least three independent biological replicates.

Immunoprecipitation Assay

Cells were rinsed twice with ice-cold PBS, harvested in P40 lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride with protease inhibitor cocktail from Sigma), and briefly sonicated. Lysates were collected by centrifugation at 12,000 × *g* for 15 minutes at 4°C. For immunoprecipitation, ratio of suspension to packed gel volume was 2:1. Resin pre-equilibration was done as per the manufacturer's protocol. Three hundred microliters of a 1:1:1 ratio of cell lysates was added and incubated for 16 hours at 4°C with gentle rotation. Beads were pelleted by centrifugation at 5,000 × *g* for 30 seconds at 4°C and washed three times with washing buffer (50 mM Tris, pH 7.4,150 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride with protease inhibitor cocktail from Sigma). Immunocomplexes were eluted by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE, and transferred to

a nitrocellulose membrane. Membranes were blocked for 2 h at room temperature in TBST buffer (20 mM Tris, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk, followed by overnight incubation at 4°C in TBST buffer containing 3% nonfat dry milk and the appropriate primary antibody. Membranes were washed three times in TBST and then incubated for 2 h at room temperature with the secondary IgG-coupled horseradish peroxidase antibody. The membranes were washed three times with TBST, and the signals were visualized with enhanced chemiluminescence reagent as described in immunoblotting.

Statistical analyses

Results from all studies were compared with unpaired two-tailed Student's t test using GraphPad Prism 5 software. P values less than 0.05 were considered significant.

SAM cleavage assay

HEK 293T cells transfected with viperin, and/or IRAK1 and TRAF6 were harvested from one 10 cm diameter tissue culture plate each, resuspended in 500 µl of anoxic Tris-buffered saline (50 mM Tris-Cl, pH 7.6, 150 mM NaCl) containing 1% Triton X-100, sonicated within an anaerobic glovebox (Coy Chamber), and centrifuged at 14,000 g for 10 min. Dithiothreitol (DTT;5 mM) and dithionite (5 mM) were added to the cell lysate. The assay mixture was incubated at room temperature for 30 min prior to starting the reaction by the addition of SAM (200 µM). The assay was incubated for 60 min at room temperature, after which the reaction stopped by heating at 95 °C for 10 min. The solution was chilled to 4 °C, and the precipitated proteins were removed by centrifugation at 14,000 rpm for 25 min. The supernatant was then extracted with acetonitrile. Samples were analyzed in triplicate by UPLC-tandem mass spectrometry as described previously [44]. For nucleotide triphosphate assays, the different nucleotides were added at 300 µM

concentration along with DTT and dithionite. For details of standard curve construction and calculations refer to Supplementary Fig.7.

Supporting information

Additional figures showing quantitation of western blots, cellular localization of transfected proteins and methodology for estimating absolute concentration of viperin in cell lysates.

Competing Interests

The authors declare no competing financial interest.

Materials & Correspondence

Correspondence and material requests should be addressed to nmarsh@umich.edu.

Author Contributions

A.B.D., S.G., A.M.P., P.A.M., K.Z. and J.D.H. performed the experiments. A.B.D., P.A.M., K.Z. and J.D.H. analyzed the results. S.G. and A.M.P. provided reagents. A.B.D., J.D.H., R.T.K. and E.N.G.M. designed the experiments. A.B.D. and E.N.G.M. wrote the paper.

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