Viperin inhibits hepatitis C virus replication by interfering with binding of NS5A to host protein hVAP-33

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Viperin is a type-I and -II interferon-inducible intracytoplasmic protein that mediates antiviral activity against several viruses. A previous study has reported that viperin could limit hepatitis C virus (HCV) replication in vitro. However, the underlying mechanism remains elusive. In the present study, we found that overexpression of viperin could inhibit HCV replication in a dose-dependent manner in both the replicon and HCVcc systems. Furthermore, through co-immunoprecipitation and laser confocal microscopic analysis, viperin was found to interact with the host protein hVAP-33. Mutagenesis analysis demonstrated that the anti-HCV activity of viperin was located to its C terminus, which was required for the interaction with the C-terminal domain of hVAP-33. Competitive co-immunoprecipitation analysis showed that viperin could interact competitively with hVAP-33, and could therefore interfere with its interactions with HCV NS5A. In summary, these findings suggest a novel mechanism by which viperin inhibits HCV replication, possibly through binding to host protein hVAP-33 and interfering with its interaction with NS5A.

INTRODUCTION

Hepatitis C virus (HCV) infection is a growing public health concern, affecting 170 million people worldwide. Seventy to eighty per cent of infected patients become chronic carriers who, in addition to being the source of most new infections, can progress to exhibiting chronic hepatic cirrhosis and hepatocellular carcinoma (Chen & Morgan, 2006). Infection with HCV is currently treated with alpha interferon (IFN-α)-based therapy (McHutchison et al., 1998). IFNs activate the inducible expression of hundreds of genes, which are the main mediators of the host antiviral response (Van Damme et al., 2008). Many steps in the viral replication cycle are potential targets of these IFN-inducible proteins. However, the mechanisms of action of the vast majority of IFN-inducible proteins remain unknown.

Viperin is an evolutionarily conserved protein that is highly inducible by both type-I and -II IFNs (Der et al., 1998). Initially identified as a human cytomegalovirus-inducible protein, it has been reported that this protein can be induced by infection by many other viruses, including vesicular stomatitis virus (Boudinot et al., 2000), yellow fever virus (Khaioullina et al., 2005) and HCV. Upon expression, viperin localizes to the cytosolic face of the ER membrane through its N-terminal amphipathic alpha-helix domain (Hinson & Cresswell, 2009b). Recently, viperin has been found to be significantly expressed in all percutaneous liver biopsies from chronic hepatitis C (CHC) patients and has been reported to exert non-cytolytic inhibition of the replication of HCV (Helbig et al., 2005; Jiang et al., 2008). These findings suggest that viperin might play an important role in controlling HCV replication and mediating IFN-induced antiviral effects against HCV.

The present study was undertaken to analyse the mechanism by which viperin limits HCV replication. We confirmed that viperin could inhibit replication of HCV in both subgenomic (genotype 1b) and full-length genomic (genotype 2a) systems. Indirect immunofluorescence and co-immunoprecipitation analysis revealed that viperin binds to the cellular protein hVAP-33 and interferes with its interaction with HCV NS5A protein, but not NS5B protein. Mutagenesis analysis also revealed that the C-terminal domain of viperin is required for its anti-HCV effect.

RESULTS

Overexpression of viperin impairs HCV replication in vitro. Previous studies have observed that the IFN-inducible protein viperin was increased significantly in percutaneous

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Two supplementary figures and a supplementary table are available with the online version of this paper.
liver biopsies from CHC patients and that it had anti-HCV activity in vitro (Helbig et al., 2005). Therefore, we first confirmed the effect of viperin against HCV in a subgenomic replicon system (genotype 1b). Levels of protein expression and intracellular HCV RNA were determined 60 h post-transfection by Western blotting and real-time PCR, respectively. Results revealed that the level of NS5A was significantly downregulated with increasing expression of viperin (Fig. 1a). Meanwhile, HCV RNA was also reduced significantly in cells transfected with viperin (Fig. 1c) compared with cells transfected with empty vector. Additionally, expression of viperin in HCV replicon-containing or replicon-free cells did not significantly affect cell growth (data not shown). Therefore, these data suggest that the reduction of HCV replication in replicon cells by expression of viperin was due to its anti-HCV activity, rather than direct cytotoxicity.

Next, we confirmed these results in an HCVcc system (genotype 2a). We transfected Huh7.5 cells with different amounts of viperin expressing plasmid 36 h before infecting with JFH-1 at an m.o.i. of 0.01 and then analysed intracellular HCV RNA and NS5A at 72 h post-infection. Similar to the data shown in Fig. 1(a, c), pre-expression of

![Fig. 1. Overexpression of viperin reduces HCV replication and infection. (a–c) Replication of HCV in replicon cells transfected with viperin. Cells were harvested with SDS loading buffer for Western blotting analysis 60 h post-transfection, followed by detection with anti-NS5A, anti-myc and anti-actin antibodies (a), and protein bands were then quantified by densitometry analysis to the levels of NS5A and β-actin and shown as a histogram (b). HCV RNA levels were then measured by real-time quantitative PCR (c). Values (means ± SD) from triplicate wells are shown. Asterisks indicate statistical significance according to Student’s t-test (*, P<0.05). Similar results were obtained in two additional independent experiments. (d–f) Replication of HCV in the HCVcc system transfected with viperin. Huh7.5 cells plated in a 12-well plate were transfected with 1 μg pCMV-vector, 0.5 or 1 μg pCMV-viperin expression plasmids or left untreated, and the cells were then replated on a 6-well plate 36 h post-transfection. After another 12 h, cells were infected with HCVcc at an m.o.i. of 0.01 and then harvested at 72 h post-infection. Intracellular HCV NS5A protein and viral RNA were quantified by Western blotting analysis (d) and real-time quantitative PCR (f), respectively. Protein bands were then quantified by densitometry analysis to the levels of NS5A and β-actin (e). Values (means ± SD) from triplicate wells are shown. Asterisks indicate statistical significance according to Student’s t-test (*, P<0.05). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. Similar results were obtained in two additional independent experiments.]

Viperin in cells could inhibit synthesis of HCV RNA (Fig. 1f) and viral NS5A protein (Fig. 1d) in a dose-dependent manner. Under these conditions, the level of intracellular β-actin was not affected (Fig. 1d). These results indicate that HCV replication was reduced in Huh7.5 cells when viperin was expressed prior to JFH-1 infection. Taken together, these results indicated that viperin exerted anti-HCV activity in vitro by downregulating HCV RNA and non-structural proteins.

**Viperin interacts with human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33).**

Products of interferon-stimulated genes (ISGs) such as LMP7, PKR and 2′,5′-oligoadenylate synthase have been demonstrated to interact with HCV non-structural proteins, thereby exerting their antiviral activities (Khu et al., 2004; Taguchi et al., 2004; Yan et al., 2007). Therefore, we first performed immunoprecipitation assays to study whether intermolecular interactions existed between viperin and HCV non-structural proteins. When plasmids expressing individual HCV non-structural proteins were cotransfected with viperin-expressing plasmid into HEK293T cells, no interactions were found by immunoprecipitation assay 48 h post-transfection (Supplementary Fig. S1, available in JGV Online). Recently, viperin was found to localize to the cytosolic face of the ER through its N-terminal amphipathic alpha-helix domain (Hinson & Cresswell, 2009a). As it is widely accepted that HCV non-structural proteins form the replication complex at the ER membrane, the possibility exists that viperin interacts with other components of the replication complex. As the protein hVAP-33 has been reported to be involved in the formation of the HCV replication complex (Gao et al., 2004), we examined the colocalization of GFP–viperin with DsRed–hVAP-33 in HEK293T cells. Results showed that GFP–viperin colocalized with DsRed–hVAP-33 (Fig. 2c). Overlap between GFP and DsRed–hVAP-33 was not observed when control GFP-expressing cells were examined, nor was there any overlap of DsRed with GFP–viperin.

To confirm further the interaction between viperin and hVAP-33, myc–viperin and FLAG–hVAP-33 and myc–vector and FLAG–hVAP-33 were co-expressed in HEK293T and Huh7 cells, respectively, followed by immunoprecipitation with an anti-myc antibody. Viperin was shown to interact specifically with hVAP-33 in lanes 6 of Fig. 2(a) (in HEK293T cells) and Fig. 2(b) (in Huh7 cells). As a negative control, no interaction was observed between myc–vector and **Fig. 2.** Viperin interacts with hVAP-33 protein. (a) In vivo co-immunoprecipitation of viperin with hVAP-33 in HEK293T cells. HEK293T cells plated in 60 mm dishes were transfected with 1 μg pCMV-viperin or pCMV-vector and 1 μg pCDNA-3.1A/3×FLAGg-hVAP-33. Cell lysates were immunoprecipitated with anti-myc mAb (IP: anti-myc) or mouse normal IgG (Control). The immunoprecipitated fractions (pellets) and 5% of the remaining supernatant fractions (5% input) were then subjected to Western blotting analysis. The blots were reacted with anti-FLAG and anti-myc antibodies. Ig HC (heavy chain) is marked. (b) In vivo co-immunoprecipitation of viperin with hVAP-33 in Huh7 cells. The methodology used was the same as for the experiments described in (a). (c) Viperin colocalizes with hVAP-33. HEK293T cells were processed for immunofluorescence analysis 48 h after being transfected with DsRed–hVAP-33, GFP–viperin, DsRed–vector or GFP–vector plasmids as indicated. The cells were fixed and labelled as described in Methods. Coverslips were mounted and observed by confocal laser scanning microscopy (Leica).
FLAG–hVAP-33 in lanes 3 of Fig. 2(a) (in HEK293T cells) and Fig. 2(b) (in Huh7 cells). These results suggested that viperin interacts with the host protein hVAP-33.

The C-terminal domains of viperin and hVAP-33 are responsible for their interaction. Having shown that there is a molecular interaction between viperin and hVAP-33, we conducted mutational analysis to define the interacting regions of hVAP-33 and viperin. We first constructed expression plasmids for truncated hVAP-33 as shown in Fig. 3(a); myc–full-length viperin (myc–viperin-FL) was co-expressed with FLAG-tagged hVAP-33 and truncated mutants FLAG–hVAP-33(1–155) and FLAG–hVAP-33(156–242) in HEK293T cells and, at 48 h post-transfection, cells were lysed and subject to immunoprecipitation assay with anti-myc antibody. hVAP-33(156–242), which includes the CC and TM domains, could be precipitated by the anti-myc antibody (Fig. 3b, lane 9). This result was further supported by indirect immunofluorescence analysis. GFP–viperin was cotransfected with DsRed–hVAP-33(156–242) and DsRed–hVAP-33(1–155) into HEK293T cells. A significant overlap between GFP–viperin and DsRed–hVAP-33(156–242) was observed around the periphery of the nucleus. No overlap of GFP and DsRed–hVAP-33(156–242) was observed when GFP-expressing cells were examined (Fig. 3c). Therefore, these results indicated that the C-terminal domain of hVAP-33 was responsible for its interaction with viperin.

Multiple sequence alignment analysis of the viperin protein has revealed that viperin contains an N-terminal variable domain (aa 1–76), followed by a highly conserved radical SAM domain (RSD; aa 77–209) in the middle and a C-terminal conserved domain (aa 210–361) (Jiang et al., 2008). Recently, the first 50 amino acid residues of the N-terminal region were demonstrated to form an amphipathic alpha-helix, mediating localization of viperin to the cytosolic face of the ER (Hinson & Cresswell, 2009a). Therefore, two truncated mutants were constructed: N217, which covers the coding region of the N-terminal 217 amino acids and includes the amphipathic alpha-helix domain as well as the RSD, and C144, which encodes the C-terminal 144 amino acids (Fig. 3d). Myc–viperin, myc–viperin-N217 and myc–viperin-C144 were cotransfected with FLAG–hVAP-33-FL into HEK293T cells. Immunoprecipitation assays with an anti-FLAG antibody showed that viperin-C144 was responsible for its interaction with hVAP-33 protein (Fig. 3e, lane 9). Similarly, colocalization of hVAP-33 with the C144 mutant of viperin was also found when DsRed–hVAP-33 and GFP–viperin-C144 expression plasmids were cotransfected into HEK293T cells (Fig. 3f). Therefore, these results indicated that the C-terminal conserved domain of viperin and the C-terminal domain of hVAP-33 were responsible for their interaction. Taken together, these data further demonstrated that viperin can interact with hVAP-33.

The C-terminal domain of viperin exerts the major anti-HCV effect. The above results demonstrated that viperin can bind to hVAP-33 through C-terminal amino acid residues. We further investigated whether the C144 region of viperin was involved in the inhibition of HCV replication in an HCV subgenomic system. Viral protein expression and the intracellular HCV RNA level were determined by Western blotting and real-time PCR, respectively, 60 h after transfection with viperin-FL, viperin-N217 or viperin-C144. The level of NS5A was significantly downregulated when viperin-FL and viperin-C144 expression plasmids were transfected into replicon cells (genotype 1b) (Fig. 4a). As a control, the level of β-actin expression was not affected. HCV RNA was also reduced in cells transfected with viperin-FL and viperin-C144 compared with cells transfected with the empty plasmid (Fig. 4c).

We further confirmed the results in the HCVcc system (genotype 2a). Similar to the data shown in the Fig. 4(a, c), pre-expression of viperin and viperin-C144 in cells could inhibit synthesis of HCV RNA (Fig. 4f, lanes 2 and 4) and viral NS5A protein (Fig. 4d, lanes 2 and 4). Under these conditions, the intracellular β-actin level was not affected (Fig. 4d). In addition, we found that viperin-N217 could also inhibit HCV replication, but not as significantly as C144. The above results suggested that the C-terminal domain of viperin exerts the major antiviral activity against HCV in hepatocytes. Similar to the report by Jiang et al. (2008), high-molecular-mass aggregates could be detected after long exposure when viperin-C144 was overexpressed (Fig. 4a, lane 4), suggesting the N-terminal region of viperin deleted in viperin-C144 was important for the proper folding and/or membrane association of the protein.

Viperin interferes with the interaction between hVAP-33 and NS5A. Previous studies have demonstrated that the C terminus of hVAP-33 binds NS5A, whereas the N terminus of hVAP-33 binds NS5B (Tu et al., 1999), and that these interactions are critical for the assembly of the HCV replication complex on ER-derived membranes. As the above results have shown that the C-terminal domain of hVAP-33 can be bound by viperin, it is possible that viperin may exert its antiviral activity against HCV through competition with NS5A for interactions with the C-terminal domain of hVAP-33.

To test this hypothesis, HEK293T cells and Huh7 cells were cotransfected with myc-tagged NS5A (genotype 1b) (myc–NS5A) and FLAG-tagged hVAP-33 expression plasmids, in the presence or absence of overexpression of viperin [haemagglutinin (HA)–viperin]. The interaction between NS5A and hVAP-33 was examined by immunoprecipitation and Western blotting 48 h after transfection. As shown in Fig. 5(a) (left), in the control group without viperin expression, myc–NS5A could be significantly immunoprecipitated by FLAG–hVAP-33. However, in the presence of viperin, the level of immunoprecipitated myc–NS5A was markedly reduced, although the total levels of FLAG–hVAP-33 and myc–NS5A were equivalent to those of the control group. Meanwhile, consistent with
previous results, a substantial amount of HA–viperin was also immunoprecipitated by FLAG–hVAP-33, as shown in Fig. 5(a) (left lower part). When the immunoprecipitation assay was undertaken with myc–NS5A, we found that the level of immunoprecipitated FLAG–hVAP-33 in cells transfected with HA–viperin was greatly reduced compared with that of cells transfected with the HA vector alone (Fig. 5a, right).

![Diagram](http://vir.sgmjournals.org)

Fig. 3. The C-terminal domain of viperin interacts with the C-terminal domain of hVAP-33 protein. (a) Schematic representations of full-length (FL) and truncated hVAP-33 constructs. (b) In vivo co-immunoprecipitation of hVAP-33 and truncated mutations with viperin. HEK293T cells plated in 60 mm dishes were cotransfected with 1 μg pCMV-viperin and 1 μg pCDNA-3.1A/3×FLAG-hVAP-33-FL, pCDNA-3.1A/3×FLAG-hVAP-33-(1–155) or pCDNA-3.1A/3×FLAG-hVAP-33-(156–242). Cell lysates were immunoprecipitated with anti-myc monoclonal antibodies or mouse normal IgG. The immunoprecipitated fractions (pellets) and 5% of the remaining supernatant fractions (5% input) were then subjected to Western blotting analysis. The blot was reacted with anti-FLAG and anti-myc antibodies. (c) Viperin colocalizes with hVAP-33(156–242). HEK293T cells were processed for immunofluorescence analysis 48 h after being transfected with DsRed–hVAP-33(1–155) and GFP–viperin or DsRed–hVAP-33(156–242) and GFP–viperin, respectively. The cells were fixed and labelled as described in Methods. Coverslips were mounted and observed by confocal laser scanning microscopy (Leica). (d) Schematic representations of full-length (FL) and truncated viperin constructs. (e) In vivo co-immunoprecipitation of viperin and truncated mutations with hVAP-33. HEK293T cells plated in 60 mm dishes were cotransfected with 1 μg pCMV-viperin-FL, pCMV-viperin-N217 or pCMV-viperin-C144 and 1 μg pCDNA-3.1A/3×FLAG-hVAP-33. Cell lysates were then immunoprecipitated with anti-FLAG mAbs or mouse normal IgG. The immunoprecipitated fractions (pellets) and 5% of the remaining supernatant fractions (5% input) were then subjected to Western blotting analysis. The blots were reacted with anti-myc and anti-FLAG antibodies, respectively. (f) Viperin-C144 colocalizes with hVAP-33. HEK293T cells were processed for immunofluorescence analysis 48 h after being transfected with different matches of DsRed–hVAP-33, viperin-N217–GFP and DsRed–hVAP-33, GFP–viperin-C144. Cells were fixed and labelled as described in Methods. Coverslips were mounted and observed by confocal laser scanning microscopy (Leica).
To exclude the possibility that the reduction in the interaction between NS5A and hVAP-33 was due to downregulation of NS5A by overexpression of viperin, we examined the levels of NS5A expression when the expression of viperin was increased incrementally. No differences were observed in the level of NS5A expression among cells transfected with different amounts of viperin, indicating that overexpression of viperin has little effect on the expression of NS5A when cotransfected in HEK293T cells (Fig. 5b). Similar results were also observed for the interaction of NS5A with hVAP-33 in Huh7 cells (Fig. 5c). These results indicate that overexpression of viperin could reduce the reciprocal immunoprecipitation efficiencies of FLAG–hVAP-33 with myc–NS5A.

This result was further supported by indirect immunofluorescence analysis. Replicon cells were transfected with the viperin-expressing plasmid and colocalization of endogenous NS5A with DsRed–hVAP-33 was examined by indirect immunofluorescence 40 h after transfection. The results showed that the distribution of DsRed–hVAP-33 fluorescence in replicon cells transfected with the empty vector overlapped significantly with endogenous NS5A stain (Fig. 5d, lower part). However, no obvious overlap of hVAP-33 with endogenous NS5A was observed when cells transfected with viperin-expressing plasmids were examined (Fig. 5d, upper part). Also, in HEK293T cells, as shown in Fig. 5(e), in the absence of viperin, hVAP-33 was colocalized with NS5A. However, in the presence of viperin, colocalization between hVAP-33 and NS5A was decreased. These data indicated that viperin could interfere with the interaction between hVAP-33 and NS5A.

Viperin does not interfere with the interaction between hVAP-33 and NS5B. In our study, we found that viperin could interfere with the interaction between hVAP-33 and NS5A. As previous studies have reported that the C terminus of hVAP-33 binds the N terminus of NS5A, whereas the N terminus of hVAP-33 binds the C terminus of NS5B (Gao et al., 2004; Tu et al., 1999), it would be worthwhile to examine further the effects of viperin on interactions between hVAP-33 and NS5B. In contrast to the above results, in the presence of viperin (HA–viperin), the amounts of immunoprecipitated hVAP-33 and NS5B were not affected when compared with the control group (Fig. 6). Combined with the observation that viperin interferes with the mutual interaction of NS5A with hVAP-33, these findings suggest strongly that viperin disrupts the interaction of NS5A with

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**Fig. 4.** The C-terminal domain of viperin exerts the major anti-HCV effect. (a–c) Replication of HCV in replicon cells transfected with viperin-FL or truncated mutation plasmids. The methodology was the same as for the experiments described in Fig. 1(a–c). (d–f) Replication of HCV in the HCVcc system transfected with viperin-FL or truncated mutation plasmids. The methodology was the same as for the experiments described in Fig. 1(d–f).
hVAP-33 through binding to hVAP-33, which is crucial for the replication of HCV RNA, and that this may account for the anti-HCV activity of viperin in vitro.

**DISCUSSION**

IFNs represent the first line of host defence against virus infection, activating the JAK-STAT cascade signal amplification system and inducing the expression of related cellular genes after binding to specific receptors on the cell surface (Der et al., 1998; Klotman & Chang, 2006; Samuel, 2001; Stetson & Medzhitov, 2006; Zhao et al., 2007). Of these gene products, only a few cellular proteins responsible for the inhibition of HCV replication have been identified. Previous studies have suggested that viperin worked together with ISG 20 and PKR to mediate IFN responses against HCV infection. However, the underlying antiviral mechanism has not been completely defined. Here, our results suggest a novel molecular mechanism by which viperin inhibits HCV replication through binding to the host protein hVAP-33 and the findings further indicate that this may result from interference with the interaction between hVAP-33 and HCV NS5A.

HCV is the prototype member of the genus Hepacivirus in the family Flaviviridae. The single-stranded, positive-sense 9.6 kb RNA genome encodes a polypeptide of 3010–3030 amino acids that is processed post-translationally by host and viral proteases into at least 10 structural and non-structural proteins (Grakoui et al., 1993). Non-structural proteins NS3 to NS5B are necessary and sufficient for HCV RNA
replication (Evans et al., 2004; Huang et al., 2007; Miyanari et al., 2003). Similar to other positive-stranded RNA viruses (Salonen et al., 2005), a hallmark of HCV replication is the formation of a membrane-associated replication complex, which is composed of viral non-structural proteins, certain cellular proteins, replicating RNA and altered ER membrane (Aizaki et al., 2004; Bartenschlager & Lohmann, 2000; Blight et al., 2000; Egger et al., 2002; El-Hage & Luo, 2003; Moradpour et al., 2007). Although it is not clear how these proteins are assembled, it has been demonstrated that hVAP-33 (also called hVAP-A) is required for the formation of this replication complex. The hVAP-33 protein interacts with HCV non-structural proteins NS5A and NS5B via its C and N termini, respectively, to anchor NS5A and NS5B to the ER membrane (Gao et al., 2004).

In this study, we found that viperin could interact with hVAP-33 via its C-terminal domain (viperin-C144). As viperin is localized at the cytosolic face of the ER (Hinson & Cresswell, 2009a), this provided the possibility that viperin interacts intracellularly with hVAP-33, and further disturbs the interactions of hVAP-33 with NS5A. Furthermore, viperin-C144 could inhibit HCV replication as significantly as full-length viperin (Fig. 4), suggesting that the C-terminal domain of viperin was involved in interactions with cellular and/or viral proteins which are important for viral replication. Through mutagenesis studies, Jiang et al. (2008) revealed that an aromatic amino acid residue in the C terminus was essential for proper antiviral function. Furthermore, addition of epitope tags at the C terminus resulted in a complete elimination of its antiviral activity. All these results indicate that there is a structural requirement for the C terminus of viperin to exert antiviral activity. It is possible that the steric hindrance effect of viperin might destroy the connection of hVAP-33 and NS5A and then prevent the formation of a complete HCV replication complex. The role of this amino acid residue in the interaction of viperin and hVAP-33 and whether this binding affects the interaction between hVAP-33 and NS5A are worth further study.

In addition to viperin-C144, viperin-N217 was found to inhibit replication of HCV to some extent (Fig. 4), in contrast to the study of Jiang et al. (2008), in which they demonstrated that the N terminus of viperin played a major antiviral role. It is conceivable that the use of different cell lines or different levels of expression might account for these differences in experimental observations as, in the study of Jiang et al. (2008), an HEK293-derived cell line was used to induce expression of viperin, while the viperin-expressing plasmid was transfected directly into Huh7 or Huh7.5 cells in this study. As the antiviral activity of viperin demonstrated by Jiang et al. (2008) depends on its radical SAM domain, which contains conserved motifs to coordinate a \([4Fe–4S]\) cluster and the cofactor SAM and is essential for its enzymic activity, differences may exist in enzymic activity of viperin between different cell lines; this question should be investigated further.

In conclusion, the data presented here suggest a possible mechanism by which viperin inhibits HCV replication through binding to host protein hVAP-33 and interfering replication (Evans et al., 2004; Huang et al., 2007; Miyanari et al., 2003). Similar to other positive-stranded RNA viruses (Salonen et al., 2005), a hallmark of HCV replication is the formation of a membrane-associated replication complex, which is composed of viral non-structural proteins, certain cellular proteins, replicating RNA and altered ER membrane (Aizaki et al., 2004; Bartenschlager & Lohmann, 2000; Blight et al., 2000; Egger et al., 2002; El-Hage & Luo, 2003; Moradpour et al., 2007). Although it is not clear how these proteins are assembled, it has been demonstrated that hVAP-33 (also called hVAP-A) is required for the formation of this replication complex. The hVAP-33 protein interacts with HCV non-structural proteins NS5A and NS5B via its C and N termini, respectively, to anchor NS5A and NS5B to the ER membrane (Gao et al., 2004).

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In conclusion, the data presented here suggest a possible mechanism by which viperin inhibits HCV replication through binding to host protein hVAP-33 and interfering
with its interaction with NS5A as a consequence. Further studies toward understanding the amino acid residue(s) of viperin that are responsible for interacting with hVAP-33 as well as the enzymic activity of the N-terminal domain in hepatocytes and its association with the effects on HCV replication should provide more valuable insights into the anti-HCV activity of IFN and may lead to the development of novel antivirals for the treatment of CHC.

**METHODS**

**Cell lines.** Huh7 (kindly provided by Professor Charles Rice), Huh7.5 and HEK293T cells were maintained in monolayer culture in Dulbecco’s modified minimal essential medium (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 U penicillin/ml−1, 100 U streptomycin/ml−1 and 10 % FCS, in a humidified atmosphere at 37 °C with 5 % CO2. BB7 HCV subgenomic replicon cells (Blight et al., 2000) were grown in conditional medium supplemented with 5 µg blasticidin ml−1 (In Vitrogen).

**Plasmids and reagents.** DNA fragments encoding N5SA and NS5B were generated from a genotype 1b strain (BB7) by PCR. The amplified DNA fragments were digested with EcoRI and XhoI (NEB) and then cloned into the pCMV-myc vector (Clontech). DNA fragments encoding viperin, viperin-N217 and viperin-C144 were amplified from the pCDNA3.1A-viperin plasmid (kindly provided by Professor Peter Cresswell), digested with EcoRI and XhoI and then cloned into the pCMV-myc or pCMV-HA vector (Clontech). PCR-generated viperin and viperin-C144 fragments were digested with XhoI and EcoRI and cloned into the pGFP-C2 vector (Clontech), while viperin-N217 was cloned into pGFP-N2 vector. pDsRed-hVAP-33 was cloned from replicon cells and then cloned into the EcoRI and XhoI sites of pDsRed-Express-C1. FLAG-hVAP-33, FLAG-hVAP-33(1-155) and FLAG-hVAP-33(156-242) were generated from pDsRed-hVAP-33, digested with EcoRI and XhoI and then cloned into the pCDNA3.1A/3 × FLAG vector.

Mouse mAb against HCV NSSA was obtained from Virogen. Mouse monoclonal anti-β-actin and anti-FLAG antibodies were from Sigma. Mouse mAb against myc was from Santa Cruz. Mouse IgG TrueBlot was from eBioscience and Alexa Fluor 488-conjugated anti-mouse IgG antibodies were from Rockland. Transfection reagents FuGENE 6 and FuGENE HP were purchased from Roche.

**Generation of infectious HCV particles and virus infection.** In vitro synthesis of HCV RNA and electroporation were performed as described previously (Lindenbach et al., 2005). Briefly, Huh7.5 cells were mixed with in vitro-transcribed RNA and electroporated with five pulses lasting 99 µs at 820 V over 1.1 s in an ECM 830 (BTX Genetronics). The culture medium was harvested 13 days post-transfection, and purified viruses were used for infection and titration.

**Immunoprecipitation.** For in vivo co-immunoprecipitation in Huh7 or HEK293T cells, cells were seeded onto 60 mm dishes 16 h before transfection. The plasmids were transfected using FuGENE 6 (Roche) in HEK293T cells and FuGENE HP (Roche) in Huh7 cells, respectively. Forty-eight hours after transfection, the cells were washed three times with ice-cold PBS and solubilized with 600 µl lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1.0 % NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl2, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 1 mM PMSF, 1 × proteinase inhibitor cocktail (Roche)] for 5 min on ice, followed by another rotating-inoculation for 30 min at 4 °C. Detergent-insoluble material was removed by centrifugation at 12,000 g for 10 min at 4 °C. After equal division, whole-cell lysates were incubated with 1 µg monoclonal antibody or mouse normal IgG at 4 °C for 2 h. Pre-equilibrated protein A/G-agarose beads were then added and, after 2 h of incubation, the beads were collected by centrifugation and then washed gently three times with 800 µl lysis buffer. Bound proteins were eluted by boiling in 2 × SDS loading buffer and subjected to Western blotting. The efficiency of transfection in Huh7 cells is shown in Supplementary Fig. S2.

**Quantitative real-time PCR.** Cells were harvested with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. After treatment with DNase I, each RNA sample was reverse-transcribed with random primers (dN6). The cDNA was mixed with SYBR Green PCR Master Mix (Toyobo) and subjected to real-time PCR using an ABI PRISM 7500 (Applied Biosystems). Cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from the same cDNA was used as an internal control to determine the number of cells and metabolic status. Primers specific for the HCV 5’UTR and GAPDH are shown in Supplementary Table S1. Forty cycles of PCR were performed with cycling conditions of 15 s at 95 °C, 20 s at 55 °C and 25 s at 72 °C.

**Western blotting.** Cells were lysed directly with 2 × SDS loading buffer (100 mM Tris/HCl, pH 6.8, 20 % glycerol, 4 % SDS, 3 % β-mercaptoethanol and 0.02 % bromophenol blue) and boiled. Samples were separated by 10 % SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience) and blocked with 5 % skimmed milk in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4 and 0.1 % Tween 20, pH 7.4). Blots were probed with different primary antibodies, followed by a secondary antibody conjugated to HRP. Protein bands were visualized with ECL Plus chemiluminescence reagent (PerkinElmer).

**Immunofluorescence staining and confocal microscopic analysis.** Cells were fixed with 3.5 % paraformaldehyde and permeabilized with 0.1 % Triton X–100 in PBS. After washing twice with PBS, samples were incubated overnight with mAb at 4 °C (9E10 NSSA; 1:200) and then washed with PBS containing 1 % FCS. Samples were then incubated at room temperature for 2 h with Alexa Fluor 488-conjugated secondary antibody (1:400). Coverslips were mounted on glass plates and the cells were observed using a confocal laser scanning microscope (Leica TCS-NT) with a ×64 NA 1.4 oil-immersion objective. Images were acquired as described previously (Yi et al., 2011).

**Statistical analysis.** All results were confirmed in at least three independent experiments in triplicate within each experiment. Data were analysed using Student’s t-test and expressed as means ± SD. A value of P<0.05 was considered to indicate statistical significance.

**ACKNOWLEDGEMENTS**

We thank Professor Peter Cresswell (Yale University, New Haven, CT, USA) for providing viperin plasmids, Professor Charles M. Rice (Rockefeller University, New York, NY, USA) for providing the 9E10 antibody and Huh7 cells and Dr Michael R. Beard (University of Adelaide, Australia) for constructive suggestions. This work was supported by the Shanghai Program for Outstanding Medical Academic Leader, a Chinese State Basic Research Foundation grant (2009CB522504) and the National Mega Project for Infectious Disease (2008ZX10002-002).

**REFERENCES**
