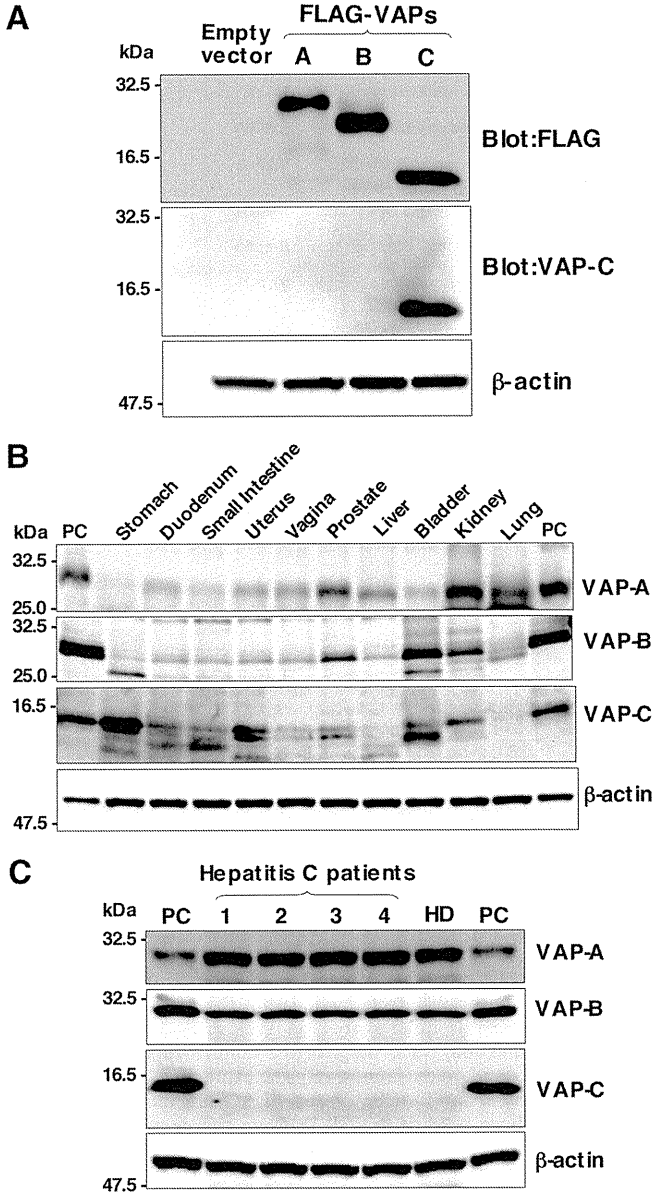

FIG. 6. Distribution of VAPs in human tissues. (A) Anti-VAP-C antibody specifically recognizes VAP-C. Human embryonic kidney 293T cells transfected with expression plasmid encoding FLAG-tagged VAP-A, VAP-B, or VAP-C or empty vector were harvested at 48 h posttransfection and examined by immunoblotting using anti-FLAG tag, anti-VAP-C, and anti-β-actin antibodies. (B) The premade human tissue lysates "Protein medleys" (20 μg each; Clonetech) were examined by immunoblotting using antibodies against VAP-A, VAP-B, VAP-C, or β-actin. (C) Expression of VAP family proteins in human liver tissues. Liver samples obtained from four hepatitis C patients (1 to 4) and one healthy donor (HD) were examined by immunoblotting as described above. The data in each panel are representative of the results of three independent experiments. PC indicates 293T cells transfected with expression plasmid encoding VAP-A, VAP-B, and VAP-C.

(15, 48). VAP-A has been detected in a detergent-resistant membrane fraction that was shown to be capable of replicating HCV RNA in vitro, and the interaction of VAP-A with NS5A is required for the efficient replication of HCV genomic RNA

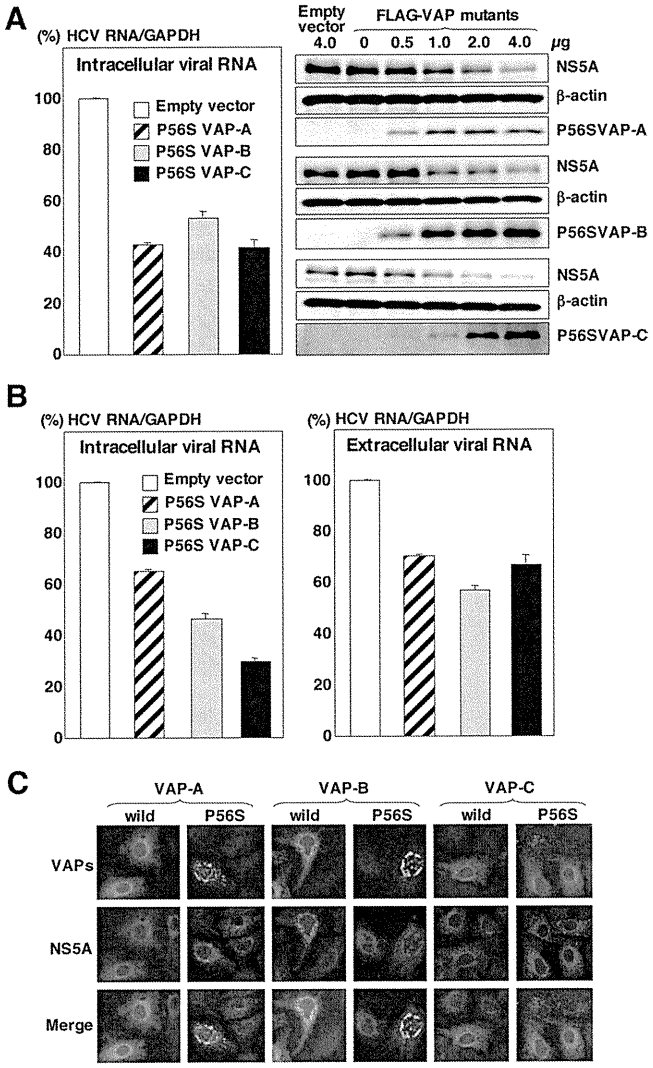

FIG. 7. Substitution of Ser for Pro[56] in VAPs leads to suppression of HCV replication. (A) Left: Huh 9-13 cells were transfected with 4 μg of the expression plasmids encoding FLAG-tagged P56S VAP mutants or empty vector, and the level of intracellular HCV RNA was determined at 72 h posttransfection by real-time PCR after normalization with GAPDH mRNA. The value for HCV RNA at 0 h posttransfection in the cell line transfected with the empty plasmid is represented as 100%. Data in this panel are shown as the means ± standard deviations. Right: Huh 9-13 cells were transfected with 0 to 4 μg of the FLAG-tagged P56S VAP mutant plasmids or empty vector, and the levels of expression of NS5A, β-actin, and the mutant VAPs were determined by immunoblotting at 72 h posttransfection. The data in each panel are representative of the results of three independent experiments. (B) Huh7OK1 cells transfected with 4 μg of the expression plasmids encoding FLAG-tagged P56S VAP mutants or empty vector were infected with strain JFH1 at an MOI of 0.05 at 14 h posttransfection, and the intracellular (left) and extracellular (right) expression levels of viral RNA were determined by real-time PCR after normalization with GAPDH mRNA at 96 h postinfection. Data in this panel are shown as the means ± standard deviations. (C) Levels of expression of wild-type VAPs, P56S mutant VAPs, and NS5A in Huh 9-13 cells at 72 h after transfection with the expression plasmids encoding FLAG-tagged VAPs or P56S VAP mutants were determined by immunofluorescent assay. The data in each panel are representative of the results of three independent experiments.

(2, 7) and is modulated by the phosphorylation of NS5A (4, 6). VAP-B also participates in HCV replication through the formation of homo- and/or heterodimers with VAP-A (9). VAP-A and VAP-B form hetero- and homodimers through their TM regions and interact with NS5A and NS5B through the coiled-coil domain and MSP domain, respectively (9, 44). VAP-C is a splicing variant of VAP-B, consisting of the N-terminal half of VAP-B and the subtype-specific amino acid residues generated by the frameshift. However, the biological significance of VAP-C in the life cycle of HCV has not been determined. In this study, we have demonstrated that VAP-C is capable of binding to HCV NS5B but not to NS5A, VAP-A, and VAP-B due to the lack of the coiled-coil and TM regions. The expression of VAP-C inhibited the interaction of VAP-A and VAP-B with NS5B, impaired the RNA replication and particle formation of HCV, and was barely detected in human liver cells. These results suggest that VAP-C acts as a negative regulator for HCV propagation and is partly involved in the determination of the tissue specificity of HCV replication.

Overexpression of VAP-A but not of VAP-B inhibited the incorporation of the vesicular stomatitis virus (VSV) envelope glycoprotein G (VSV-G) into ER vesicles in CHO cells, resulting in impairment of membrane protein transport from the ER to the Golgi apparatus (37). VAP-B was shown to be involved in the unfolded protein response, which is an ER reaction to suppress the accumulation of misfolded proteins, and the expression of the P56S VAP-B mutant was suggested to nullify the unfolded protein response induced by VAP-B, to produce a large aggregation of ER, and to be involved in the development of ALS (17, 37). These data suggest that VAP-A and VAP-B possess different physiological functions; however, the contributions of the proteins to the life cycle of HCV have not been characterized. The expression of VAP-B but not of VAP-A resulted in an enhancement of the replication of the subgenomic HCV RNA of the genotype 1b strain Con1, whereas the expression of either VAP-A or VAP-B clearly enhanced viral RNA replication in cells infected with the genotype 2a strain JFH1 virus, suggesting that the contributions of VAP-A and VAP-B to viral RNA replication might differ among the genotypes of HCV. The expression of VAP-B or VAP-A enhanced RNA replication in the HCV replicon cells and the secretion of viral RNA, core protein, and infectious particles into the culture supernatants of Huh7OK1 cells infected with JFH1 virus, whereas the expression of these proteins had no effect on the expression of NS5A or on IRES-dependent translation. Thus, further studies will be needed to clarify the molecular mechanisms underlying the posttranslational enhancement of HCV production by the expression of VAP-A and VAP-B. In contrast to the expression of VAP-A and VAP-B, the expression of VAP-C clearly suppressed the RNA replication of both the genotype 1b RNA replicon cells and the genotype 2a strain JFH1 virus, by which both the expression of the viral proteins and the viral particle production were drastically impaired. Furthermore, the expression of the P56S mutants of VAP-A and VAP-B reduced RNA replication in HCV replicon cells and propagation of the JFH1 virus, probably due to the induction of aggregation of the ER. The reason why ER aggregation was induced by the expression of the P56S VAP-A mutant in Huh7 cells but not in CHO cells (17, 37) is not known at the moment.

The phosphorylation state of NS5A was suggested to control the interaction between VAP-A and NS5A and the replication efficiency of HCV RNA (6). Introduction of the adaptive mutations originally identified in the genotype 1b strain Con1 into NS5A of genotype 1a suppressed the hyperphosphorylation of NS5A, potentiated interaction with VAP-A, and enhanced the RNA replication (6). However, we have previously shown that NS5A of genotype 1a could bind to VAP-A and VAP-B at a level similar to that of genotype 1b despite the adaptive mutations (9). In this study, overexpression of each of the VAP proteins exhibited no effect on the mobility of NS5A in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3 and 5), suggesting that there is no correlation between the VAP-dependent regulation of HCV propagation and the phosphorylation state of NS5A.

FKBP8 exhibits peptidyl prolyl *cis-trans* isomerase activity and interacts with NS5A and Hsp90 through the tetratricopeptide repeat (TPR) domain, and these interactions are suggested to be involved in the correct folding of the HCV replication complex (34). Treatment of cells with inhibitors of the ATPase activity of Hsp90, such as geldanamycin and its derivatives, impairs the RNA replication and particle production of HCV (28, 34, 45). The MSP domain of VAP-A was shown to interact with the TPR1 protein, which has a TPR domain and forms the chaperone complex with Hsp90 (22). Knockdown of the TPR1 protein or treatment with Hsp90 inhibitors in mammalian cells has been shown to inhibit the transport of VSV-G, leading to accumulation of the glycoprotein in the Golgi apparatus (22). The VAP-A- or VAP-B-induced enhancement of virus production might be attributable to the recruitment of Hsp90 into the replication complex through the interaction with the MSP domain.

VAP-A is well known to interact through the MSP domain with a number of mammalian and yeast proteins sharing the FFAT motif, including OSBPs, ORPs (20), and CERT (10, 19), and to be involved in the regulation of biosynthesis or trafficking of sterols and lipids. HCV replication and infection have been shown to be regulated by lipid components and to be capable of being inhibited by treatment with several inhibitors targeting lipid biosynthesis (14, 18). The intracellular membranous web structure observed in HCV replicon cells was shown to be resistant to detergent treatment, suggesting that the lipid raft-like structure abundant in cholesterol and sphingolipid is generated by the replication of HCV RNA (2, 24). Therefore, it might be feasible to speculate that VAP-A and VAP-B are involved in the construction of the HCV replication complex consisting of viral proteins and host cellular lipid components and that VAP-C interrupts the VAP-A and VAP-B functions and negatively regulates HCV propagation. Although the molecular mechanisms and the biological significance remain to be clarified, the MSP domain of VAP proteins was processed in human leukocytes and secreted into human serum (43). Further studies are needed to clarify the biogenesis and biological functions of the truncated VAP proteins in the replication of HCV.

In summary, we have shown that VAP-C is capable of suppressing the RNA replication and particle production of HCV by inhibiting the binding of VAP-A and VAP-B to NS5B through the N-terminal half of its MSP domain. The clear suppression of HCV propagation by the expression of VAP-C

further suggests the possibility of developing a novel therapeutic measure to eliminate HCV by the exogenous expression of VAP-C in the hepatocytes of chronic hepatitis C patients.

ACKNOWLEDGMENTS

We thank H. Murase for her secretarial work. We also thank R. Bartenschlager and T. Wakita for providing cell lines and plasmids.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

REFERENCES

1. **Abe, T., Y. Kaname, I. Hamamoto, Y. Tsuda, X. Wen, S. Taguwa, K. Moriishi, O. Takeuchi, T. Kawai, T. Kanto, N. Hayashi, S. Akira, and Y. Matsuura.** 2007. Hepatitis C Virus nonstructural protein 5A modulates Toll-like receptor-MyD88-dependent signaling pathway in the macrophage cell lines. J. Virol. **81:**8953–8966.
2. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology **324:**450–461.
3. **Behrens, S. E., L. Tomei, and R. De Francesco.** 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. **15:**12–22.
4. **Blight, K. J., A. A. Kolykhalov, and C. M. Rice.** 2000. Efficient initiation of HCV RNA replication in cell culture. Science **290:**1972–1974.
5. **Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. **76:**5974–5984.
6. **Evans, M. J., C. M. Rice, and S. P. Goff.** 2004. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc. Natl. Acad. Sci. USA **101:**13038–13043.
7. **Gao, L., H. Aizaki, J.-W. He, and M. M. C. Lai.** 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J. Virol. **78:**3480–3488.
8. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. **67:**2832–2843.
9. **Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Moriishi, and Y. Matsuura.** 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J. Virol. **79:**13473–13482.
10. **Hanada, K., K. Kumagai, S. Yasuda, Y. Miura, M. Kawano, M. Fukasawa, and M. Nishijima.** 2003. Molecular machinery for non-vesicular trafficking of ceramide. Nature **426:**803–809.
11. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
12. **Hoofnagle, J. H.** 2002. Course and outcome of hepatitis C. Hepatology **36:**S21–S29.
13. **Huang, D. C., S. Cory, and A. Strasser.** 1997. Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. Oncogene **14:**405–414.
14. **Ikeda, M., K. Abe, M. Yamada, H. Dansako, K. Naka, and N. Kato.** 2006. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology **44:**117–125.
15. **Inoue, K., T. Umehara, U. T. Ruegg, F. Yasui, T. Watanabe, H. Yasuda, J. M. Dumont, P. Scalfaro, M. Yoshiba, and M. Kohara.** 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology **45:**921–928.
16. **Kaiser, S. E., J. H. Brickner, A. R. Reilein, T. D. Fenn, P. Walter, and A. T. Brunger.** 2005. Structural basis of FFAT motif-mediated ER targeting. Structure **13:**1035–1045.
17. **Kanekura, K., I. Nishimoto, S. Aiso, and M. Matsuoka.** 2006. Characterization of amyotrophic lateral sclerosis-linked P56S mutation of vesicle-associated membrane protein-associated protein B (VAPB/ALS8). J. Biol. Chem. **281:**30223–30233.
18. **Kapadia, S. B., and F. V. Chisari.** 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc. Natl. Acad. Sci. USA **102:**2561–2566.
19. **Kawano, M., K. Kumagai, M. Nishijima, and K. Hanada.** 2006. Efficient trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus requires a VAMP-associated protein-interacting FFAT motif of CERT. J. Biol. Chem. **281:**30279–30288.
20. **Loewen, C. J., A. Roy, and T. P. Levine.** 2003. A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. EMBO J. **22:**2025–2035.
21. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113.
22. **Lotz, G. P., A. Brychzy, S. Heinz, and W. M. Obermann.** 2008. A novel HSP90 chaperone complex regulates intracellular vesicle transport. J. Cell Sci. **121:**717–723.
23. **McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. **21:**3980–3988.
24. **Miyanari, Y., M. Hijikata, M. Yamaji, M. Hosaka, H. Takahashi, and K. Shimotohno.** 2003. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. J. Biol. Chem. **278:**50301–50308.
25. **Moriishi, K., and Y. Matsuura.** 2007. Host factors involved in the replication of hepatitis C virus. Rev. Med. Virol. **17:**343–354.
26. **Moriishi, K., and Y. Matsuura.** 2003. Mechanisms of hepatitis C virus infection. Antivir. Chem. Chemother. **14:**285–297.
27. **Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura.** 2003. Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. **77:**10237–10249.
28. **Nakagawa, S., T. Umehara, C. Matsuda, S. Kuge, M. Sudoh, and M. Kohara.** 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem. Biophys. Res. Commun. **353:**882–888.
29. **Nishimura, A. L., M. Mitne-Neto, H. C. Silva, A. Richieri-Costa, S. Middleton, D. Cascio, F. Kok, J. R. Oliveira, T. Gillingwater, J. Webb, P. Skehel, and M. Zatz.** 2004. A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. Am. J. Hum. Genet. **75:**822–831.
30. **Nishimura, Y., M. Hayashi, H. Inada, and T. Tanaka.** 1999. Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. Biochem. Biophys. Res. Commun. **254:**21–26.
31. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199.
32. **Okamoto, K., Y. Mori, Y. Komoda, T. Okamoto, M. Okochi, M. Takeda, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2008. Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. J. Virol. **82:**8349–8361.
33. **Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura.** 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J. Virol. **78:**6370–6380.
34. **Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. **25:**5015–5025.
35. **Okamoto, T., H. Omori, Y. Kaname, T. Abe, Y. Nishimura, T. Suzuki, T. Miyamura, T. Yoshimori, K. Moriishi, and Y. Matsuura.** 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J. Virol. **82:**3480–3489.
36. **Pennetta, G., P. R. Hiesinger, R. Fabian-Fine, I. A. Meinertzhagen, and H. J. Bellen.** 2002. Drosophila VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. Neuron **35:**291–306.
37. **Prosser, D. C., D. Tran, P. Y. Gougeon, C. Verly, and J. K. Ngsee.** 2008. FFAT rescues VAPA-mediated inhibition of ER-to-Golgi transport and VAPB-mediated ER aggregation. J. Cell Sci. **121:**3052–3061.
38. **Skehel, P. A., R. Fabian-Fine, and E. R. Kandel.** 2000. Mouse VAP33 is associated with the endoplasmic reticulum and microtubules. Proc. Natl. Acad. Sci. USA **97:**1101–1106.
39. **Skehel, P. A., K. C. Martin, E. R. Kandel, and D. Bartsch.** 1995. A VAMP-binding protein from *Aplysia* required for neurotransmitter release. Science **269:**1580–1583.
40. **Taguwa, S., T. Okamoto, T. Abe, Y. Mori, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2008. Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. J. Virol. **82:**2631–2641.
41. **Tellinghuisen, T. L., J. Marcotrigiano, and C. M. Rice.** 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature **435:**374–379.
42. **Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica.** 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J. Virol. **67:**4017–4026.
43. **Tsuda, H., S. M. Han, Y. Yang, C. Tong, Y. Q. Lin, K. Mohan, C. Haueter, A. Zoghbi, Y. Harati, J. Kwan, M. A. Miller, and H. J. Bellen.** 2008. The amyotrophic lateral sclerosis 8 protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. Cell **133:**963–977.
44. **Tu, H., L. Gao, S. T. Shi, D. R. Taylor, T. Yang, A. K. Mircheff, Y. Wen, A. E. Gorbalenya, S. B. Hwang, and M. M. Lai.** 1999. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology **263:**30–41.

45. **Ujino, S., S. Yamaguchi, K. Shimotohno, and H. Takaku.** 2009. Heat-shock protein 90 is essential for stabilization of the hepatitis C virus non-structural protein NS3. J. Biol. Chem. **284:**6841–6846.
46. **Wang, C., M. Gale, Jr., B. C. Keller, H. Huang, M. S. Brown, J. L. Goldstein, and J. Ye.** 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol. Cell **18:**425–434.
47. **Wasley, A., and M. J. Alter.** 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. **20:**1–16.
48. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell **19:**111–122.
49. **Weir, M. L., A. Klip, and W. S. Trimble.** 1998. Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. Biochem. J. **333:**247–251.
50. **Weir, M. L., H. Xie, A. Klip, and W. S. Trimble.** 2001. VAP-A binds promiscuously to both v- and tSNAREs. Biochem. Biophys. Res. Commun. **286:**616–621.
51. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102:**9294–9299.

Involvement of PA28γ in the Propagation of Hepatitis C Virus

Kohji Moriishi,[1] Ikuo Shoji,[2] Yoshio Mori,[1] Ryosuke Suzuki,[3] Tetsuro Suzuki,[3] Chikako Kataoka,[1] and Yoshiharu Matsuura[1]

We have reported previously that the proteasome activator PA28γ participates not only in degradation of hepatitis C virus (HCV) core protein in the nucleus but also in the pathogenesis in transgenic mice expressing HCV core protein. However, the biological significance of PA28γ in the propagation of HCV has not been clarified. PA28γ is an activator of proteasome responsible for ubiquitin-independent degradation of substrates in the nucleus. In the present study, knockdown of PA28γ in cells preinfection or postinfection with the JFH-1 strain of HCV impaired viral particle production but exhibited no effect on viral RNA replication. The particle production of HCV in PA28γ knockdown cells was restored by the expression of an small interfering RNA (siRNA)-resistant PA28γ. Although viral proteins were detected in the cytoplasm of cells infected with HCV, suppression of PA28γ expression induced accumulation of HCV core protein in the nucleus. HCV core protein was also degraded in the cytoplasm after ubiquitination by an E3 ubiquitin ligase, E6AP. Knockdown of PA28γ enhanced ubiquitination of core protein and impaired virus production, whereas that of E6AP reduced ubiquitination of core protein and enhanced virus production. Furthermore, virus production in the PA28γ knockdown cells was restored through knockdown of E6AP or expression of the siRNA-resistant wild-type but not mutant PA28γ incapable of activating proteasome activity. *Conclusion:* Our results suggest that PA28γ participates not only in the pathogenesis but also in the propagation of HCV by regulating the degradation of the core protein in both a ubiquitin-dependent and ubiquitin-independent manner. (HEPATOLOGY 2010;52:411-420)

Over 170 million individuals worldwide are infected with hepatitis C virus (HCV), which is a major etiological agent of liver diseases, including hepatic steatosis, cirrhosis, and hepatocellular carcinoma (HCC).[1] HCV is classified into the genus Hepacivirus of the *Flaviviridae* family and has a positive, single-strand RNA genome that encodes a single polyprotein consisting of about 3,000 amino acids.[2] The N-terminal one-third of the polyprotein is occupied by the structural proteins, and the remaining portion consists of nonstructural proteins involved in viral replication and assembly. Host and viral proteases cleave the appropriate sites of the polyprotein, resulting in generation of at least 10 viral proteins. The capsid (core), E1 and E2 proteins, and p7 are cleaved off by signal peptidase from the polyprotein. Furthermore, the C-terminal signal sequence of the core protein is processed by signal peptide peptidase.[3] Our recent data indicate that signal peptide peptidase cleaves the polyprotein between Phe^{177} and Leu^{178} in the signal sequence, and this processing is required for HCV propagation.[4] The mature core proteins make nucleocapsid with viral RNA, and HCV particles bud into the lumen of the endoplasmic reticulum bearing E1 and E2 glycoproteins on the host lipid components, and are released from the host cells.

Several reports suggest that HCV core protein plays an important role in the development of various outcomes of liver failure, including steatosis and HCC.[5,6]

Abbreviations: HA, hemagglutinin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; moi, multiplicity of infection; shRNA, short hairpin RNA; siRNA, small interfering RNA.

From the [1]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; the [2]Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo, Japan; and the [3]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Received February 3, 2010; accepted March 13, 2010.

Supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

Potential conflict of interest: Nothing to report.

Address reprint requests to: Yoshiharu Matsuura, D.V.M., Ph.D., Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: matsuura@biken.osaka-u.ac.jp; fax: (81)-6-6879-8269.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23680

Additional Supporting Information may be found in the online version of this article.

We have reported previously that HCV core protein specifically interacts with a proteasome activator PA28γ/REGγ in the nucleus and is digested by a PA28γ-dependent proteasome activity.[7] *In vivo* experiments in a mouse model suggest that PA28γ plays a critical role in the pathogenesis induced by HCV core protein.[8,9] PA28γ forms a homoheptamer in the nucleus and enhances the proteasome-mediated cleavage after basic amino acid residues, whereas PA28α and PA28β exhibit 41% and 34% homology to PA28γ, respectively, and form a heteroheptamer in the cytoplasm to activate cleavage after hydrophobic, acidic, or basic amino acid residues.[10] Recently, several groups reported that PA28γ interacts with steroid receptor coactivator-3 and cell cycle suppressors such as $p21^{WAF1/CIP1}$, $p16^{INK4A}$, and $p19^{ARF}$, and enhances the degradation of these proteins in a ubiquitin- and adenosine triphosphate–independent manner.[11-13] Furthermore, other mechanisms of ubiquitin-independent degradation have been considered for cell cycle regulation, summarized in the review of Jariel -Encontre et al.[14] However, the precise physiological functions of PA28γ are largely unknown *in vivo*, because PA28γ-knockout mice exhibit only mild growth retardation and live approximately as long as their control littermates.[15,16]

HCV core protein is degraded in a PA28γ-dependent and ubiquitin-independent manner in the nucleus,[7,17] while E6AP is also involved in the degradation of the core protein in a ubiquitin-dependent manner.[17,18] E6AP is a member of E3 ligases, which catalyze ubiquitin ligation of host and foreign proteins. Knockdown of E6AP suppressed degradation of HCV core protein and enhanced the release of infectious particles, suggesting that E6AP negatively regulates HCV propagation.[18] However, the role of PA28γ in the propagation of HCV has not yet been characterized. In this study, we examined the biological significance of PA28γ in the propagation of HCV.

Materials and Methods

Transfection, Immunoblotting, and RNA Interference. Plasmid DNA was transfected into Huh7OK1 cells by way of liposome-mediated transfection using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA). Expression of HCV core protein was determined by way of enzyme-linked immunosorbent assay as described.[19] Immunoblotting was performed as described.[8] The small interfering RNAs (siRNAs) targeted to the PA28γ gene were purchased from Ambion (Austin, TX) and were introduced into the cell lines using Lipofectamine RNAiMax (Invitrogen). siRNAs with the Ambion siRNA ID numbers 138669 and 138670 were designated as siPA28γ1 and siPA28γ2, respectively. Antibodies and plasmids are described in the Supporting Information.

Cell Lines and Virus Infection. All cell lines were cultured at 37°C under the conditions of humidified atmosphere and 5% CO_2. The human hepatoma cell line Huh7OK1 and derivative cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. The Huh7-derived cell line harboring a subgenomic or a full-length HCV replicon RNA[20] was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and 1 mg/mL G418 (Nakarai Tesque, Kyoto, Japan). Huh7OK1 cells were transfected with pSilencer-shPA28γ4 or a control plasmid, pSilencer 2.1 U6 hygro negative control (Ambion), and drug-resistant clones were selected by treatment with hygromycin (Wako, Tokyo, Japan) at a final concentration of 100 μg/mL. Huh7OK1 cells transfected with the control plasmid were selected with puromycin and designated as shCntrl, whereas those transfected with pSilencer-shPA28γ4 were established by limited dilution,[8] and two of the resulting cell lines were designated as KD5 and KD7. Plasmids encoding wild-type or mutant PA28γ complementary DNAs resistant to siRNA against PA28γ were prepared by using the silent mutations as reported.[8] These plasmids were transfected into Huh7OK1 cells and cultivated in medium containing 0.1 μg/mL of puromycin for 2 days. The surviving cells were used for virus infection. The shCtrl and KD5 cells were transformed with pSilencer shE6AP or pSilencer 3.1 H1 puro negative control (Ambion) and treated with 0.1 μg/mL of puromycin for 2 days. The surviving cells were infected with JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al.[21] The infectivity of JFH1 strain was determined using a focus-forming assay[21] and is expressed in focus-forming units. The Huh7 cell line harboring subgenomic replicon RNA of the Con1 or JFH1 strain was prepared according to the method of Pietschmann et al.[22] The infectivity of the Japanese encephalitis virus (JEV) was determined by an immunostaining focus assay as described[23] and is expressed in focus-forming units. Colony formation and replication assays, quantitative

reverse-transcription polymerase chain reaction, and estimation of cell growth was performed as described in the Supporting Information.

Immunofluorescent Staining. Huh7OK1-derived cells were seeded at 0.5×10^4 cells/well in an eight-well chamber slide, infected with JFH-1 virus at an moi of 0.3 after incubation at 37°C for 24 hours, stained with Bodipy 558/568 C_{12} according to the method of Targett-Adams et al.[24] at 4 days postinfection, and then fixed at 4°C for 30 mintues with 4% paraformaldehyde in phosphate-buffered saline. After treatment of cells with 1 μg/mL of RNase A, nuclei were stained with 50 μM Heachest 33258. The fixed cells were permeabilized with 20 mM Tris-HCl containing 1% Nonidet P-40 and 135 mM NaCl at room temperature for 5 minutes, reacted with rabbit anti-core or anti-NS5A antibody followed by Alexa Fluor 488-goat antibody to rabbit immunoglobulin G, washed three times with phosphate-buffered saline, and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using Image-Pro software (Media Cybernetics). The percentage of the nuclear core protein to the total core protein was examined randomly in 10 fields of every three wells. The percentage of the nuclear NS5A to total NS5A was estimated by the same method as the ratio of the core protein.

Results

Transient Knockdown of PA28γ Prior to or After Infection With HCV Reduces Particle Production. We reported previously that Huh7OK1 cells are as permissive to JFH-1 virus infection as Huh7.5.1 cells.[25] The Huh-7OK1 cell line retained the ability to produce type I IFNs through the RIG-I–dependent signaling pathway upon infection with RNA viruses and exhibited a cell surface expression level of human CD81 comparable to that of the parental cell line. However, the mechanism through which the Huh7 OK1 cell line exhibits highly permissive to JFH-1 virus infection has not been clarified yet. Two siRNAs were used to knock down PA28γ, but only one, siPA28γ1, was used because the other had off-target effects (Supporting Fig. 1). To examine the effect of PA28γ on the propagation of HCV, siPA28γ1 was introduced into Huh7OK1 cells 24 hours before infection. The levels of viral RNA, core protein, and infectious viral titer were determined at 48 and 96 hours postinfection. Viral RNA in the culture supernatant and cells was clearly reduced by the knockdown of PA28γ at 48 and 96 hours postinfection, respectively (Fig. 1A), whereas a significant reduction of core protein expression was detected at 96 hours but not at 48

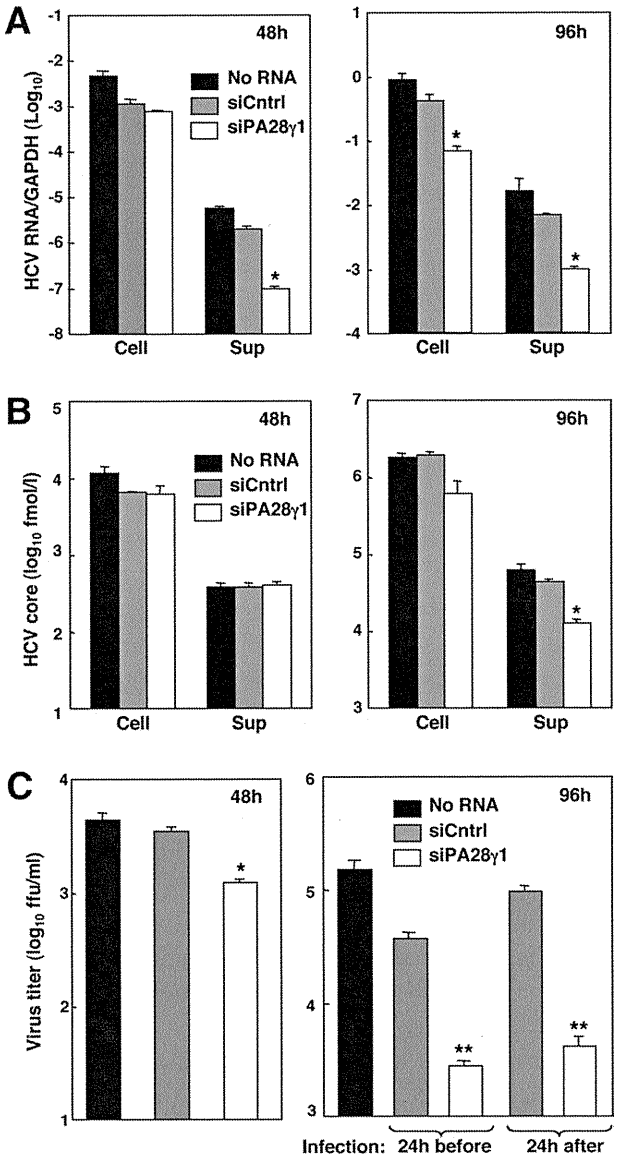

Fig. 1. Transient knockdown of PA28γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (right panel). The quantity of HCV RNA in cells and supernatants was determined by way of quantitative reverse-transcription polymerase chain reaction. (B) The expression of HCV core protein in cells and supernatants at 48 hours (left panel) and 96 hours (right panel) postinfection was determined by ELISA. (C) Huh7OK1 cells that were transfected with siCntrl or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection. The infectivity of the virus in the culture supernatant was determined by a focus-forming assay at 48 hours postinfection (left panel). Those transfected with the siRNAs at 24 hours before and after infection with JFH-1 virus were determined similarly at 96 hours postinfection (right panel). $*P < 0.05$, $**P < 0.01$ versus control siRNA-transfected cells. Data are representative of three independent experiments.

hours postinfection (Fig. 1B). Infectious viral titer in the culture supernatant was significantly reduced at 48 and 96 hours postinfection by the PA28γ knockdown (Fig. 1C), consistent with the suppression of the viral RNA in the supernatant. Furthermore, a comparable suppression of the production of infectious particles in the supernatant was also achieved by introducing siPA28γ1 into cells even at 24 hours postinfection (Fig. 1C, right panel). These results suggest that PA28γ participates in the regulation of HCV propagation in postentry steps.

Stable Knockdown of PA28γ Impairs Viral Propagation. To establish the PA28γ knockdown cell lines, Huh7OK1 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to PA28γ and selected with hygromycin, resulting in two clones—KD5 and KD7—that exhibited a clear reduction of PA28γ expression (Fig. 2A). Although the suppression of PA28γ expression in KD7 cells was slightly more efficient than that in KD5 cells, the growth of KD7 cells was impaired (Fig. 2B). Viral production in the culture supernatants in cells infected with the JFH-1 virus was significantly impaired in PA28γ knockdown KD5 cells compared with control cells (Fig. 2C). The viral RNA and core protein in the supernatant were also reduced in KD5 cells (Fig. 2D). Expression of siRNA-resistant PA28γ in PA28γ knockdown KD5 and KD7 cells recovered virus production in the supernatant to a level similar to that in the control cells transfected with an empty vector, and overexpression of siRNA-resistant PA28γ in control cells slightly enhanced virus production (Fig. 2E). Our previous data suggest that capsid protein of JEV does not bind to PA28γ.[7] To examine whether PA28γ regulates JEV propagation, KD5 and shCntrl cells were infected with JEV at an moi of 0.5. The infectivity of JEV in KD5 cells was similar to that in shCntrl cells (Fig. 2F), suggesting that PA28γ does not participate in the virus production pathway of JEV. These results further support the notion that PA28γ participates in HCV propagation.

Knockdown of PA28γ Exhibits No Effect on Viral RNA Replication. Although knockdown of PA28γ resulted in the suppression of viral particle and RNA production in the culture supernatant at 48 hours postinfection with JHF-1 virus, viral RNA in the cells was not reduced (Fig. 1), suggesting that PA28γ does not participate in viral replication. To gain more insight on this point, we examined the effect of PA28γ knockdown on RNA replication in replicon cells. Transient knockdown of PA28γ through introduction of siPA28γ into the subgenomic HCV replicon cells derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28γ

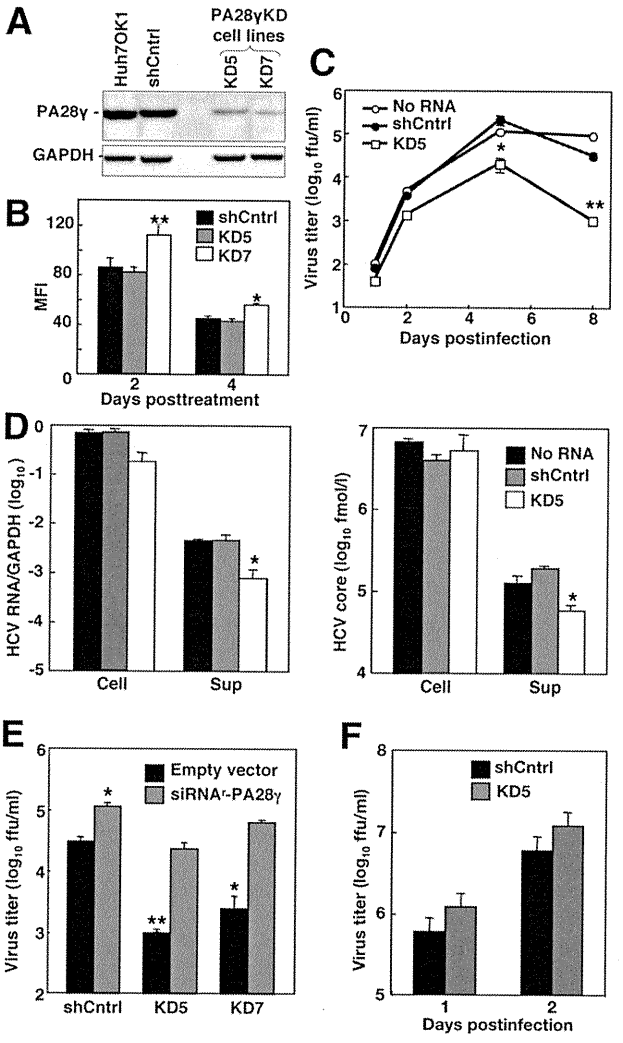

Fig. 2. Establishment of PA28γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, and PA28γ knockdown was confirmed by way of immunoblotting. (B) Growth of the cell lines was determined by staining with carboxyfluorescein succinimidyl ester. (C,D) KD5 and shCntrl cell lines were infected with the JFH-1virus at an moi of 0.05. The infectious virus titers in the culture supernatants (C) was determined by way of focus-forming assay. The virus RNA (D, left panel) and the core protein (D, right panel) in both cell and the supernatant were determined at 5 days postinfection by way of ELISA and quantitative reverse-transcription polymerase chain reaction, respectively. (E) The plasmid encoding an siRNA-resistant PA28γ or empty vector was transfected into the cell lines, seeded at 5×10^4 cells into a six-well plate after cultivation in the presence of puromycin for 2 days, and infected with JFH-1 virus at an moi of 0.05. The viral titers were determined at 5 days postinfection. $^*P < 0.05$, $^{**}P < 0.01$ versus shCntrl cells transfected with an empty vector. (F). KD5 and shCntrl cell lines were infected with the JEV virus at an moi of 0.5. The infectivity of JEV in the supernatant was determined at 1 and 2 days postinfection. Data are representative of three independent experiments.

knockdown cell line KD5 and the control cell line transfected with the subgenomic replicon RNA (WT) were gradually increased until 4 days posttransfection, whereas luciferase activities in the same two cell lines transfected with the polymerase-dead replicon RNA (GND) were decreased in a time-dependent manner (Fig. 3B). Next, to explore the effect of PA28γ knockdown on the viral replication over a longer period, replicon RNA encoding the neomycin-resistance gene was transfected into the cell lines for a colony formation assay. The numbers of colonies in the KD5 cell line after 4 weeks of selection with G418 were similar to those in the control cell line (Fig. 3C). To further clarify the roles of PA28γ on the postreplication steps, *in vitro* transcribed full-length viral RNA was transfected into Huh7OK1 cells, and siPA28γ1 was then introduced into the cells at 24 hours posttransfection of viral RNA. Intracellular core protein was increased in a time-dependent manner, but no significant difference was observed between cells transfected with control siRNA and those transfected with siPA28γ1 (Fig. 3D, left panel). However, infectious virus titers in the supernatant were significantly decreased by the transient and stable knockdown of PA28γ compared with control cells (Fig. 3D, middle and right panels). Furthermore, PA28γ did not contribute to the virus production of JEV (Fig. 2F), suggesting that the general sorting pathway of the flavivirus is functional under

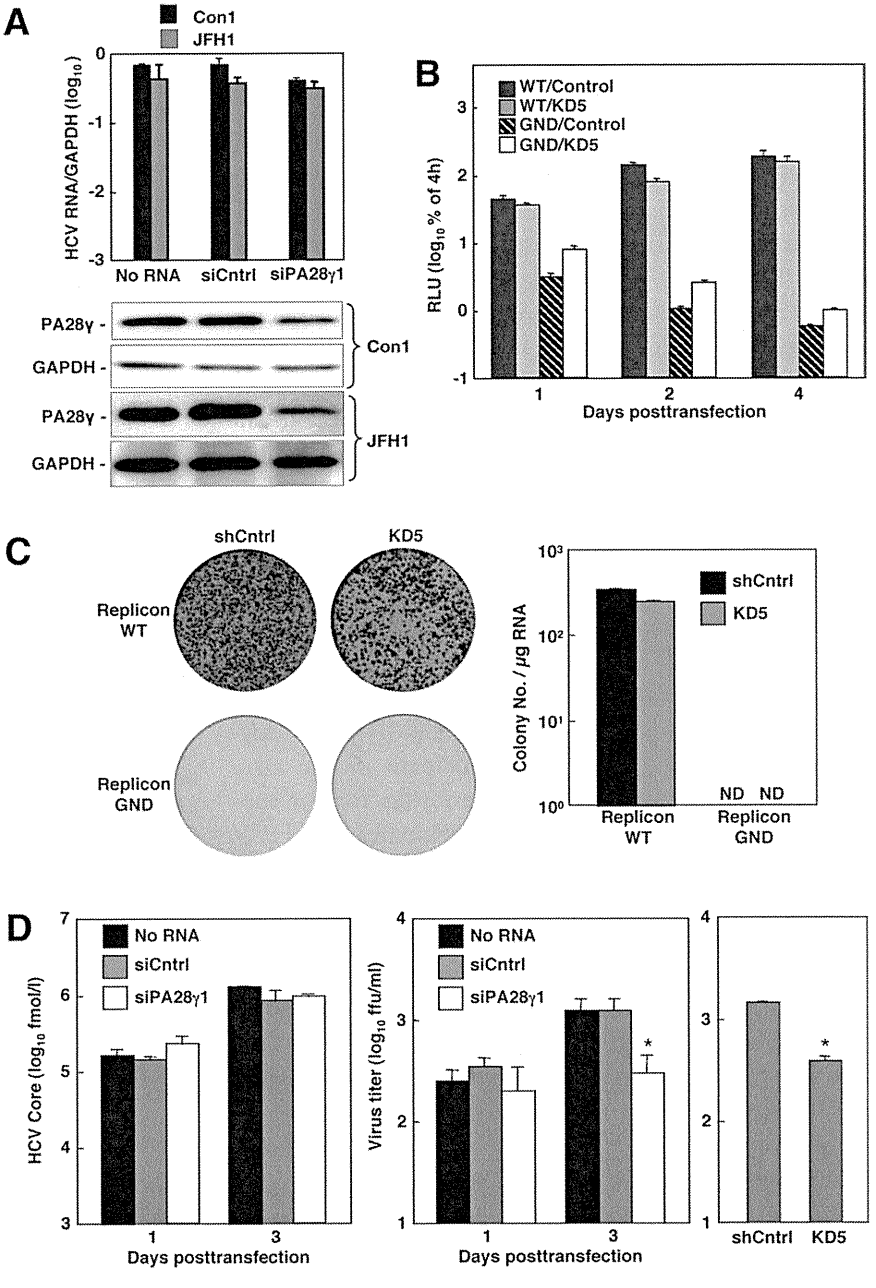

Fig. 3. Effect of PA28γ knockdown on HCV RNA replication. (A) The siCntrl or siPA28γ1 (10 nM) was transfected into the subgenomic HCV replicon cells derived from Con1 and JFH-1 strains. The transfected cells were harvested at 72 hours posttransfection. The replicon RNA was determined by quantitative reverse-transcription polymerase chain reaction at 72 hours posttransfection (upper). PA28γ or glyceraldehyde 3-phosphate dehydrogenase was detected by way of immunoblotting. Cell lysates were subjected to western blotting using antibodies to PA28γ and glyceraldehyde 3-phosphate dehydrogenase (lower). (B) The HCV replicon RNA encoding luciferase gene (WT) or the HCV replicon RNA that has a replication-deficient mutation (GND) was transfected into the shCntrl (Control) and KD5 cell lines. Relative luciferase activity was determined using the activity at 4 hours post-electroporation as a transfection efficiency. (C) Colony formation assay. Replicon RNA encoding the neomycin-resistance gene was transfected into the shCntrl and KD5 cell lines, and the remaining colonies were fixed with 4% paraformaldehyde at 4 weeks posttransfection and then stained with crystal violet. The number of colonies was counted (right). (D) Huh7OK1 cells transfected with 10 μg of *in vitro*-transcribed full-length JFH-1 viral RNA were further transfected with siCntrl or siPA28γ1 at 24 hours posttransfection of viral RNA. The level of HCV core protein in the cells was determined by way of ELISA at 1 and 3 days posttransfection (left). Infectious virus titers in the culture supernatants at 1 and 3 days posttransfection were determined by way of focus-forming assay (middle). Infectious viral titers in the shCntrl or KD5 cells transfected with 10 μg of the infectious viral RNA were determined at 5 days posttransfection (right). $^*P < 0.05$, $^{**}P < 0.01$ versus the control cells or cells transfected with siCntrl. Data are representative of three independent experiments.

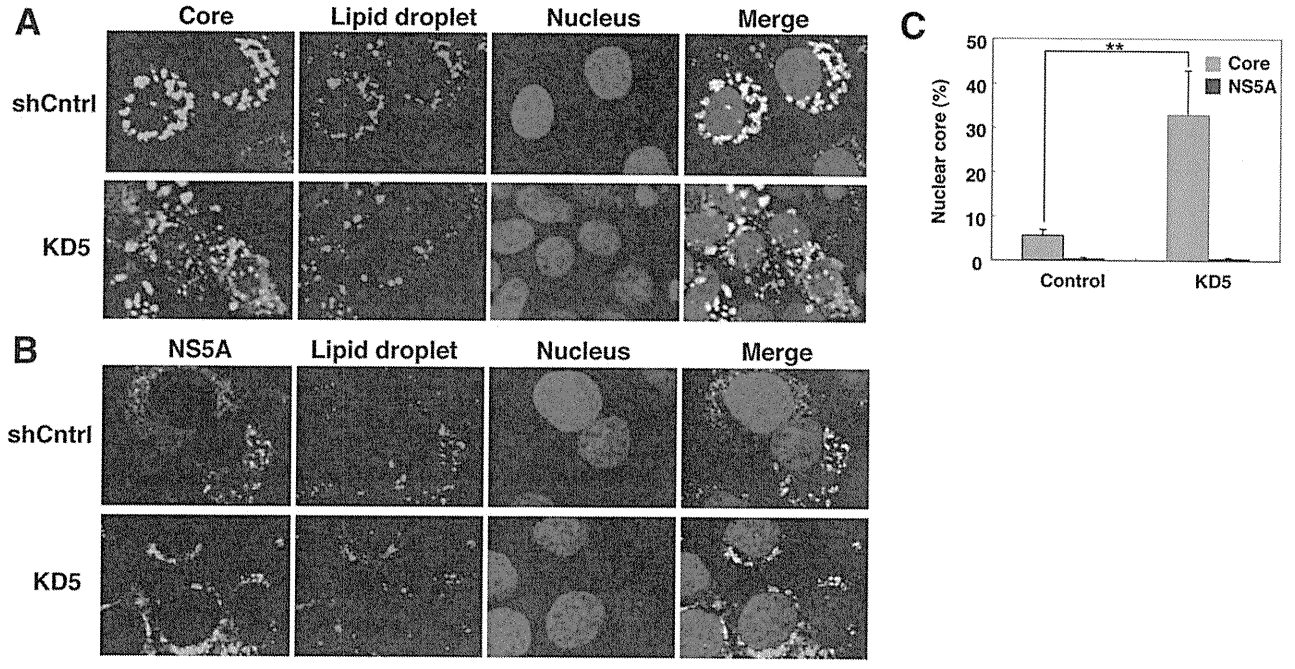

Fig. 4. Effect of PA28γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies raised against the proteins and Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G antibody. Lipid droplets were stained with Bodipy 558/568 C12. Nuclei were stained with 50 μM Heachest 33258 after treatment with 1 μg/mL of RNase A. Data are representative of three independent experiments. (C) The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using the method described in Materials and Methods. The percentage of the nuclear NS5A to total NS5A was estimated by the same way as the ratio of the core protein. $^{**}P < 0.01$ versus control siRNA-transfected cells.

the PA28γ knockdown condition. These results suggest that PA28γ specifically regulates the postreplication steps in the life cycle of HCV.

Core Protein Is Partially Accumulated in the Nucleus of PA28γ Knockdown Cells. We reported previously that some fraction of HCV core protein migrates into the nucleus and is then degraded by a PA28γ-dependent proteasome pathway.[7] Furthermore, we have demonstrated that HCV core protein is clearly accumulated in the nucleus of the liver cells of PA28γ-knockout mice.[8] However, the role of PA28γ on the intracellular localization of HCV core protein in the infected HCV cells has not been characterized. HCV core protein was chiefly detected in cytoplasm of the control cell line infected with the JFH-1 virus, where it appeared around lipid droplets after staining with Bodipy 558/568 C12 (Fig. 4A, upper panels). In contrast, the core protein was detected not only in the cytoplasm around the surface of lipid droplets, but also in the nucleus in the KD5 cell line (Fig. 4A, lower panels). The NS5A protein was detected in the cytoplasm but not in the nucleus in both the shCntrl and KD5 cell lines (Fig. 4B). The percentage occupied by nuclear core protein to total core protein was increased by about six time levels in the KD5, while the ratio of nuclear NS5A to total NS5A exhibited no difference (Fig. 4C). These results suggest that PA28γ participates in the degradation of HCV core protein in the nucleus.

PA28γ Positively Regulates HCV Propagation by Inhibiting Ubiquitin-Dependent Degradation of Core Protein in Cytoplasm. We reported previously that HCV core protein is degraded by at least two distinct pathways: a ubiquitin-dependent proteasome pathway and a ubiquitin-independent proteasome pathway.[17] The ubiquitin E3 ligase, E6AP, can catalyze ubiquitin ligation of the core protein for ubiquitin-dependent degradation in the cytoplasm,[18] whereas PA28γ participates in the degradation of the core protein through a ubiquitin-independent pathway in the nucleus.[17] We have also demonstrated that PA28γ knockdown leads to enhanced ubiquitination of HCV core protein.[8] However, the interplay between these two pathways in cells infected with HCV has not been determined. To address this point, we examined the effects of knockdown of E6AP or PA28γ on the virus propagation and the ubiquitination of the core protein. JFH-1 virus was inoculated into E6AP- and/or PA28γ knockdown cell lines (Fig. 5A). Transfection of the plasmid encoding shRNA to E6AP into the control cells (shCntrl) increased virus production (Fig. 5A [C-E]) in comparison with that of the

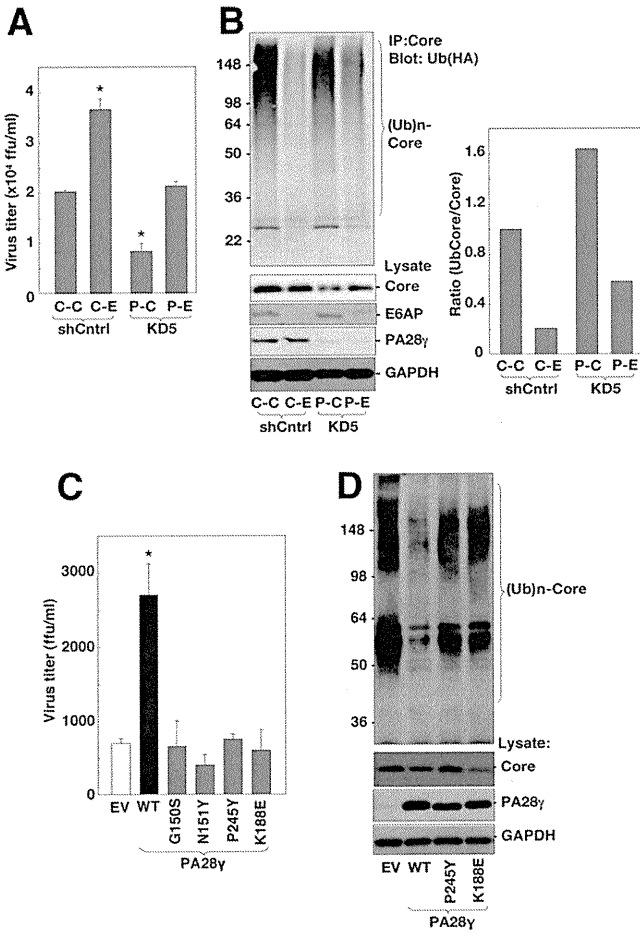

Fig. 5. PA28γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 cells in a 24-well plate were infected with the JFH-1 virus at an moi of 0.05, and infectious virus titers in the supernatants were determined at 72 hours postinfection by way of focus-forming assay. (B) The cells transfected and infected as in (A) were further transfected with a plasmid encoding HA-tagged ubiquitin at 48 hours postinfection. The cells were treated with 10 μM MG132 for 5 hours at 72 hours postinfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. The ratio of ubiquitination of HCV core protein was assessed by the densitometries of the ubiquitinated and unubiquitinated core proteins. (C) KD5 cells transfected with plasmids encoding wild-type or mutant PA28γ were infected with the JFH-1 virus at an moi of 0.05 at 24 hours posttransfection, and the infectious titers in the supernatant were determined at 72 hours postinfection by way of focus-forming assay. (D) KD5 cells transfected with plasmids encoding HCV core protein and HA-tagged ubiquitin, together with wild-type or mutant PA28γ, were treated with 10 μM MG132 for 5 hours at 24 hours posttransfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. EV, empty vector; WT, plasmid encoding wild-type PA28γ. $^*P < 0.05$ versus shCntrl or KD5 cells transfected with the negative control or empty vector. Data are representative of three independent experiments.

control cells transfected with the plasmid encoding control shRNA (Fig. 5A [C-C]). Furthermore, the impaired virus production in the PA28γ knockdown cells (KD5) was restored by the transfection of the plasmid encoding shRNA to E6AP (Fig. 5A [P-E]). Cells expressing hemagglutinin (HA)-tagged ubiquitin infected with the JFH-1 virus were immunoprecipitated by the anti-core antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-HA antibody (Fig. 5B). E6AP knockdown decreased the ratio of ubiquitination of HCV core protein, in contrast to the increase of that by PA28γ knockdown (Fig. 5B, lanes C-E and P-C). Furthermore, E6AP knockdown in the PA28γ knockdown cells restored the ubiquitination of the core protein to a certain extent (Fig. 5B, lane P-E). It was shown that Pro^{245} of PA28γ is critical for binding to the 20S proteasome, and that Gly^{150} and Asn^{151} of PA28γ are important for activation of the proteasome.[26] To further examine the functional significance of PA28γ on HCV propagation, expression plasmids encoding siRNA-resistant PA28γ mutants in which Gly^{150}, Asn^{151}, and Pro^{245} were replaced with Ser (G150S), Tyr (N151Y), and Tyr (P245Y), respectively, were transfected into KD5 cells and inoculated with JFH-1 virus at 24 hours posttransfection. The infectious virus titers in the culture supernatant were determined at 3 days postinfection (Fig. 5C). KD5 cells transfected with the plasmid encoding wild-type PA28γ exhibited a partial recovery of virus production, although those transfected with the plasmid encoding PA28γ G150S, N151Y, or P245Y or with an empty vector exhibited no effect on virus production. Replacing Lys^{188} with Glu in PA28γ (PA28γ K188E) confers the capability of proteasome-mediated cleavage after hydrophobic, acidic, and basic residues such as those exhibited by PA28α.[27] Expression of siRNA-resistant PA28γ K188E in KD5 cells could not restore virus production (Fig. 5D). The ubiquitination of HCV core protein was inhibited by expression of the wild-type PA28γ but not expression of the PA28γ mutants (P245Y or K188E) in KD5 cells (Fig. 5D). Collectively, these results suggest that PA28γ positively regulates HCV propagation by inhibiting degradation of HCV core protein by an E6AP/ubiquitin-dependent proteasome.

Discussion

To explore the role of PA28γ on the life cycle of HCV, we examined the effects of knockdown of PA28γ in Huh7OK1 cells infected with the JFH-1 virus. Knockdown of PA28γ in Huh7OK1 cells before or after infection with the JFH-1 virus impaired

production of infectious particles but did not impair viral RNA replication. However, PA28γ knockdown did not affect the production of JEV, of which the capsid protein does not interact with PA28γ, suggesting that PA28γ knockdown does not affect the general sorting pathway of flavivirus. These results suggest that PA28γ is specifically involved in the postreplication steps of HCV life cycle. Our previous report indicated that HCV core protein was accumulated in the nucleus of the hepatocytes of HCV core transgenic/PA28γ knockout mice.[8] PA28γ is located mainly in the nucleus, although a small portion is also located in the cytoplasm[7,28] and can up-regulate trypsin-like proteasome activity, which cleaves after basic amino acid residues.[27] Previous studies have shown that some fraction of HCV core protein is translocated into the nucleus and quickly degraded in the PA28γ-dependent proteasome pathway.[7,8,29] Miyanari et al.[30] demonstrated that the core protein is localized on the surface of lipid droplets and is surrounded by nonstructural proteins, suggesting that HCV particles are assembled near the surface of the lipid droplets. In the present experiments, although HCV core protein was detected on the surface of the lipid droplets in both control and PA28γ knockdown cell lines, it was partially localized in the nucleus in PA28γ knockdown cells but not control cells. Furthermore, localization of HCV core protein on the surface of lipid droplets was impaired in PA28γ knockdown cells (Fig. 4). These results suggest that HCV core protein is partially translocated into the nucleus and degraded in the PA28γ-dependent proteasome pathway in HCV-infected cells and that PA28γ does not directly participate in the particle formation of HCV.

HCV core protein is degraded by at least two proteasome pathways: a ubiquitin-dependent pathway and a ubiquitin-independent and PA28γ-dependent pathway.[17] The E3 ligase E6AP catalyzes ubiquitin ligation to HCV core protein, resulting in enhanced degradation of the core protein in the cytoplasm.[18] Knockdown of E6AP up-regulated virus production in cells infected with the JFH-1 virus,[18] suggesting that E6AP/ubiquitin-dependent degradation of the core protein contributes to an antiviral response. In contrast, knockdown of PA28γ induced up-regulation of the ubiquitination of HCV core protein and down-regulation of the viral production, suggesting that PA28γ-dependent proteasome activity contributes to the proviral response by suppressing E6AP-dependent degradation of the core protein, thereby enhancing viral particle formation. The wild-type PA28γ enhances the trypsin-like activity of proteasome that cleaves peptide bonds after basic residues of the substrates, whereas the PA28γ K188E mutant enhances the proteasome activity that cleaves peptide bonds after hydrophobic, acidic, and basic residues in the manner of PA28α.[27] Therefore, the sizes of fragments produced by the PA28γ-dependent proteasome should be different from those produced by the PA28α/β- or ubiquitination-mediated proteasome. It might be feasible to speculate that the peptide fragments of HCV core protein generated by the PA28γ-dependent proteasome or PA28γ *per se* may be directly or indirectly involved in the suppression of the E6AP-dependent ubiquitination of the core protein. Further studies will be needed to clarify the relationship between E6AP and PA28γ in the degradation and ubiquitination of HCV core protein. Figure 6 shows a schematic diagram of our hypothesis of the regulation of HCV propagation by PA28γ.

HCV core protein was found in not only nuclei but also cytoplasm of the infected KD5 cells (Fig. 4). The down-regulation of virus production should potently reduce a total amount of the core protein in KD5 cells before a clear accumulation of the core protein in nuclei. Furthermore, a small amount of PA28γ was found in the PA28γ knockdown cells, suggesting that E6AP-dependent degradation of HCV core protein is not potently suppressed in the PA28γ knockdown cells. If HCV core protein is constitutively expressed under the PA28γ knockout cells regardless of an amount of infected virus, a clear accumulation of the core protein in nuclei should be found without cytoplasmic expression of the core protein under the PA28γ knockout condition. We reported previously that HCC and liver steatosis in mouse are induced by the HCV core protein in the presence, but not the absence, of PA28γ.[8] Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of $PA28\gamma^{+/+}$ mice,[8,31] HCV core protein was clearly accumulated in the nuclei, but clearly reduced in cytoplasm, of liver cells of $PA28\gamma^{-/-}$ mouse.[8] In addition, ubiquitination of HCV core protein was increased by PA28γ knockdown in the 293T cell line.[8] These results and the data in Fig. 5 suggest that the suppression of PA28γ function enhances the E6AP-dependent degradation of HCV core protein. Hence, the reason there is no difference between $PA28\gamma^{+/+}$ and $PA28\gamma^{-/-}$ mice with respect to the amount of core protein may be due to the competitive regulation of the core protein by E6AP- and PA28γ-dependent degradation mechanisms. E6AP-dependent degradation of HCV core protein in cytoplasm may be enhanced *in vivo* under the PA28γ knockout condition.

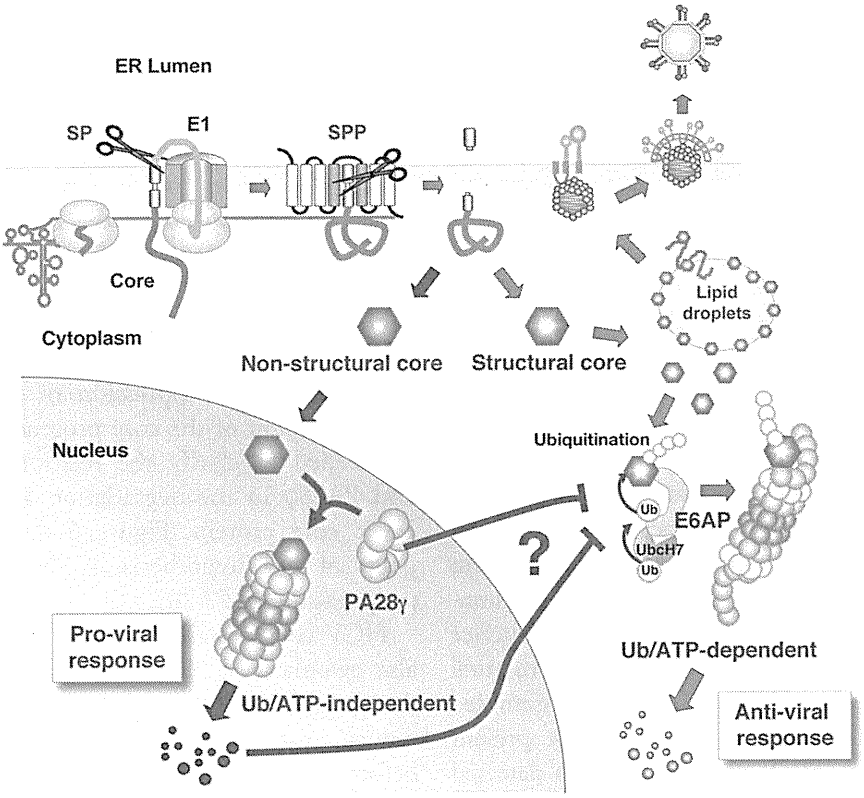

Fig. 6. Schematic diagram of the potential roles of PA28γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid droplets close to the endoplasmic reticulum to form a nucleocapsid with the viral RNA genome and is incorporated into virus particles as a structural protein. In addition to the structural protein of HCV, the core protein has characteristics of a nonstructural protein. HCV core protein is degraded through ubiquitin-dependent and ubiquitin-independent proteasome pathways. E6AP catalyzes ubiquitin ligation to HCV core protein and promotes degradation in the cytoplasm, which contributes to the antiviral response. In contrast, the core protein partially migrates into the nucleus and is degraded through a ubiquitin-independent and PA28γ-dependent proteasome pathway, and the core protein fragments generated by the PA28γ pathway or PA28γ *per se* were suggested to participate in the suppression of E6AP-dependent ubiquitination of HCV core protein, which contributes to the proviral response.

In conclusion, in this study we demonstrated that the proteasome activator PA28γ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28γ plays a crucial role in the development of liver pathology induced by HCV core protein.[8] PA28γ knockout mice exhibit only mild growth retardation.[15,16] Therefore, PA28γ may be a novel and promising antiviral target not only for elimination of HCV from hepatitis C patients but also for intervention in the progression of liver diseases induced by chronic HCV infection.

Acknowledgment: We thank H. Murase for her secretarial work. We also thank R. Bartenschlager and T. Wakita for providing cell lines and plasmids.

References

1. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000;20:1-16.
2. Moriishi K, Matsuura Y. Host factors involved in the replication of hepatitis C virus. Rev Med Virol 2007;17:343-354.
3. Hussy P, Langen H, Mous J, Jacobsen H. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology 1996;224:93-104.
4. Okamoto K, Mori Y, Komoda Y, Okamoto T, Okochi M, Takeda M, et al. Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. J Virol 2008;82:8349-8361.
5. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci U S A 1997;94:1200-1205.
6. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997;78:1527-1531.
7. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, et al. Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237-10249.
8. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A 2007;104:1661-1666.
9. Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, et al. Involvement of PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. J Virol 2007;81:1727-1735.

10. Li X, Lonard D, Jung SY, Malovannaya A, Feng Q, Qin J, et al. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. Cell 2006;124: 381-392.
11. Zhang Z, Zhang R. Proteasome activator PA28 gamma regulates p53 by enhancing its MDM2-mediated degradation. EMBO J 2008;27: 852-864.
12. Chen X, Barton LF, Chi Y, Clurman BE, Roberts JM. Ubiquitin-independent degradation of cell-cycle inhibitors by the REGgamma proteasome. Mol Cell 2007;26:843-852.
13. Li X, Amazit L, Long W, Lonard DM, Monaco JJ, O'Malley BW. Ubiquitin- and ATP-independent proteolytic turnover of p21 by the REGgamma-proteasome pathway. Mol Cell 2007;26:831-842.
14. Jariel-Encontre I, Bossis G, Piechaczyk M. Ubiquitin-independent degradation of proteins by the proteasome. Biochim Biophys Acta 2008; 1786:153-177.
15. Barton LF, Runnels HA, Schell TD, Cho Y, Gibbons R, Tevethia SS, et al. Immune defects in 28-kDa proteasome activator gamma-deficient mice. J Immunol 2004;172:3948-3954.
16. Murata S, Kawahara H, Tohma S, Yamamoto K, Kasahara M, Nabeshima Y, et al. Growth retardation in mice lacking the proteasome activator PA28gamma. J Biol Chem 1999;274:38211-38215.
17. Suzuki R, Moriishi K, Fukuda K, Shirakura M, Ishii K, Shoji I, et al. Proteasomal turnover of hepatitis C virus core protein is regulated by two distinct mechanisms: a ubiquitin-dependent mechanism and a ubiquitin-independent but PA28gamma-dependent mechanism. J Virol 2009;83:2389-2392.
18. Shirakura M, Murakami K, Ichimura T, Suzuki R, Shimoji T, Fukuda K, et al. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J Virol 2007;81:1174-1185.
19. Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, et al. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 1999;37:1802-1808.
20. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
21. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
22. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002;76:4008-4021.
23. Mori Y, Okabayashi T, Yamashita T, Zhao Z, Wakita T, Yasui K, et al. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J Virol 2005;79:3448-3458.
24. Targett-Adams P, Chambers D, Gledhill S, Hope RG, Coy JF, Girod A, et al. Live cell analysis and targeting of the lipid droplet-binding adipocyte differentiation-related protein. J Biol Chem 2003;278: 15998-16007.
25. Okamoto T, Omori H, Kaname Y, Abe T, Nishimura Y, Suzuki T, et al. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J Virol 2008;82: 3480-3489.
26. Zhang Z, Clawson A, Realini C, Jensen CC, Knowlton JR, Hill CP, et al. Identification of an activation region in the proteasome activator REGalpha. Proc Natl Acad Sci U S A 1998;95:2807-2811.
27. Li J, Gao X, Ortega J, Nazif T, Joss L, Bogyo M, et al. Lysine 188 substitutions convert the pattern of proteasome activation by REGgamma to that of REGs alpha and beta. EMBO J 2001;20:3359-3369.
28. Nikaido T, Shimada K, Nishida Y, Lee RS, Pardee AB, Nishizuka Y. Loss in transformed cells of cell cycle regulation of expression of a nuclear protein recognized by SLE patient antisera. Exp Cell Res 1989; 182:284-289.
29. Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, et al. Molecular determinants for subcellular localization of hepatitis C virus core protein. J Virol 2005;79:1271-1281.
30. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089-1097.
31. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.

Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules

Xiaoyu Wen[1☯], Takayuki Abe[1☯], Hiroshi Kukihara[1], Shuhei Taguwa[1], Yoshio Mori[1], Hideki Tani[1], Nobuyuki Kato[2], Tetsuro Suzuki[3], Masashi Tatsumi[4], Kohji Moriishi[1], Yoshiharu Matsuura[1]*

1 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, **2** Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, **3** Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan, **4** AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Abstract

To eliminate hepatitis C virus (HCV) from infected hepatocytes, we generated two therapeutic molecules specifically activated in cells infected with HCV. A dominant active mutant of interferon (IFN) regulatory factor 7 (IRF7) and a negative regulator of HCV replication, VAP-C (Vesicle-associated membrane protein-associated protein subtype C), were fused with the C-terminal region of IPS-1 (IFNβ promoter stimulator-1), which includes an HCV protease cleavage site that was modified to be localized on the ER membrane, and designated cIRF7 and cVAP-C, respectively. In cells expressing the HCV protease, cIRF7 was cleaved and the processed fragment was migrated into the nucleus, where it activated various IFN promoters, including promoters of IFNα6, IFNβ, and IFN stimulated response element. Activation of the IFN promoters and suppression of viral RNA replication were observed in the HCV replicon cells and in cells infected with the JFH1 strain of HCV (HCVcc) by expression of cIRF7. Suppression of viral RNA replication was observed even in the IFN-resistant replicon cells by the expression of cIRF7. Expression of the cVAP-C also resulted in suppression of HCV replication in both the replicon and HCVcc infected cells. These results suggest that delivery of the therapeutic molecules into the liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

Citation: Wen X, Abe T, Kukihara H, Taguwa S, Mori Y, et al. (2011) Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules. PLoS ONE 6(1): e15967. doi:10.1371/journal.pone.0015967

Editor: Paul Digard, University of Cambridge, United Kingdom

Received September 29, 2010; **Accepted** December 7, 2010; **Published** January 6, 2011

Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: matsuura@biken.osaka-u.ac.jp

☯ These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma [1,2]. Although the proportion of patients achieving a sustained virological response (SVR) has been increased by the recent used of combination therapy with pegylated-interferon-α (PEG-IFNα) and ribavirin (RBV), half of patients still exhibit no response to this therapy, suggesting that the IFN signaling pathway is modulated by HCV infection. In addition, various side effects have been reported in more than 20% of patients treated with this combination therapy [3].

HCV belongs to the family *Flaviviridae* and possesses a single positive-stranded RNA genome that encodes a single polyprotein composed of about 3,000 amino acids. The HCV polyprotein is processed into 10 viral proteins by host and viral proteases. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one third of the polyprotein, followed by nonstructural proteins. The NS2 protease cleaves its own carboxyl terminus and NS3 cleaves the downstream positions to produce NS4A, NS4B, NS5A and NS5B. Although laboratory strains of HCV propagating in cell culture (HCVcc) have been established based on the full-length genome of the genotype 2a JFH1 strain [4], establishment of a robust cell culture system capable of propagating serum-derived HCV from hepatitis C patients has not yet been achieved.

Type I IFN exhibits potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs) which encode proteins involved in the establishment of antiviral state in cells [5]. IFNs induce transcription of ISGs through activation of the Jak-STAT pathway [6]. Binding of type I IFN to the IFN receptor induces phosphorylation of the receptor-associated tyrosine kinases, Jak1 and Tyk2, and then these kinases activate STAT1 and STAT2. The phosphorylated STATs migrate into the nucleus and activate ISG promoters through binding to the specific responsible elements. HCV infection has been suggested to impair the IFN production through multiple pathways. The IFN-induced Jak-STAT signaling is inhibited in cells and transgenic mice expressing HCV proteins and in the liver biopsy samples of chronic hepatitis C patients [7–9].

Induction of type I IFN upon infection with pathogens is crucial for innate immunity, and it is mediated by the activation of pattern-recognition receptors, including Toll-like receptors (TLRs) and cytosolic receptors, such as RIG-I and MDA5 [10–12]. The induction of type I IFN is primarily controlled at the gene transcriptional level, wherein a family of transcription factors known as IFN regulatory factors (IRFs) play a pivotal role. IRF3

and IRF7 are known to be essential for the RIG-I-, MDA5-, and TLR-mediated type I IFN production pathways. IRF3 is induced primarily by a response to initiate IFNβ production, whereas IRF7 is induced by IFNβ and participates in the late phase for IFNβ induction [13]. All TLRs, except for TLR3, activate the MyD88-dependent pathway, whereas TLR3 and TLR4 activate the TRIF-dependent pathway. HCV NS3/4A protease has been shown to impair the production of IFNβ as well as the subsequent IFN-inducible genes through the inactivation of the adaptor molecules involved in the TLR-dependent and -independent signaling pathways [14–18]. On the other hand, Vilasco *et al.* suggested that impairment of IKKi - which, along with TBK1, is one of the important factors participating in IRF3 phosphorylation and activation - in the HCV replicon cells plays at least a partial role in the restoration of type I IFN signaling pathways [19]. In addition, IRF7 was shown to participate in the positive feedback of type I IFN signaling through the IFN receptor [13]. Therefore, we tried to examine the effect of exogenous expression of IRF7 under the assumption that IRF7 is a potent type I IFN inducer and capable of modulating the viral propagation in hepatocytes infected with HCV.

In this study, we generated two therapeutic molecules consisting of a dominant active mutant of IRF7 or VAP-C, a negative regulator of HCV replication [20], followed by the C-terminal region of IFN promoter stimulator 1 (IPS-1), including the cleavage site of the HCV NS3/4A protease, which was modified so that the cleavage site localized on the ER membrane [21]. The expression of the plasmids encoding these molecules in the HCV replicon and HCVcc-infected cells resulted in a substantial suppression of HCV propagation, suggesting the possibility that these or other similar molecules could be used therapeutically to eliminate HCV from hepatocytes infected with HCV.

Results

IRF7m, a dominant active mutant of IRF7, activates the IFN promoters in cells replicating HCV

Previous studies have shown that an IRF7 mutant, IRF7m, lacking the amino acid residues from 284 to 454, a region that includes the auto-inhibitory domain (from amino acid residue 305 to 467), and an IRF3 mutant, IRF3m, carrying the substitution of Ser^{396} to Asp in the carboxyl terminal region (Fig. 1A), induced a potent activation of type I IFN promoter in non-hepatic cell lines irrespective of viral infection [22–25]. We first examined the effect of the expression of the IRF dominant active mutants on the inhibition of HCV RNA replication through the production of type I IFN. HCV replicon cells and Huh7OK1 cells infected with HCVcc were transfected with the plasmids encoding either wild-type or dominant active mutant of IRF3 or IRF7 together with the reporter plasmids encoding a luciferase gene under the control of the promoters of IFNα6, IFNβ and ISRE, respectively. Among these examined constructs, we observed significant activation of the promoters of IFNα6 and ISRE in the replicon and HCVcc-infected cells compared with naïve and mock-infected cells upon expression of IRF7m, while we observed no activation of the IFNα6 promoter in cells expressing IRF3m (Figs. 1 B and 1C). Potent stimulation of the IFNβ promoter was observed in the replicon cells expressing IRF7m but not in cells infected with HCVcc. Next we examined the antiviral activity of the IRF constructs in both replicon (Fig. 1D) and HCVcc-infected cells (Fig. 1E). The expression of the plasmid encoding IRF7m resulted in potent suppression of viral protein and viral RNA syntheses in both cell types. Although expression of IRF3m induced a slight suppression of viral propagation in cells infected with HCVcc, expression of the IRF constructs except for IRF7m did not induce the significant suppression of viral replication and propagation. These results suggest the possibility of elimination of HCV through a specific induction of type I IFN by the expression of IRF7m in HCV-infected cells.

cIRF7, a chimeric construct of IRF7m, specifically activates the IFN promoters in cells replicating HCV

To induce IFNs in cells infected with HCV but not in uninfected cells through a selective activation of IRF7m, we constructed a chimeric IRF7 (cIRF7) consisting of the IRF7m fused with FLAG-tag and the C-terminal amino acid residues from 503 to 540 of IPS-1 modified to be localized on ER (Fig. 2A upper) [21]. HCV NS3/4A protease cleaves the carboxyl site of Cys^{508} in the C-terminal domain of IPS-1. Although cIRF7 is anchored in the ER and exhibits no activation in uninfected cells, cIRF7 would be cleaved by the NS3/4A protease in cells infected with HCV and the released N-terminal fragment would migrate into the nucleus and activate various IFN promoters (Fig. 3). Immunoblot analyses revealed that cIRF7 was cleaved in 293T cells expressing HCV NS3/4A protease of a wild type but not in those expressing the mutant protease NS3/4A(S139A), and a mutant cIRF7(C508A) which has a substitution of Cys^{508} to Ala, exhibited resistance to the cleavage by the HCV protease (Fig. 2A bottom). To assess a specific activation of the IFN promoters after cleavage of the cIRF7 by HCV NS3/4A, 293T cells expressing FLAG-tagged HCV proteases were transfected with the plasmids encoding the luciferase gene under the control of the promoter of IFNα6, IFNβ or ISRE together with the plasmid encoding either cIRF7 or cIRF7(C508A). Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFNα6, IFNβ and ISRE promoters in cells expressing HCV NS3/4A protease but not in those expressing the mutant protease NS3/4A(S139A) (Fig. 2B). Next we examined the activation of the IFN promoters associated with the expression of the plasmid encoding cIRF7 in the replicon and HCVcc-infected cells. Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFN promoters in both cell types (Figs. 2C and 2D). On the other hand, these promoters were not activated by the expression of cIRF7 in the replicon cells harboring subgenomic RNA of Japanese encephalitis virus (JEV) and Huh7 cells infected with JEV (Fig. 2E). These results suggest that the cIRF7 expression is a feasible method for specifically activating the IFN promoters in cells infected with HCV.

Specificity of activation of the IFN promoters by the expression of cIRF7

To further examine the specificity of the activation of the IFN promoters by the expression of cIRF7 in cells replicating HCV, a plasmid encoding either cIRF7 or IRF7m was co-transfected with that encoding the luciferase gene under the ISRE promoter into the HCV replicon or HCVcc-infected cells and cultured in the presence or absence of inhibitors for HCV replication. Treatment with an HCV protease inhibitor (BILN2061) or cyclosporine A (CsA) inhibited the activation of the ISRE promoter by the expression of cIRF7 in the HCV replicon and HCVcc-infected cells in a dose-dependent manner, in contrast to the resistance to the treatments in cells expressing the IRF7m (Fig. 4A and Fig. 4B). Recently, it was shown that an NS3/4A protease of GB virus B (GBV-B), which is the virus genetically related most closely to HCV, also impairs the dsRNA-induced IFN production through a cleavage of IPS-1[26]. Therefore, to assess the possibility of activation of cIRF7 by other flaviviral proteases, cleavage of cIRF7

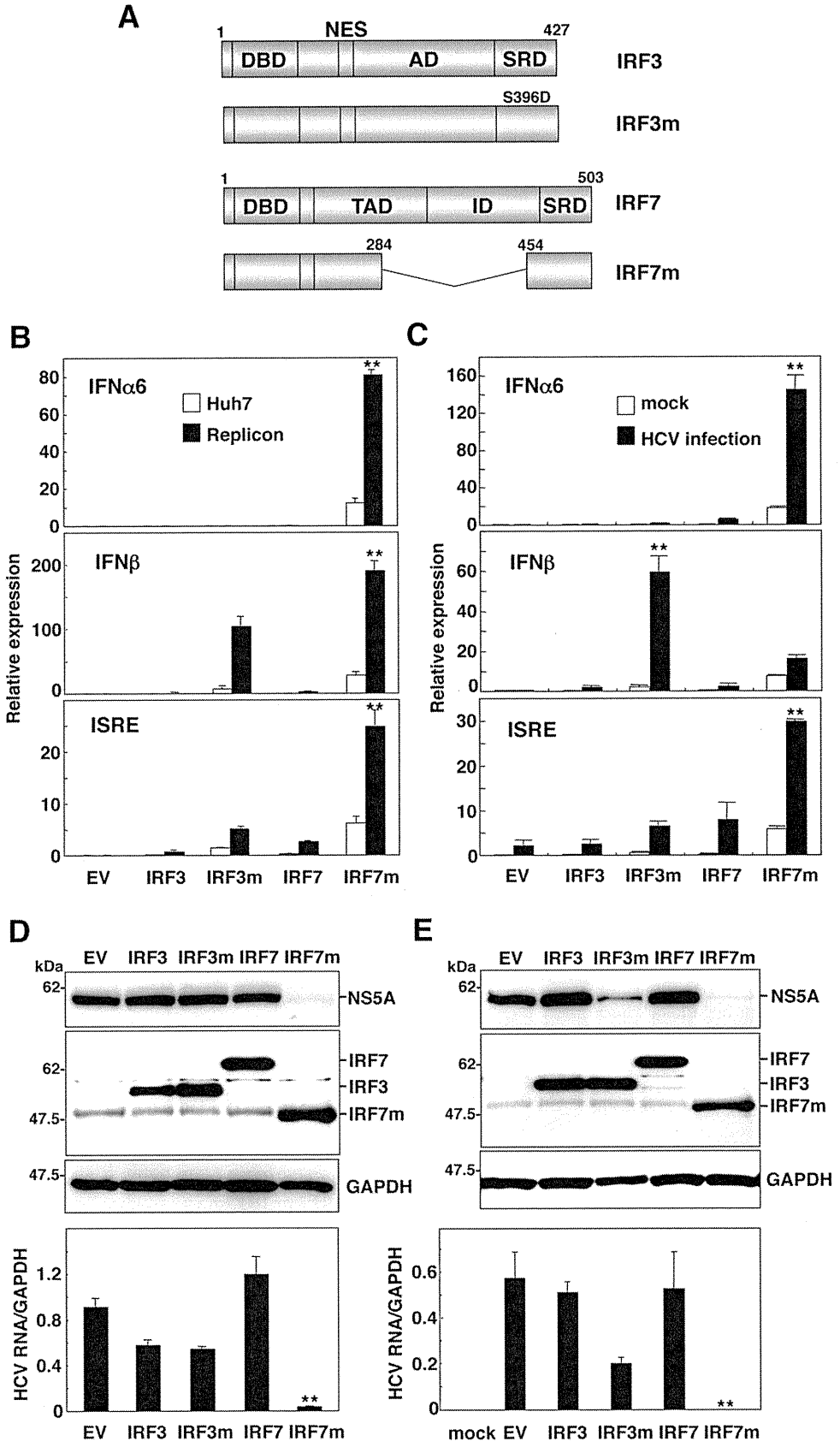

Figure 1. Dominant active mutant of IRF7 activates IFN promoters in cells replicating HCV. (A) Structures of IRF3, IRF7 and the dominant active mutants, IRF3m and IRF7m. The DNA-binding domain, nuclear export sequence, transactivation domain, association domain, inhibitory domain, and signal response domain are indicated as DBD, NES, TAD, AD, ID, and SRD, respectively. Huh7 cells and HCV replicon cells (1×10^5 cells/

well) (B), and Huh7OK1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (C) were transfected with 100 ng of plasmid encoding the luciferase gene under the control of the IFNα6, IFNβ, or ISRE promoter together with an empty vector (EV) or a plasmid encoding each of the IRF constructs. The relative luciferase activity of cell lysates was determined at 24 h post-transfection. HCV replicon cells (3×10^5 cells/well) (D) and Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (E) were transfected with EV or a plasmid encoding each of the IRF constructs and the expressions of NS5A, IRFs, and GAPDH (upper panel) and synthesis of viral RNA (lower panel) at 72 h post-transfection were determined by immunoblotting and real-time PCR after standardization with GAPDH, respectively. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (**$P<0.01$) versus the control cells or mock-infected cells.
doi:10.1371/journal.pone.0015967.g001

and activation of the IFN promoters were evaluated in 293T cells expressing the viral proteases of HCV, GBV and JEV. Immunoblot analyses revealed that cIRF7 was processed by the viral proteases of HCV and GBV but not by that of JEV and the activation of the IFN promoters was well correlated with the cleavability of the cIRF7 (Fig. 4C). Although the GBV protease exhibited an efficient activation of cIRF7 comparable to HCV protease, processing of cIRF7 and activation of the IFN promoters by the GBV protease was not inhibited by the pretreatment with the HCV protease inhibitor (Figs. 4D and 4E). These results indicate that cIRF7 is capable of activating the IFN promoters through a specific cleavage by the protease in cells infected with HCV.

Nuclear localization of cIRF7 in cells expressing HCV protease

From these results, it was suggested that cIRF7 is cleaved by the HCV protease and the processed fragment migrates into the nucleus and activates IFN promoters (Fig. 3). To confirm the nuclear localization of the cleaved cIRF7, we constructed an EGFP-cIRF7 and determined its subcellular localization in cells expressing the HCV protease and in the HCV replicon cells by confocal microscopy. Nuclear accumulation of the cIRF7 was observed in cells expressing EGFP-cIRF7 together with NS3/4A, but not in those with NS3/4A(S139A) or NS5A and also not in cells co-expressing EGFP-cIRF7(C508A) and NS3/4A (Fig. 5A). Furthermore, expression of EGFP-cIRF7 but not of EGFP-cIRF7(C508A) induced a nuclear accumulation of cIRF7 in the HCV replicon cells, and nuclear localization of the cIRF7 abrogated the expression of viral antigen (NS3), in contrast to the co-localization of EGFP-cIRF7(C508A) and the ER marker PDI, which had no discernible antiviral effect (Fig. 5B). These results suggest that cIRF7 is capable of suppressing HCV replication through an HCV protease-dependent cleavage, migration into the nucleus and activation of the IFN promoters.

Suppression of HCV replication by the expression of cIRF7

To examine the inhibitory effect of the expression of cIRF7 on HCV replication, a plasmid encoding either cIRF7 or cIRF7(C508A) was transfected into the HCV replicon and HCVcc-infected cells, and HCV replication was evaluated by immunoblotting and real-time PCR. The expression of cIRF7 but not of cIRF7(C508A) resulted in cleavage by the HCV protease, and a clear reduction of viral protein and RNA syntheses in both replicon and HCVcc-infected cells (Figs. 6A and 6B). In addition, we examined the effect of cIRF7 on the replication of HCV in the 4βR replicon cells [27,28], which have been shown to exhibit more resistant to the IFNα treatment than Con1 replicon cells (Fig. 6C upper left). Expression of the cIRF7 in the 4βR replicon cells but not in those cured HCV RNA (4βRc cells) induced an activation of the ISRE promoter (Fig. 6C lower left). Expression of cIRF7 but not of cIRF7(C508A) also resulted in processing by the HCV protease and suppression of viral protein and RNA syntheses in the 4βR replicon cells (Fig. 6C right panels).

Suppression of HCV replication by the expression of cVAP-C

Human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and B (VAP-B) are known to be involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response [29]. VAP-A and VAP-B have been shown to be involved in the replication of HCV, and we have shown recently that human VAP-C, a splicing variant of VAP-B, negatively regulates HCV replication by interfering with the interaction of VAP-A and VAP-B with HCV NS5B [20]. We next examined the possibility of using a selective activation of VAP-C to suppress HCV replication in cells infected with HCVcc. We generated expression plasmids encoding a chimeric VAP-C fused with the IPS-1 sequence (cVAP-C), a cVAP-C(C508A) which is made resistant to the HCV protease by a substitution in the cleavage site similar to the substitutions made in cIRF7(C508A), or VAP-C (Fig. 7A). The cVAP-C was cleaved in cells infected with HCVcc, and expression of cVAP-C and VAP-C suppressed expression of NS5A, in contrast to the weak reduction of NS5A in the infected cells expressing cVAP-C(C508A), probably due to a slight cleavage of cVAP-C(C508A) (Fig. 7B, top). Furthermore, the production of viral RNA and infectious particles in the culture supernatants of cells infected with HCVcc was also impaired by the expression of cVAP-C and VAP-C, but not of cVAP-C(C508A) in a dose-dependent manner (Fig. 7B, middle and bottom). Collectively, these results suggest that delivery of the therapeutic molecules into liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

Discussion

An effective prophylactic vaccine against HCV has not been developed yet. Although combination therapy consisting of PEG-IFNα and RBV has been introduced for the treatment of hepatitis C patients, and 50% of individuals infected with genotype 1 achieved a SVR, this treatment is sometimes associated with serious side effects, including depression and anemia [3]. Therefore, new anti-HCV drugs targeted to HCV protease and polymerase and capable of optimizing therapy are currently in the early stages of the development [30,31]. However, it is difficult to achieve a complete removal of viruses by antiviral drugs targeted to the viral enzymes from patients persistently infected with RNA viruses that exhibit a quasispecies nature, such as human immunodeficiency virus (HIV) and HCV. Viral quasispecies are not a simple collection of diverse mutants but a group of interactive variants capable of adapting to new environments [32]. Furthermore, introduction of antiviral drugs may induce an emergence of drug-resistant breakthrough viruses as seen in the case of HIV infection. Therefore, a novel therapeutic approach for hepatitis C patients in addition to the current chemotherapies is required to overcome serious adverse effects and improve the ratio of patients achieving SVR.

In this study, we have generated two therapeutic molecules, cIRF7 and cVAP-C, which are selectively activated in cells

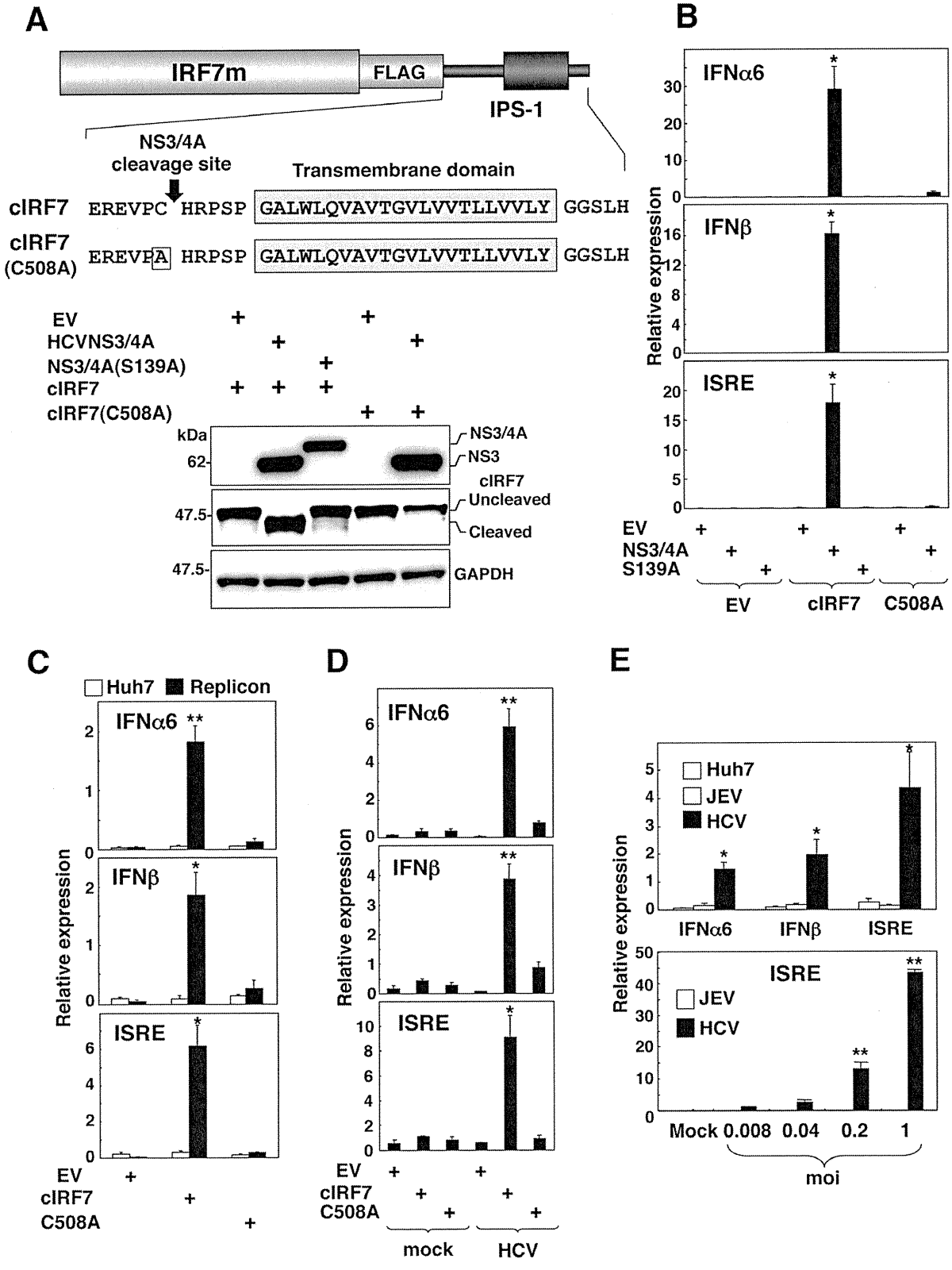

Figure 2. Construction of cIRF7 capable of activating the IFN promoters in cells replicating HCV. (A top) Schematic representation of the cIRF7 constructs. cIRF7 consists of IRF7m, FLAG-tag, and IPS-1 (503 to 540 amino acid residues) sequences containing a cleavage site by HCV NS3/4A protease, a transmembrane domain and a cytoplasmic region modified to localize on the ER. cIRF7(C508A) has a substitution of Cys508 to Ala which renders it resistant to the cleavage by the HCV protease. (A bottom) Immunoblot analyses of 293T cells transfected with a plasmid encoding either cIRF7 or cIRF7(C508A) together with either an empty vector (EV) or a plasmid encoding either FLAG-tagged HCVNS3/4A or FLAG-tagged HCVNS3/4A (S139A). (B) 293T cells (2×10^5 cells/well) were transfected with a plasmid of EV, FLAG-tagged HCVNS3/4A or FLAG-tagged HCVNS3/4A(S139A) in combination with a plasmid of EV, cIRF7 or cIRF7 (C508A) together with 100 ng of the reporter plasmid encoding the luciferase gene under the

control of the IFNα6, IFNβ or ISRE promoter, and luciferase activity was determined at 24 h post-transfection. (C) HCV replicon cells (1.5×10^5 cells/well) and (D) Huh7OK1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were transfected with 100 ng of each of the reporter plasmids together with plasmid of EV, cIRF7 or cIRF7(C508A) and luciferase activity was determined at 24 h post-transfection. (E) Huh7 cells, HCV subgenomic replicon cells, and JEV subgenomic replicon cells (1×10^5 cells/well) (top) and Huh7OK1 cells (7.5×10^4 cells/well) infected with JEV and HCV (bottom) at an moi of 0.008, 0.04, 0.2, and 1 and incubated for 24 h and 72 h, respectively, were transfected with 100 ng of each of the reporter plasmids together with cIRF7 and the luciferase activity was determined at 24 h post-transfection. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$) versus the control cells or mock-infected cells.
doi:10.1371/journal.pone.0015967.g002

replicating HCV. To tightly regulate activation of the molecules in HCV- infected cells, we employed the C-terminal amino acid sequence of human IPS-1, which has been identified as an adaptor molecule involved in the RIG-like receptor (RLR) signaling pathways. It has been demonstrated that HCV NS3/4A protease efficiently cleaves the upstream position of the transmembrane region of IPS-1 on the mitochondrial outer membrane and disrupts the IFN signaling pathway [15–18]. Furthermore, to avoid induction of mitochondrial dysfunction and cell death due to the expression of the therapeutic molecules on the mitochondria, we replaced three arginine residues among the C-terminal five residues of IPS-1 with non-charged amino acid glycine residues (RRRLH to GGGLH) so that these three residues would be localized on the ER membrane [21]. HCV is suggested to replicate on the ER membrane, and therefore subcellular localization and distance of the cleavage site of the substrates from the membrane could be crucial for an efficient processing. The tightly regulated activation of the therapeutic molecules in cells replicating HCV observed in this study might be largely attributable to the ER localization of the therapeutic molecules.

Irrespective of IFN sensitivity, the expression of cIRF7 in the HCV replicon cells induced the activation of type I IFN promoter and inhibited the viral RNA replication, suggesting the possibility that cIRF7 could be used for the treatment of hepatitis C patients who are infected with HCV resistant to IFNα therapy. The expression of IRF3m in cells infected with HCVcc induced a higher antiviral response than that in the Con1 replicon cells in spite of the comparable transcription of IFNβ mRNA between the two cell types Fig. 1), suggesting that differences among HCV genotypes might be caused to the difference to the sensitivity of IFNβ. To assess the real efficacy of cIRF7 for suppression of HCV replication, we must await the establishment of robust cell culture systems capable of propagating various genotypes of HCV derived from the sera of hepatitis C patients.

It has been shown previously that HCV interferes with the induction of type I IFN through the cleavage of IPS-1 by NS3/4A protease [15–18], the interaction of NS5A with MyD88, a major adaptor molecule of TLRs [33], and the intervention of the IFNα-activated Jak-STAT signaling pathway by HCV proteins [7–9]. After cleavage by the HCV protease, the processed cIRF7 migrates into the nucleus and activates various IFN promoters, and it may participate in regulation of the expression of hundreds of ISGs, suggesting that cIRF7 is capable of inducing an antiviral response through the Jak-STAT-independent pathway. Although it has been reported previously that the basal expression of IRF7 and the IRF7-induced activation of the IFNα promoter are impaired in the HCV replicon cells [34], in this study we have shown that cIRF7 is activated in cells infected with HCVcc and capable of inducing type I IFN. Collectively, these results suggest that cIRF7 is capable of eliminating HCV that persistently infects

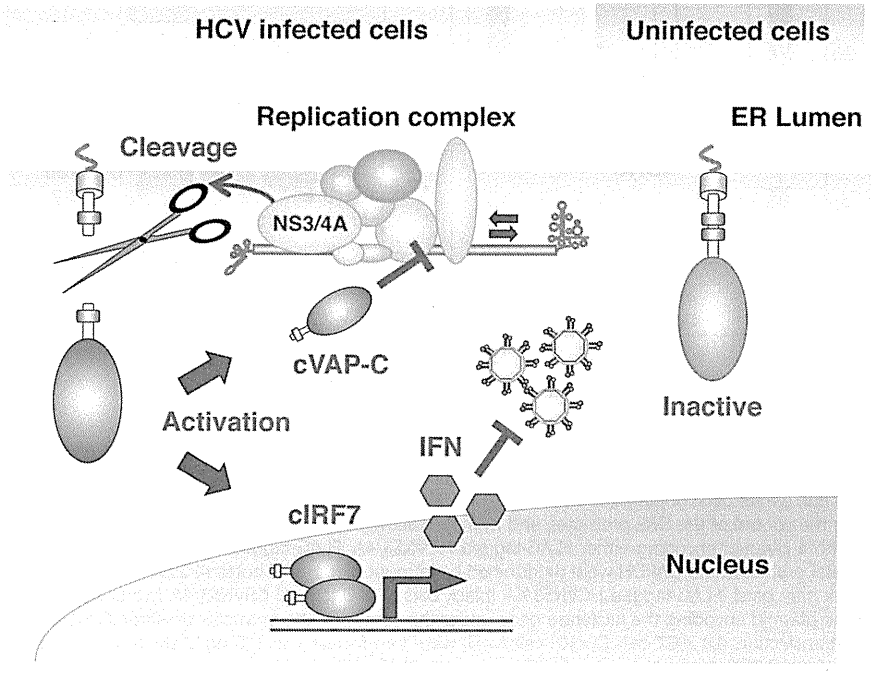

Figure 3. Scheme of activation of the therapeutic molecule in cells infected with HCV. The chimeric molecules are cleaved by HCV NS3/4A protease and the released fragments inhibit propagation of HCV through induction of IFN after translocation into the nucleus (cIRF7) or disruption of the replication complex (cVAP-C), whereas the molecule is stably anchored in the ER within uninfected cells.
doi:10.1371/journal.pone.0015967.g003