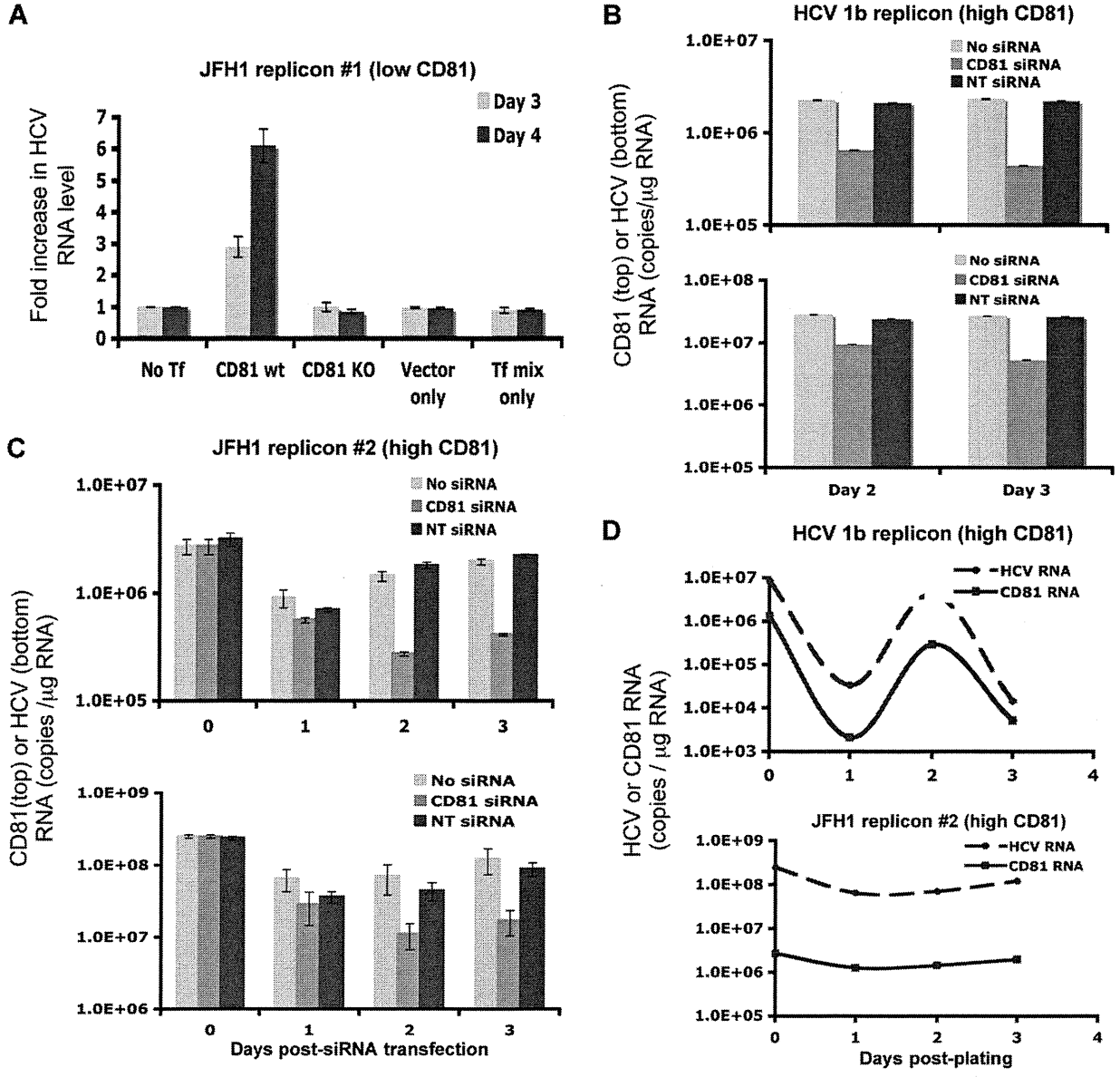

FIG. 7. Modulation of the CD81 level changes HCV RNA replication efficiency. (A) JFH1 subgenomic replicon cells with low CD81 levels (pSGR-JFH1-C4/1) were transfected with a CD81 expression plasmid, and HCV RNA levels were determined on days 3 and 4 after transfection. The fold increase in the HCV RNA level after transfection is shown. CD81 KO, CD81 plasmid with a mutated start codon. (B and C) Two HCV subgenomic replicon cell lines, Huh7/Rep-Feo (B) and SGR-JFH1-FLuc/Neo (C), both of which have high levels of CD81 expression, were treated with CD81 siRNA to silence CD81 expression. CD81 RNA (top) and HCV RNA (bottom) levels were determined 2 and 3 days later. NC, negative control (nontargeted 2) siRNA. (D) CD81 and HCV RNA levels were determined in 1b (top) and JFH 2 (bottom) subgenomic replicon cells at various time points after plating. Mean values ± SD are shown. Data from one of three independent experiments are shown.

of CD81 expression. CD81 levels in those cells fluctuated, and so did the HCV RNA levels. A positive correlation between the two kinetics was observed (Fig. 7D), suggesting the importance of high levels of CD81 for efficient HCV replication.

CD81 in HCV RNA template function. Like other positive-strand RNA viruses, the use of the same viral RNA as templates by viral protein translation and RNA transcription in the HCV life cycle should be mutually exclusive. The experimental data supporting this notion for positive-strand RNA viruses were generated from an *in vitro* cell-free system (4, 15). The template pool is constantly replenished as *in vivo* infection proceeds, and the two processes are expected to be asynchronous. However, a clear pattern for a single dominant template function at a time would also be predicted if the template function is subjected to control by a cellular factor(s) allowing coordinated protein translation and RNA transcription. Indeed, we observed a pattern for the mutually exclusive use of HCV RNA as templates for viral protein translation versus RNA replication in infected cell cultures when the kinetics of HCV RNA and core protein levels were analyzed. As shown in Fig. 8, the kinetics of the intracellular HCV RNA and core protein levels were opposite at each time point: an increasing

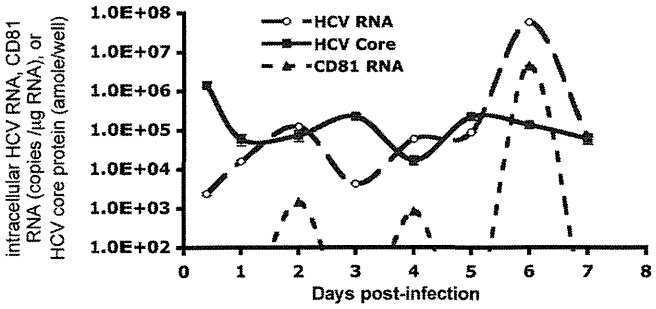

FIG. 8. Relationship of intracellular HCV RNA and core protein levels to CD81 levels during viral replication. CD81-H cells were infected with HCV and harvested at various time points. Intracellular HCV RNA, CD81 RNA, and core protein levels were determined. amole/well, attomoles/well. Mean values ± SD are shown. Data from one of two independent experiments are shown.

intracellular HCV RNA level was accompanied by a decreasing core protein level and vice versa. Our data not only support the mutually exclusive use of HCV RNA for each template function *in vivo* but also suggest that cellular factors may direct HCV RNA to one template function at a time. When the kinetics of the intracellular HCV RNA, core protein, and CD81 levels were analyzed, changing CD81 RNA levels were positively correlated with the changing HCV RNA level but were inversely related to the core protein level (Fig. 8). The positive and inverse correlations among CD81, HCV RNA, and viral protein kinetics suggest that CD81 may be one of the cellular factors directing HCV RNA to the replication process. The viral protein level appears normal while the HCV RNA level is decreasing when the CD81 level is low, suggesting that templates can still be used for viral protein translation but not for RNA synthesis. This is probably why no difference in the luciferase activities or core protein levels in CD81-L cells from those in CD81-H was detected at the early phase of transfection or infection. Thus, CD81 may control HCV RNA replication, possibly through directing HCV RNA template function toward RNA replication.

DISCUSSION

CD81 is known to mediate viral entry in HCV infection (10, 30, 34, 49) and was also implicated in the cell-to-cell transmission of HCV infection (45, 47). Our study showed that significant differences existed in HCV RNA levels after HCV infection among CD81-H, CD81-L1, and CD81-L2 populations and could not be explained simply by the CD81 entry function. This observation prompted us to investigate whether CD81 is required for additional steps in the HCV life cycle, such as RNA replication. Using a variety of techniques and cell lines, we uncovered a novel function of CD81 in the HCV life cycle that is important for HCV RNA replication. CD81 is a tetraspanin family member and is enriched in the lipid rafts of membranous compartments of the cell, where HCV RNA replication is believed to take place (9, 16). The requirement for CD81 participation by the HCV replication process can be facilitated by the physical proximity of CD81 to the HCV replicating site.

To explain our data and the proposed dual functions of CD81 in the context of HCV infection and replication, we reason that a low threshold amount of CD81 is required for the HCV entry function but that a much higher level of CD81 is necessary for efficient HCV replication subsequent to viral entry. Koutsoudakis et al. (26) previously reported that about 70,000 CD81 molecules in a cell appear to be the threshold for viral entry. Our data suggest that the three cell lines with very different levels of CD81 allow similar viral entry but appear to support divergent efficiencies of HCV replication that correlate well with CD81 levels. It is interesting that CD81-L2 cells, despite having very low levels of CD81, can still support viral entry although at a somewhat lower level than the higher-level-CD81-expressing cells. The CD81 expression level of the L2 cells is probably just around the "threshold level" for viral entry.

Our data provide evidence for the mutually exclusive use of HCV RNA as templates for either RNA replication or protein synthesis in infected cell cultures. Two lines of evidence support that the use of HCV RNA for RNA replication is subjected to cellular factor control, such as CD81. One line of evidence is the absence of efficient RNA replication after viral protein translation in HCV-infected and RNA-transfected CD81-L1 and -L2 cells, suggesting that RNA replication could not occur efficiently when the CD81 level was low. The other evidence is that a clear pattern for one dominant template function at a time was shown for infected CD81-H cells, suggesting that there is a coordinated process that directs HCV RNA molecules toward RNA replication function. It is likely that cellular factors are involved in directing viral RNA molecules toward two distinct template functions. On the other hand, viral protein synthesis is negatively correlated with CD81 and HCV RNA levels. These data suggest that the template function of HCV RNA is controlled by cellular factors like CD81, which directs HCV RNA toward its replication function instead of protein translation. However, it is not clear how CD81 exerts this function.

CD81 may assist directly in the assembly of the HCV replicase complex, including NS5B, contributing to viral RNA replication. Alternatively, CD81 may be linked indirectly to a cellular pathway that is critical for efficient viral replication. In a recent study, Brazzoli et al. showed that the engagement of CD81 during HCV infection activates the Raf/MEK/extracellular signal-regulated kinase (ERK) signaling cascade and that this pathway affects postentry events of the HCV life cycle, presumably at the replication step (8). Further experiments are needed to elucidate the molecular mechanism of this novel CD81 function.

CD81 was previously reported to have diverse functions in biological process. For instance, CD81 is implicated in the metastasis of cancer cells (21). CD81 can influence the adhesion, morphology, activation, proliferation, and differentiation of B, T, and other cells (22). In parasite infections, hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity (39). CD81 was also implicated in the modulation of infectivity, enhancement of viral gene expression, and promotion of virus assembly, budding, and cell-to-cell spread in the HIV life cycle (19, 38, 44). The identification of this novel CD81 function in HCV replication indicates that CD81 plays a more pleiotropic role in the HCV life cycle besides its well-defined role in viral entry. Our data

suggest that CD81 has dual functions in HCV infection: a low threshold level of CD81 required for viral entry and a higher level of CD81 necessary for efficient HCV RNA replication. The dependence of HCV replication on CD81 creates an inherent vulnerability for HCV replication. Thus, CD81 functions could be explored for potential therapeutic development because of the multiple roles of CD81 in HCV infection, as was explored in a recent study of the Alb-uPA/SCID mouse model engrafted with human hepatocytes (29).

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VIROLOGY JOURNAL

SHORT REPORT Open Access

Inhibitory effects on HAV IRES-mediated translation and replication by a combination of amantadine and interferon-alpha

Lingli Yang[1,4†], Tomoko Kiyohara[2†], Tatsuo Kanda[1*†], Fumio Imazeki[1], Keiichi Fujiwara[1], Verena Gauss-Müller[3], Koji Ishii[2], Takaji Wakita[2], Osamu Yokosuka[1]

Abstract

Hepatitis A virus (HAV) causes acute hepatitis and sometimes leads to fulminant hepatitis. Amantadine is a tricyclic symmetric amine that inhibits the replication of many DNA and RNA viruses. Amantadine was reported to suppress HAV replication, and the efficacy of amantadine was exhibited in its inhibition of the internal ribosomal entry site (IRES) activities of HAV. Interferon (IFN) also has an antiviral effect through the induction of IFN stimulated genes (ISG) and the degradation of viral RNA. To explore the mechanism of the suppression of HAV replication, we examined the effects of the combination of amantadine and IFN-alpha on HAV IRES-mediated translation, HAV replicon replication in human hepatoma cell lines, and HAV KRM003 genotype IIIB strain replication in African green monkey kidney cell GL37. IFN-alpha seems to have no additive effect on HAV IRES-mediated translation inhibition by amantadine. However, suppressions of HAV replicon and HAV replication were stronger with the combination than with amantadine alone. In conclusion, amantadine, in combination of IFN-alpha, might have a beneficial effect in some patients with acute hepatitis A.

Short report

Hepatitis A virus (HAV), a member of the family Picornaviridae, causes acute hepatitis and occasionally fulminant hepatitis, a life-threatening disease. As the broad epidemiological picture of hepatitis A changes, the public health importance of this disease is being increasingly recognized [1]. It is a significant cause of morbidity worldwide, although the mortality rate due to hepatitis A is low (improved intensive care and transplantation have contributed to a reduction in deaths). Improved sanitation and living standards mean that fewer countries remain highly endemic, but the risk of HAV infection is present in countries lacking HAV immunity or where the endemicity of hepatitis A is low or intermediate [1]. In such situations, these outbreaks can prove to be long and difficult to control. Vaccination and informing the general public about good hygienic measures are important for the prevention of HAV infection, but new therapeutic options are also desirable.

Amantadine, a tricyclic symmetric amine, inhibits HAV replication *in vitro* [2]. We previously reported that amantadine inhibits hepatitis A virus internal ribosomal entry site (IRES)-mediated translation in human hepatoma cells [2]. Interferons (IFNs) also exhibit antiviral effects against HAV infection [2,3]. In the present study, we examined the effects of amantadine with or without IFN-alpha, on HAV IRES activities, HAV subgenomic replicon replication and HAV replication *in vitro* as a proof of concept for the development of a more effective treatment to control HAV infection.

First, we evaluated the cytotoxicity of amantadine and IFN-alpha by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Amantadine concentrations in a range of 1 - 125 μg/mL and those of 1 - 150 μg/mL for 12-h incubation were non-toxic for Huh7 cells and for HuhT7 cells, respectively (Figures 1A and 1B). Amantadine could be incubated for a short time, e.g., 12 h, with the cells, and then the dose of amantadine could be

* Correspondence: kandat-cib@umin.ac.jp
† Contributed equally
[1]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
Full list of author information is available at the end of the article

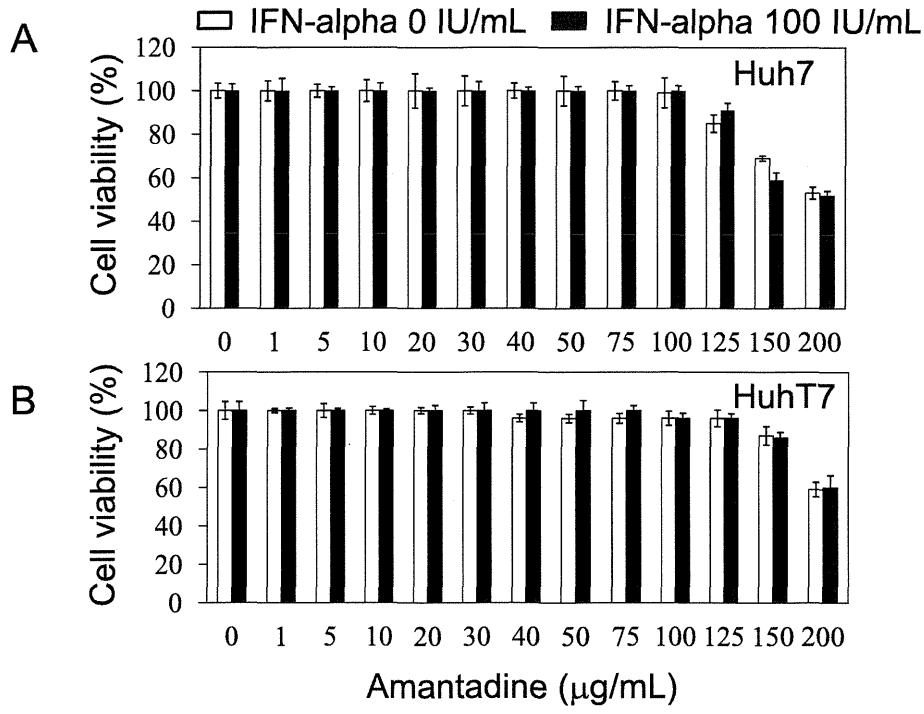

Figure 1 Effects of amantadine on cell growth and viability. MTS assays of cells 12 h after treatment with amantadine with or without 100 U/mL interferon (IFN)-alpha. (A) Huh7 cells. (B) HuhT7 cells. Data are expressed as mean ± SD.

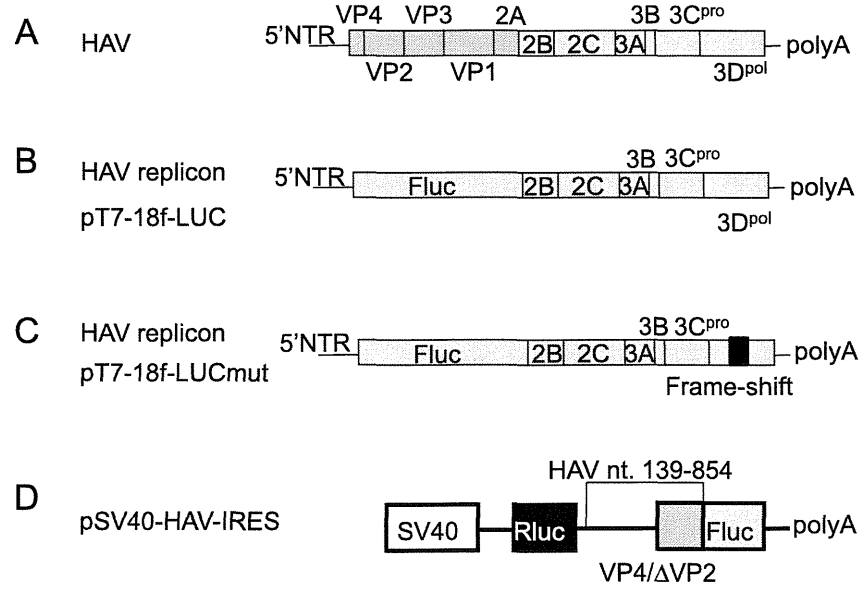

Figure 2 Structures of reporter constructs used in this study. (A) Structure of HAV genome. **(B)** Structure of the replication-competent HAV replicon (HAV replicon) pT7-18f-LUC, which contains an open-reading frame of firefly luciferase (Fluc) flanked by the first four amino acids of HAV polyprotein and by 12 C-terminal amino acids of VP1. This segment is followed by P2 and P3 domains of HAV polyprotein (HAV strain HM175 18f) [9,10]. **(C)** Structure of replication-incompetent HAV replicon (mut) (mut-HAV replicon) pT7-18f-LUCmut, which contains a frame-shift mutation in the polymerase 3 D [9,10]. **(D)** Bicistronic reporter constructs: pSV40-HAV IRES was described previously [2,4]. It encodes the Renilla luciferase genes (Rluc), the internal ribosomal entry site (IRES) HAV HM175, and the firefly luciferase gene (Fluc) under the control of the simian virus 40 promoter (SV40).

increased to higher than 100 μg/mL. With the combination of amantadine and 100 IU/mL IFN-alpha, we did not observe increased cytotoxicity compared with amantadine alone.

We previously reported that the introduction of siRNA targeted against the 5'NTR region of HAV HM175 inhibits HAV IRES-mediated translation and HAV replication [4]. Interestingly, amantadine and IFN also inhibited HAV IRES-mediated translation and HAV replication [2,3,5-8]. Accordingly, we planned to identify more effective strategies for suppressing HAV IRES-mediated translation and HAV replication. IRES is an attractive target for antivirals because HAV IRES is located in the 5'NTR region, the most conserved region among HAV strains. In the present study, we evaluated the HAV antiviral activity of amantadine and IFN-alpha. We initially examined the effects of this combination on HAV IRES-mediated translation using a luciferase reporter assay. Huh7 cells were transfected with pSV40-HAV IRES reporter vector, encoding SV40 promoter driven-*Renilla reniformis* and firefly luciferase, separated by HAV-IRES (Figure 2) [2], and treated with amantadine and/or IFN-alpha. Inhibition of luciferase activity at different levels was observed with amantadine with or without 100 IU/mL IFN-alpha (Figure 3A). Although the strongest suppression was noted with the combination of 10 μg/mL amantadine and 100 IU/mL IFN-alpha, IFN-alpha showed no additive effect on the translation inhibition by 50-100 μg/mL amantadine. This finding prompted us to examine whether IFN-alpha has additive suppression of HAV replicon replication by amantadine. We have reported that RNA replication of HAV can be analyzed in a DNA-based replicon system using HuhT7 cells that stably express T7-RNA polymerase in the cytoplasm (Figure 1) [9-11]. The luciferase activities determined after transfection of replicon DNA are a direct measure of RNA translation and replication. This is because replication in positive-stranded RNA viruses can be easily assessed with a viral replicon carrying the luciferase gene in place of viral structural genes. Moreover, luciferase activity due to translation or translation and replication can be evaluated when the transfection of a replication-competent replicon (HAV replicon) is compared with that of a replication-incompetent replicon (mut) (mut-HAV replicon) [8].

To further determine the effects of the combination of amantadine and IFN-alpha on HAV replication, we transfected the HAV replicon or mut-HAV replicon into HuhT7 cells, and the drugs were added 24 h later. Reporter assays were performed 48 or 72 h after transfection. The transfection efficacy of HAV replicon was estimated as 20-30% in our systems. Luciferase activity was normalized with respect to the protein concentration of cell

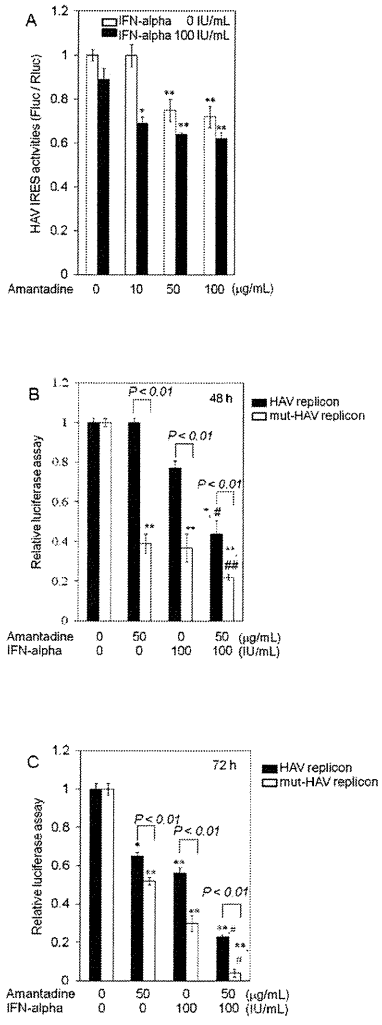

Figure 3 (A) Effects of amantadine with or without interferon on the hepatitis A virus (HAV) internal ribosomal entry site (IRES) activities in Huh7 cells. Approximately 2 × 10^5 cells were seeded on a 6-well tissue culture plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. pSV40-HAV-IRES (0.3 μg) was transfected into Huh7 cells using the Effectene transfection reagent (Qiagen, Tokyo, Japan). 24 h after transfection, amantadine and/or IFN in various concentrations was added to cells. 48 h after transfection, cell extracts were prepared, and luciferase assays were performed using the Dual Luciferase assay system (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions [2]. For controlling the variations in transcription, IRES activity was assessed by measuring the ratio of *Renilla* and firefly luciferases. All samples were run in triplicate. *Renilla* and firefly luciferase activities were measured as relative light units using a luminescencer (JNRII-AB-2300; ATTO, Tokyo, Japan). **(B, C) Effects of amantadine with or without interferon on the HAV subgenomic replicon replication in HuhT7 cells. (B)** 48 h after transfection and **(C)** 72 h after transfection. Black columns, replication-competent HAV replicon; white columns, replication-incompetent HAV replicon (mut). Relative luciferase activities without any treatments were set at 1. Data are expressed as mean (columns) ± SD (vertical lines). *P < 0.05 and **P < 0.01, compared with untreated control by Student's t test. #P < 0.01 and ## P < 0.05, compared with amantadine alone or IFN-alpha alone by Student's t test.

lysates. In this DNA-based system, 48 h after transfection, the replication rates of the HAV replicon were 100%, 77%, and 44% compared to those of control when treated with amantadine alone, IFN alone, and their combination, respectively (Figure 3B). On the other hand, since the mut-HAV replicon cannot replicate, the luciferase activity (39%, 37%, and 22% compared to those of control for the same test conditions, respectively) is due to translation of the viral RNA and not replication. Amantadine alone showed 52% at 72 h, higher than 37% at 48 h, supporting the notion that amantadine might suppress translation of the viral RNA. Suppression effects of these treatments were stronger in the mut-HAV replicon than in the HAV replicon. These findings support our observation of the suppression of HAV IRES-mediated translation by amantadine and IFN-alpha. Suppression effects at 48 h after transfection by the combination of amantadine and IFN-alpha against HAV replication were stronger than those by amantadine or IFN-alpha monotreatment. IFN-alpha was more effective than amantadine against the HAV replicon ($P = 0.0027$) (Figure 3B).

Seventy-two hours after transfection, the replication rates of the HAV replicon were 65%, 56%, and 23% compared to those of control when treated with amantadine alone, IFN-alpha alone, and their combination, respectively (Figure 3C). The replication rates of the mut-HAV replicon were 52%, 30%, and 4% of those of control, respectively. IFN-alpha was more effective than amantadine against the replication of HAV replicon or mut-HAV replicon ($P < 0.001$ or $P < 0.001$). Suppression effects of the combination of amantadine and IFN-alpha at 72 h post-transfection were stronger than those of amantadine or IFN-alpha monotreatment. Suppression effects of these treatments were stronger in the mut-HAV replicon than in the HAV replicon. Moreover, it is important to note that the effects of this combination were observed at earlier time points (Figure 3C).

Next, we performed an infectivity assay using the virus to investigate the effects of combination of amantadine and IFN-alpha on tissue culture-adapted HAV strain KRM003 (genotype IIIB, accession no. L20536) propagation in African green monkey kidney GL37 cells [12-14]. GL37 cell monolayers in 96-well culture plates were infected with HAV at a multiplicity of infection (MOI) of 5 or 50 for 1 h at 37°C in a CO_2 incubator. Without removing the inoculum, drug-containing media were added to appropriate wells. The final concentrations of amantadine, IFN-alpha, and their combination were 50 μg/ml, 100 IU/ml and 50 μg/ml of amantadine and 100 IU/ml of IFN-alpha, respectively. After incubation for 72 h, infected cells were evaluated with ELISA. Suppression of HAV replication by the combination of amantadine and IFN-alpha was stronger than those of amantadine alone, IFN-alpha alone, and untreated control (Figure 4).

IFNs are proteins induced by lymphocytes and other cells including hepatocytes in response to viruses such as HAV. In virus-infected cells, dsRNA activates antiviral interferon pathways and the production of IFN type I. The secreted IFN type I induces a positive feedback loop that results in the expression of interferon-stimulated genes (ISGs), including RNase L and protein kinase R (PKR) [15]. Our study supports the fact that the administration of IFN-alpha suppresses HAV replication through HAV IRES mediated-translation and other mechanisms and that, on the other hand, amantadine suppresses HAV replication mainly through HAV IRES mediated-translation.

There are several reports concerning HAV suppressing intracellular dsRNA-induced retinoic acid-inducible gene I (RIG-I)-mediated IFN regulatory factor 3 (IRF-3) activation to block induction of IFN [16,17]. Yang et al. reported that HAV proteins interact with mitochondrial antiviral signaling protein, an essential component of virus-activated signaling pathways that induce protective IFN responses [18]. However, in this study, the

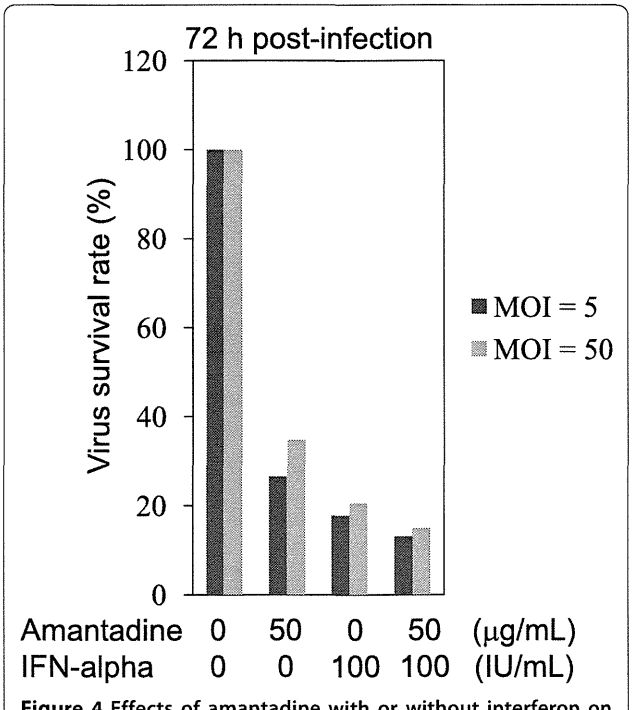

Figure 4 Effects of amantadine with or without interferon on HAV KRM003 genotype IIIB strain replication in African green monkey kidney cell GL37. GL37 cell monolayers in 96-well culture plates were infected with HAV [at a multiplicity of infection (MOI) of 5 or 40] for 1 h at 37°C in a CO_2 incubator. Amantadine and/or IFN was added to cells. After the incubation for 72 h, infected cells were evaluated with ELISA. The rate of virus survival was measured using this equation: Virus survival rate (%) = 100 × Absorbance with drug/Absorbance without drug.

administration of exogenous IFN-alpha could suppress HAV replication, although endogenous IFNs produced by cells also may play an important role in inhibiting viral replication. Further studies will be needed.

Amantadine inhibits the replication of many DNA and RNA viruses and is also used as a drug for the treatment of Parkinson's disease [2]. It is known that the M2 protein of influenza A virus is a target of amantadine [19]. Furthermore, it has been reported to inhibit HAV IRES-mediated translation and replication by our group and other researchers [2,3,5-8].

Therefore, we examined the possibilities of the combination of amantadine and IFN-alpha against HAV because these two drugs were previously reported to be effective against HAV [2,3,5-8]. To our knowledge, this is the first study demonstrating that a combination of amantadine and IFN-alpha can suppress HAV replication more effectively than amantadine or IFN-alpha alone.

Abbreviations

HAV: hepatitis A virus; **IRES**: internal ribosomal entry site; **IFN**: interferon; **MTS**: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

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Author details

[1]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. [2]Department of Virology II, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashi-Murayama, Tokyo 280-0011, Japan. [3]Institute of Medical Molecular Biology, University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. [4]Department of Dermatology, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan.

Authors' contributions

LY, Tatsuo Kanda, FI and OY conceived and designed the study. LY, Tomoko Kiyohara and Tatsuo Kanda performed the experiments. LY, Tomoko Kiyohara, Tatsuo Kanda and FI analyzed data and wrote the manuscript. Tomoko Kiyohara, KI and TW contributed to experiments using a whole HAV virus. Tomoko Kiyohara, Tatsuo Kanda and VG contributed to the interpretation of the interpretation of the results and took part to the critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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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.

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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.

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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

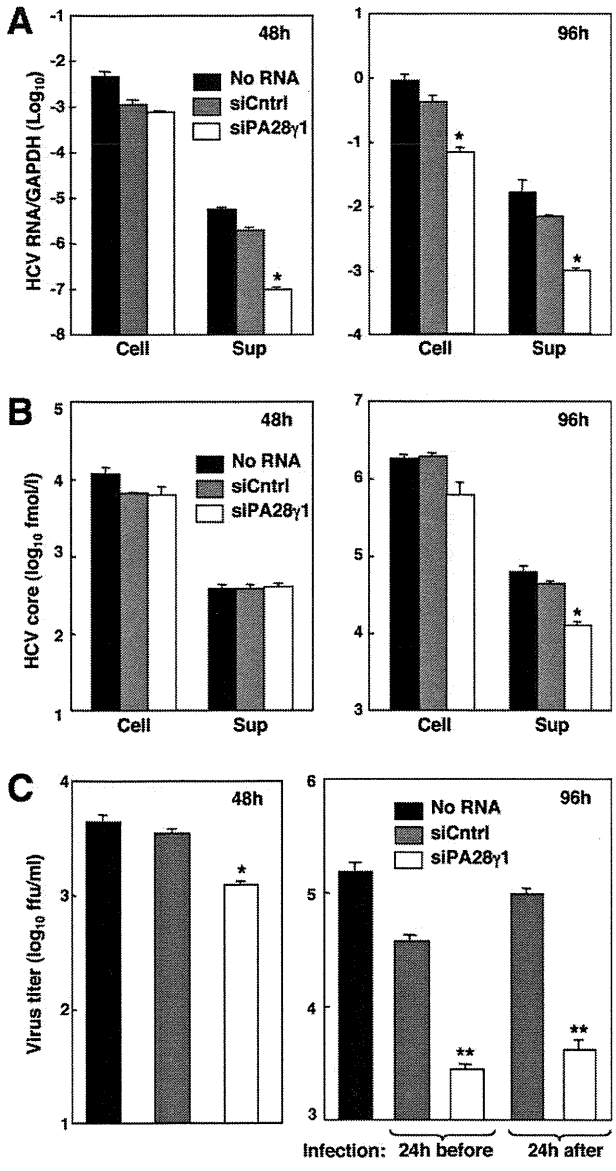

Fig. 1. Transient knockdown of PA28γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (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

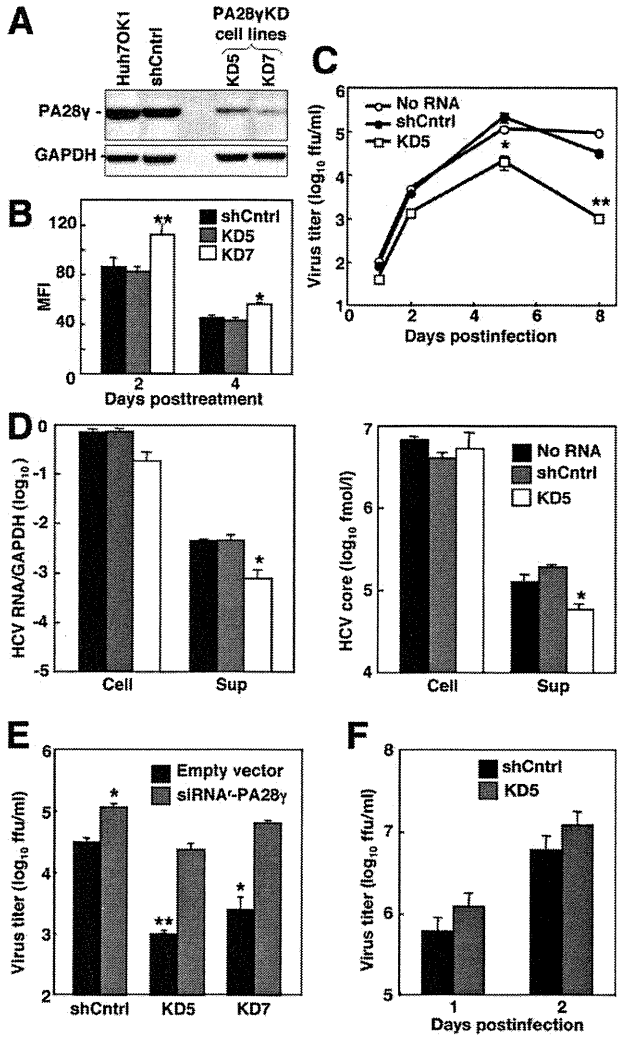

Fig. 2. Establishment of PA28γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, 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.

derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28γ

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

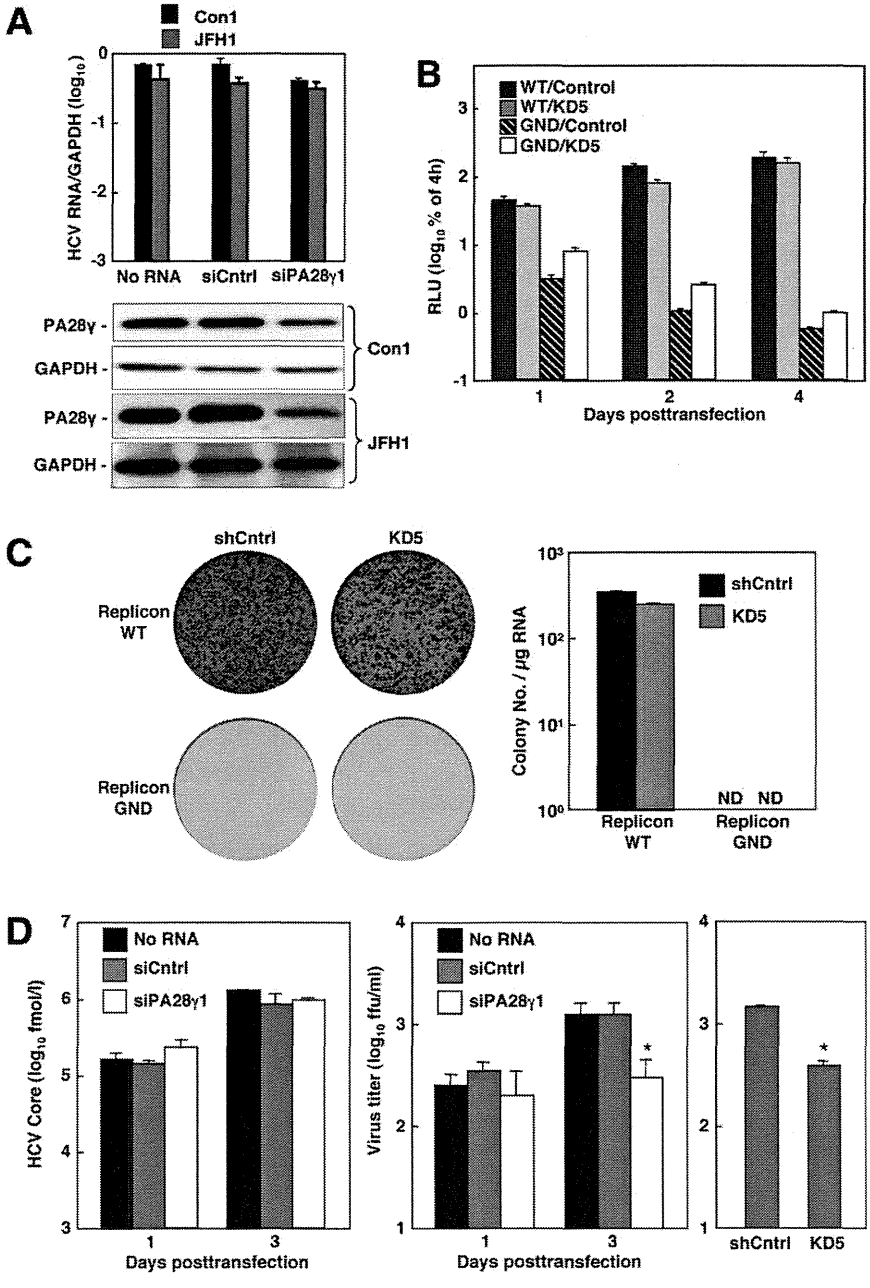

Fig. 3. Effect of PA28γ knockdown on HCV RNA replication. (A) The siCntrl or siPA28γ1 (10 nM) was transfected into the subgenomic HCV replicon cells derived from Con1 and JFH-1 strains. The transfected cells were harvested at 72 hours posttransfection. The replicon RNA was determined by quantitative 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.

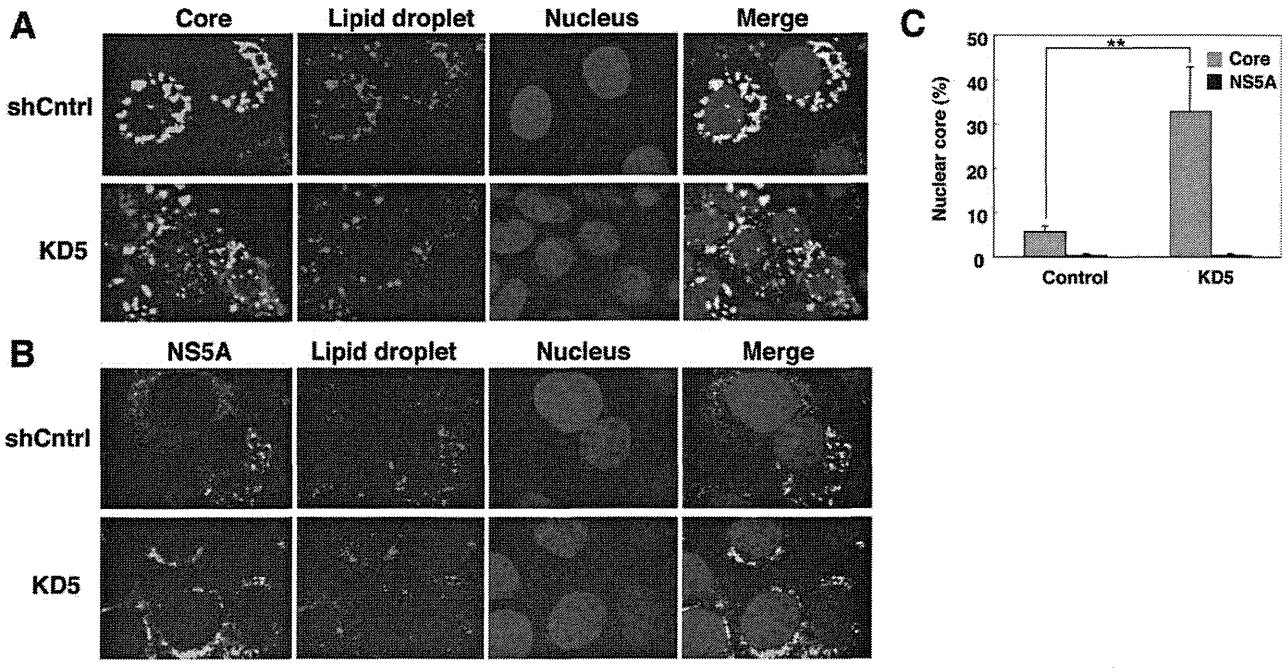

Fig. 4. Effect of PA28γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies 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

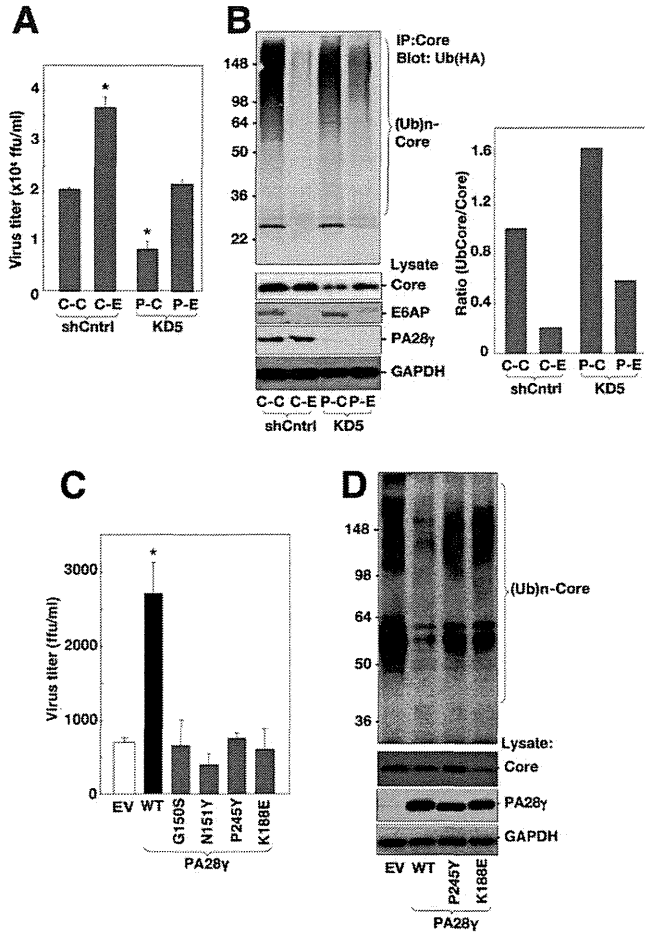

Fig. 5. PA28γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 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.

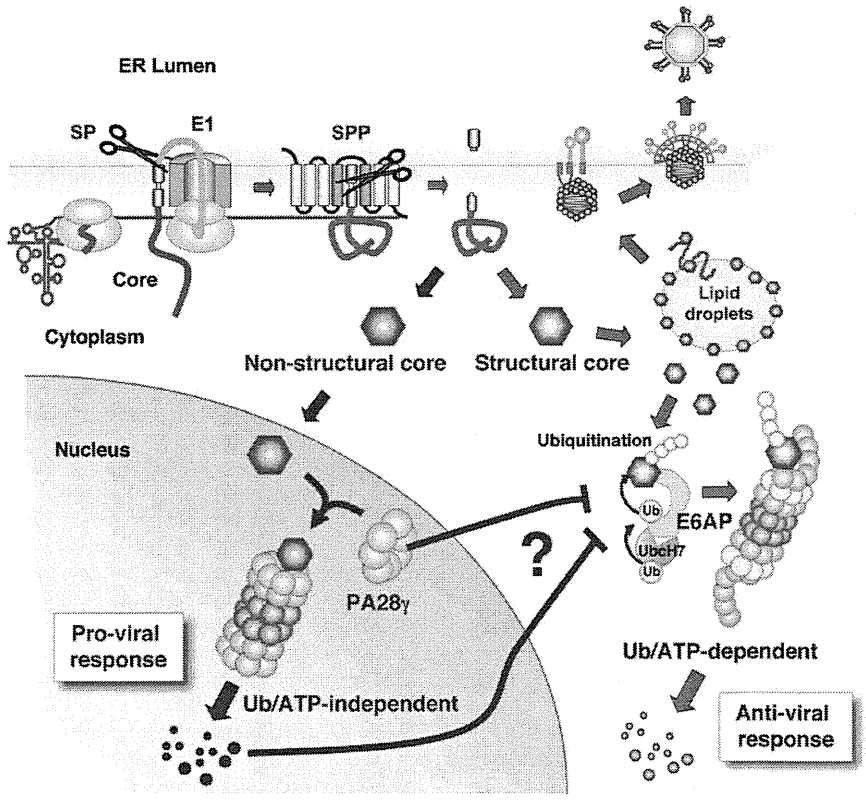

Fig. 6. Schematic diagram of the potential roles of PA28γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid droplets 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.

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ARTICLE
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E6AP Ubiquitin Ligase Mediates Ubiquitin-Dependent Degradation of Peroxiredoxin 1

Junichi Nasu,[1,2] Kyoko Murakami,[1] Shoji Miyagawa,[3] Ryosuke Yamashita,[3] Tohru Ichimura,[4] Takaji Wakita,[1] Hak Hotta,[3] Tatsuo Miyamura,[1] Tetsuro Suzuki,[1] Tazuko Satoh,[2] and Ikuo Shoji[1,3*]

[1]*Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan*

[2]*Department of Oral and Maxillofacial Surgery, School of Life Dentistry at Tokyo, the Nippon Dental University, Chiyoda-ku, Tokyo, Japan*

[3]*Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan*

[4]*Department of Applied Chemistry, National Defense Academy, Yokosuka, Kanagawa, Japan*

ABSTRACT

E6-associated protein (E6AP) is a cellular ubiquitin protein ligase that mediates ubiquitylation and degradation of tumor suppressor p53 in conjunction with the high-risk human papillomavirus E6 protein. We previously reported that E6AP targets annexin A1 protein for ubiquitin-dependent proteasomal degradation. To gain a better understanding of the physiological function of E6AP, we have been seeking to identify novel substrates of E6AP. Here, we identified peroxiredoxin 1 (Prx1) as a novel E6AP-binding protein using a tandem affinity purification procedure coupled with mass spectrometry. Prx1 is a 25-kDa member of the Prx family, a ubiquitous family of antioxidant peroxidases that regulate many cellular processes through intracellular oxidative signal transduction pathways. Immunoprecipitation analysis showed that E6AP binds Prx1 in vivo. Pull-down experiments showed that E6AP binds Prx1 in vitro. Ectopic expression of E6AP enhanced the degradation of Prx1 in vivo. In vivo and in vitro ubiquitylation assays revealed that E6AP promoted polyubiquitylation of Prx1. RNAi-mediated downregulation of endogenous E6AP increased the level of endogenous Prx1 protein. Taken together, our data suggest that E6AP mediates the ubiquitin-dependent proteasomal degradation of Prx1. Our findings raise a possibility that E6AP may play a role in regulating Prx1-dependent intracellular oxidative signal transduction pathways. J. Cell. Biochem. 111: 676–685, 2010.

KEY WORDS: E6AP; Prx1; UBIQUITIN; DEGRADATION

E6-associated protein (E6AP) is the prototype of a family of ubiquitin ligases called HECT domain ubiquitin ligases, all of which contain a domain homologous to the E6AP carboxyl terminus [Huibregtse et al., 1995]. E6AP was initially identified as the cellular factor that stimulates ubiquitin-dependent degradation of the tumor suppressor p53 in conjunction with the E6 protein of cervical cancer-associated human papillomavirus (HPV) types 16 and 18 [Huibregtse et al., 1993; Scheffner et al., 1994]. The E6–E6AP complex functions as an E3 ubiquitin ligase in the ubiquitylation of p53 [Scheffner et al., 1993]. Known substrates of the E6–E6AP complex include the tumor suppressor p53 [Scheffner et al., 1993], the PDZ domain-containing protein Scribble [Nakagawa and Huibregtse, 2000], and NFX1-91, a transcriptional repressor of the gene encoding hTERT [Gewin et al., 2004]. The ability of E6 to

Abbreviations: E6AP, E6-associated protein; Prx, peroxiredoxin; HPV, human papillomavirus; MS, mass spectrometry; MAb, monoclonal antibody; PAb, polyclonal antibody; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; CHX, cycloheximide.

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*Correspondence to: Dr. Ikuo Shoji, MD, PhD, Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan.
E-mail: ishoji@med.kobe-u.ac.jp

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