

FIG. 6. Inhibition of autophagosome formation induces severe cytoplasmic vacuolations leading to cell death in the HCV replicon cells. (A) SGR^{Con1} and SGR^{cured} cells transfected with pStrawberry-Atg4B^{C74A} or empty vector pStrawberry (EV) were fixed at 48 h posttransfection and examined by fluorescence microscopy. The boxed areas in the phase-contrast images are magnified. (B) SGR^{Con1} and SGR^{cured} cells transfected with pEGFP-Atg16L or EV were examined by fluorescence microscopy at 48 h posttransfection. The boxed areas in the phase-contrast images are magnified. (C) SGR^{cured}, SGR^{Con1},

was not observed in the cured cells infected with HCVcc, an infectious HCV strain derived from strain JFH1 (Fig. 5D, bottom). Collectively, these results suggest that the dysfunction of lysosomal acidification contributes to the impairment of autophagy in the HCV replicon cells of strain Con1.

Autophagy induced in cells replicating HCV is required for cell survival. Finally, we examined the pathological significance of autophagy during HCV replication. Atg4B is known as an LC3-processing protease, and overexpression of its protease-inactive mutant (Atg4B^{C74A}) results in inhibition of the autophagosome formation (7). To our surprise, severe cytoplasmic vacuolation was observed in the SGR^{Con1} cells expressing Atg4B^{C74A} (Fig. 6A). These vacuolations were also observed in the SGR^{Con1} cells by the expression of Atg16L (Fig. 6B), a molecule that is an essential component of the autophagy complex and that, if expressed in excess amounts, can disrupt the autophagosome formation (8). Expression of Atg4B^{C74A} induced a higher level of vacuole formation in the Con1 replicon cells than in cells infected with JFH1 virus but not in the cured cells (Fig. 6C). Along with these vacuolations, cell viability was significantly decreased by the expression of Atg4B^{C74A} in SGR^{Con1} cells and slightly in JFH1 virus-infected cells (Fig. 6D). These results suggest that autophagy induced by the RNA replication of HCV is required for host cell survival.

DISCUSSION

In the present study, we demonstrated that two genotypes of HCV induce autophagy, whereas intact autophagy flux is required for the host cell to survive. The cell death characterized by cytoplasmic vacuolation that was induced in the HCV replicon cells by the inhibition of the autophagosome formation is similar to type III programmed cell death, which is distinguishable from apoptosis and autophagic cell death (4). Type III programmed cell death has been observed in the neurodegenerative diseases caused by the deposit of cytotoxic protein aggregates (15).

We previously reported that HCV hijacks chaperone complexes, which regulates quality control of proteins into the membranous web for circumventing unfolded protein response during efficient genome replication (53); in other words, the replication of HCV exacerbates the generation of proteins associated with cytotoxicity. In the experiments using a chimpanzee model, HCV of genotype 1 was successfully used to reproduce acute and chronic hepatitis similar to that in the human patients (3, 57), and transgenic mice expressing viral proteins of HCV of genotype 1b have been shown to develop

and SGR^{cured} cells infected with JFH1 virus were transfected with pStrawberry-Atg4B^{C74A}, and the number of vacuole-positive cells in each of nine fields of view was counted at 48 h posttransfection. (D) SGR^{cured}, SGR^{Con1}, and SGR^{cured} cells infected with JFH1 virus were transfected with pStrawberry-Atg4B^{C74A} (black bars) or EV (white bars), and cell viability was determined at 6 days posttransfection by using CellTiter-Glo (Promega) according to the manufacturer's protocol. The asterisks indicate significant differences ($P < 0.05$) versus the control value. The data shown are representative of three independent experiments.

Sjögren syndrome, insulin resistance, hepatic steatosis, and hepatocellular carcinoma (27, 28). In contrast, HCVcc, based on the genotype 2a strain JFH1 isolated from a patient with fulminant hepatitis C (33, 56), was unable to establish chronic infection in chimpanzees (56) or to induce cell damage and inflammation in chimeric mice xenotransplanted with human hepatocytes (17). These results imply that the onset of HCV pathogenesis could be dependent not only upon an amount but also on a property of deposited proteins, and they might explain the aggravated vacuolations under the inhibition of autophagosome formation in strain Con1 compared to that in strain JFH1. Interestingly, the overexpression of Atg4B^{C74A} or Atg16L causes eccentric cell death in the Con1 replicon cells in which autophagy flux is already disturbed. Thus, we speculated that the quarantine of undefined abnormalities endowed with high cytotoxicity by the engulfing of the autophagic membrane might be sufficient for the amelioration of HCV-induced degeneration. The autophagosomal dysfunction observed in the Con1 replicon cells may suggest that a replicant of strain Con1 was more sensitive to the lysosomal vacuolation than that of strain JFH1. Because a limitation of our study was that we were unable to use infectious HCV of other strains, it is still unclear whether the autophagic degradation can be impaired only in the replicon of HCV strain Con1 or genotype 1.

We also demonstrated that HCV replication of strain Con1 but not that of strain JFH1 facilitates the secretion of pro-CTSB. It has been well established that the secretion of pro-CTSB is enhanced in several types of tumors (26, 50). The secretion of CTSB, like the secretion of matrix metalloproteases, is a marker of the progression of the proteolytic degradation of the extracellular matrix, which plays an important part in cancer invasion and metastasis. Since infection with HCV of genotype 1 is clinically considered a risk factor for the development of hepatocellular carcinoma (14, 51), the enhanced secretion of pro-CTSB by the replication of genotype 1 strains might synergistically promote infiltration of hepatocellular carcinoma.

As shown elsewhere (see movies in the supplemental material), although most degradations of the autophagosome were impaired due to a dislocalization of a V-ATPase subunit, some autophagic degradation was achieved in the SGR^{Con1} cells similar to that in the starved Huh7 cells. Moreover, the stagnated autophagy flux was rescued by the treatment of alpha interferon accompanied by elimination of HCV (Fig. 1C and D). Interestingly, we observed neither a significant impairment of lysosomal degradation nor the intracellular activity of cathepsins in the replicon cells of HCV strain Con1 (data not shown). Therefore, there might be a specific dysfunction within the autolysosome during the replication of HCV strain Con1. Detailed studies are needed to elucidate how HCV strain Con1 disturbs the sorting of V-ATPase.

A close relationship between autophagy and the immune system has been gradually unveiled (47). Autophagy assists not only in the direct elimination of pathogens by hydrolytic degradation but also in antigen processing in antigen-presenting cells such as macrophage and dendritic cells (DC) for presentation by major histocompatibility complex (MHC) I and II (11). Moreover, autophagy plays important roles in T lymphocyte homeostasis (44). As such, in some instances, interruptions of autophagy can allow microorganisms to escape from

the host immune system. Indeed, the immune response against herpes simplex virus was suppressed by blocking the autophagy (6). With regard to HCV, functionally impaired DC dysfunctions marked by poor DC maturation, impaired antigen presentation, and attenuated cytokine production have been reported in tissue culture models and chronic hepatitis C patients (1, 22, 46). In addition, reduction of cell surface expression of MHC-I in HCV genotype 1b replicon cells has been reported (55). We confirmed that levels of cell surface expression of MHC-I in the replicon cells of genotype 1b, but not of genotype 2a, were reduced in comparison with those in the cured cells (data not shown). Hence it might be feasible to speculate that the replication of HCV RNA of genotype 1 induces an incomplete autophagy for attenuating antigen presentation to establish persistent infection. In contrast, autophagy is known to serve as a negative regulator of innate immunity (21, 54). A recent report demonstrated that autophagy induced by infection with strain JFH1 or dengue virus attenuates innate immunity to promote viral replication (23), indicating that an HCV genotype 2a strain may facilitate autophagy to evade innate immunity.

In this study, we demonstrated that HCV utilizes autophagy to circumvent the cell death induced by vacuole formation for its survival. This unique strategy of HCV propagation may provide new clues to the virus-host interaction and, ultimately, to the pathogenesis of infection by various genotypes of HCV.

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

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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 (IFN β 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 IFN α 6, IFN β , 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.

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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-IFN α) 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 IFN β production, whereas IRF7 is induced by IFN β and participates in the late phase for IFN β 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 IFN β 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 Ser³⁹⁶ 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 IFN α 6, IFN β and ISRE, respectively. Among these examined constructs, we observed significant activation of the promoters of IFN α 6 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 IFN α 6 promoter in cells expressing IRF3m (Figs. 1B and 1C). Potent stimulation of the IFN β 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 Cys⁵⁰⁸ 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 Cys⁵⁰⁸ 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 IFN α 6, IFN β 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 IFN α 6, IFN β 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

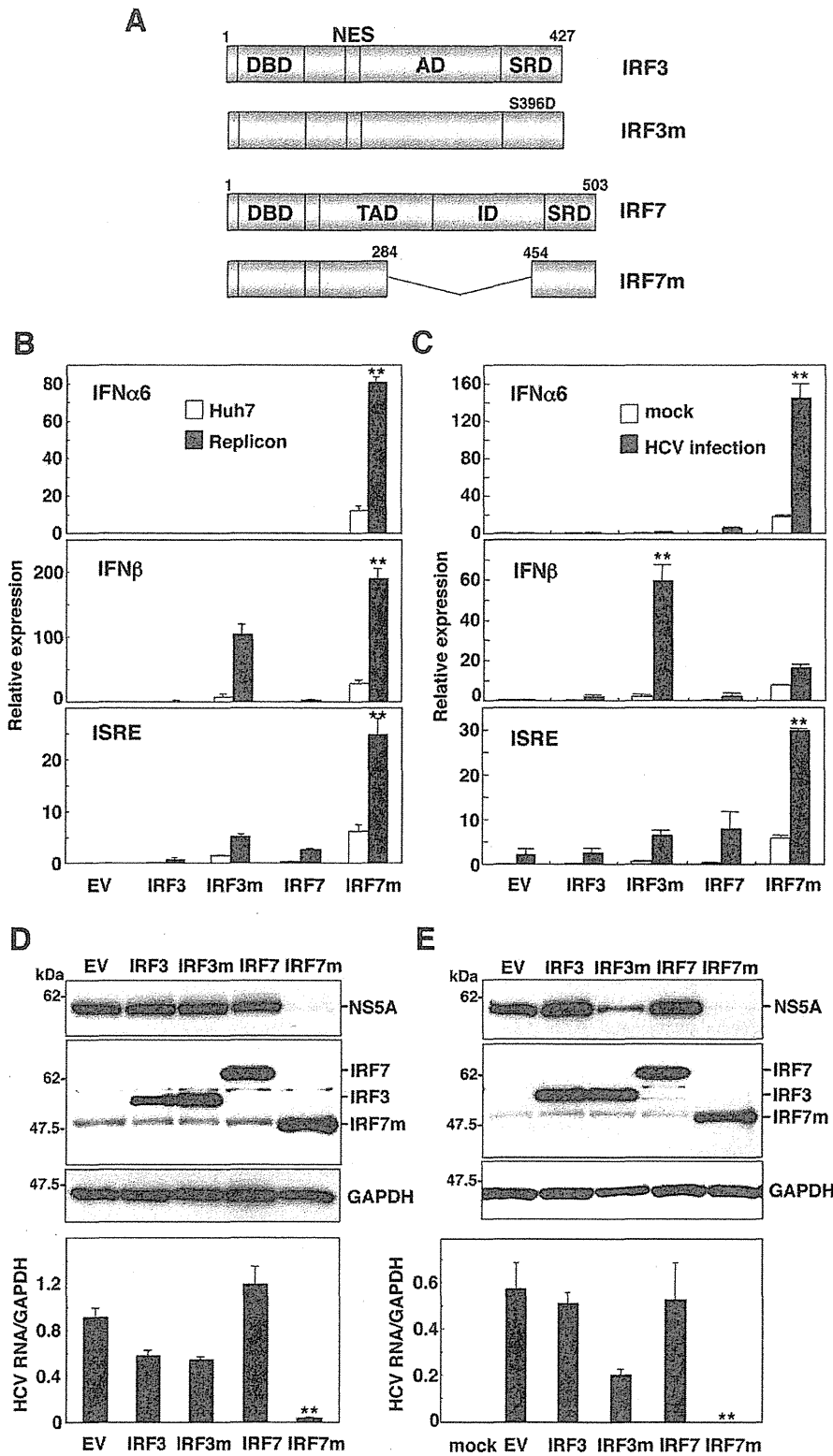


Figure 1. Dominant active mutant of IRF7 activates IFN promoters in cells replicating HCV. (A) Structures of IRF3, IRF7 and the dominant active mutants, IRF3m and IRF7m. The DNA-binding domain, nuclear export sequence, transactivation domain, association domain, inhibitory domain, and signal response 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 IFN α 6, IFN β , 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 4 β R replicon cells [27,28], which have been shown to exhibit more resistant to the IFN α treatment than Con1 replicon cells (Fig. 6C upper left). Expression of the cIRF7 in the 4 β R replicon cells but not in those cured HCV RNA (4 β Rc 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 4 β R 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-IFN α 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 inter-active 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

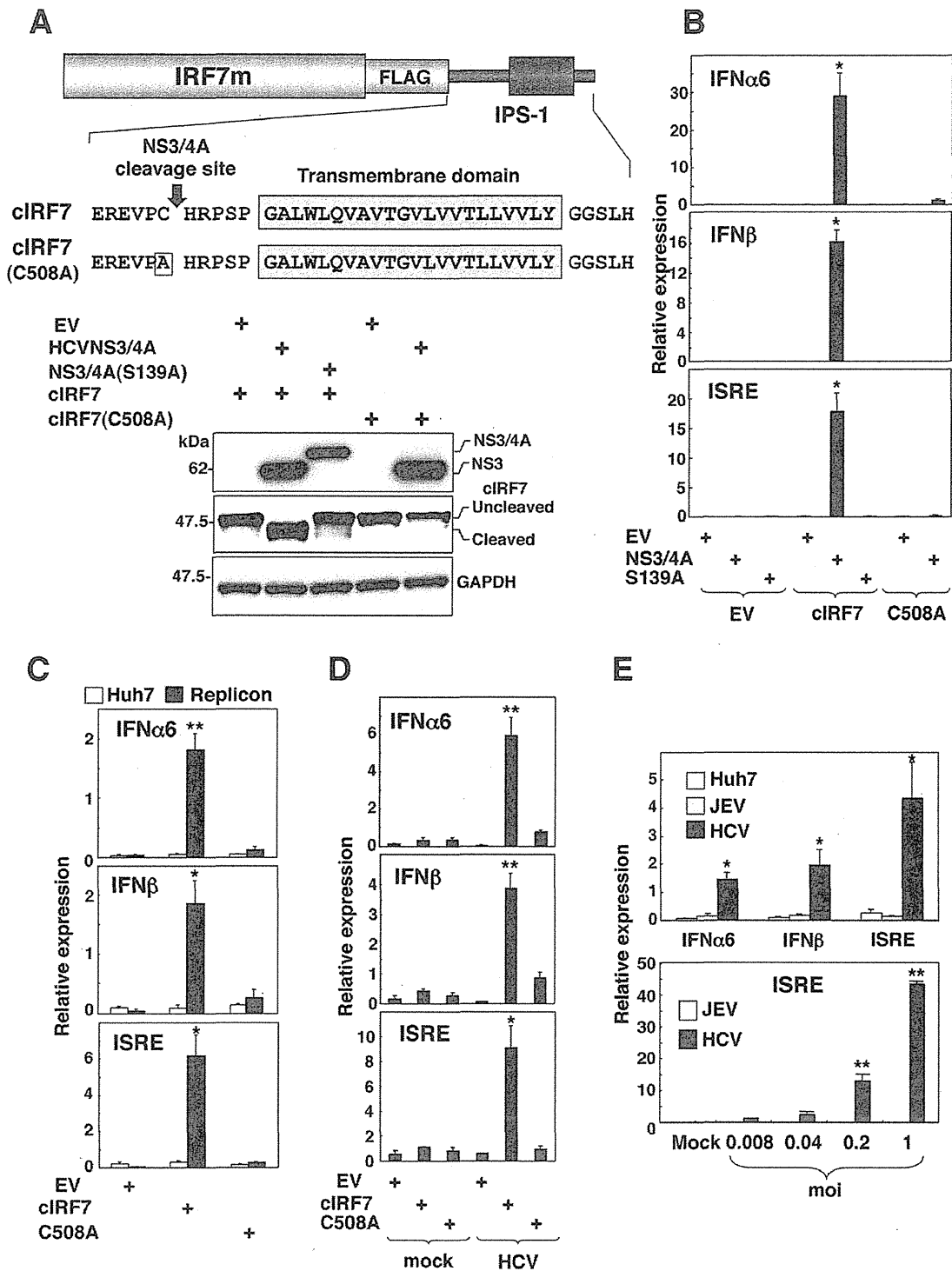


Figure 2. Construction of cIRF7 capable of activating the IFN promoters in cells replicating HCV. (A top) Schematic representation of the cIRF7 constructs. cIRF7 consists of IRF7m, FLAG-tag, and IPS-1 (503 to 540 amino acid residues) sequences containing a cleavage site by HCV NS3/4A protease, a transmembrane 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 IFN α 6, IFN β 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.
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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 IFN α 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 IFN β mRNA between the two cell types (Fig. 1), suggesting that differences among HCV genotypes might be caused to the difference to the sensitivity of IFN β . 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 IFN α -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 IFN α 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

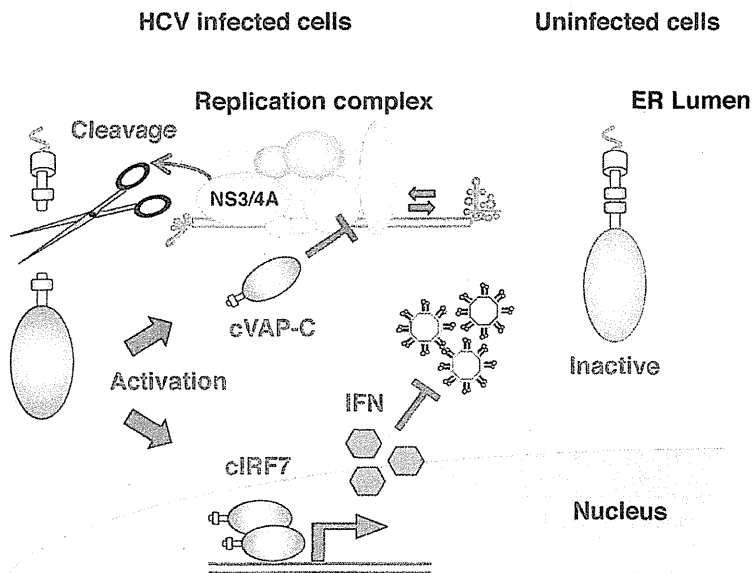


Figure 3. Scheme of activation of the therapeutic molecule in cells infected with HCV. The chimeric molecules are cleaved by HCV NS3/4A protease and the released fragments inhibit propagation of HCV through induction of IFN after translocation into the nucleus (cIRF7) or disruption of the replication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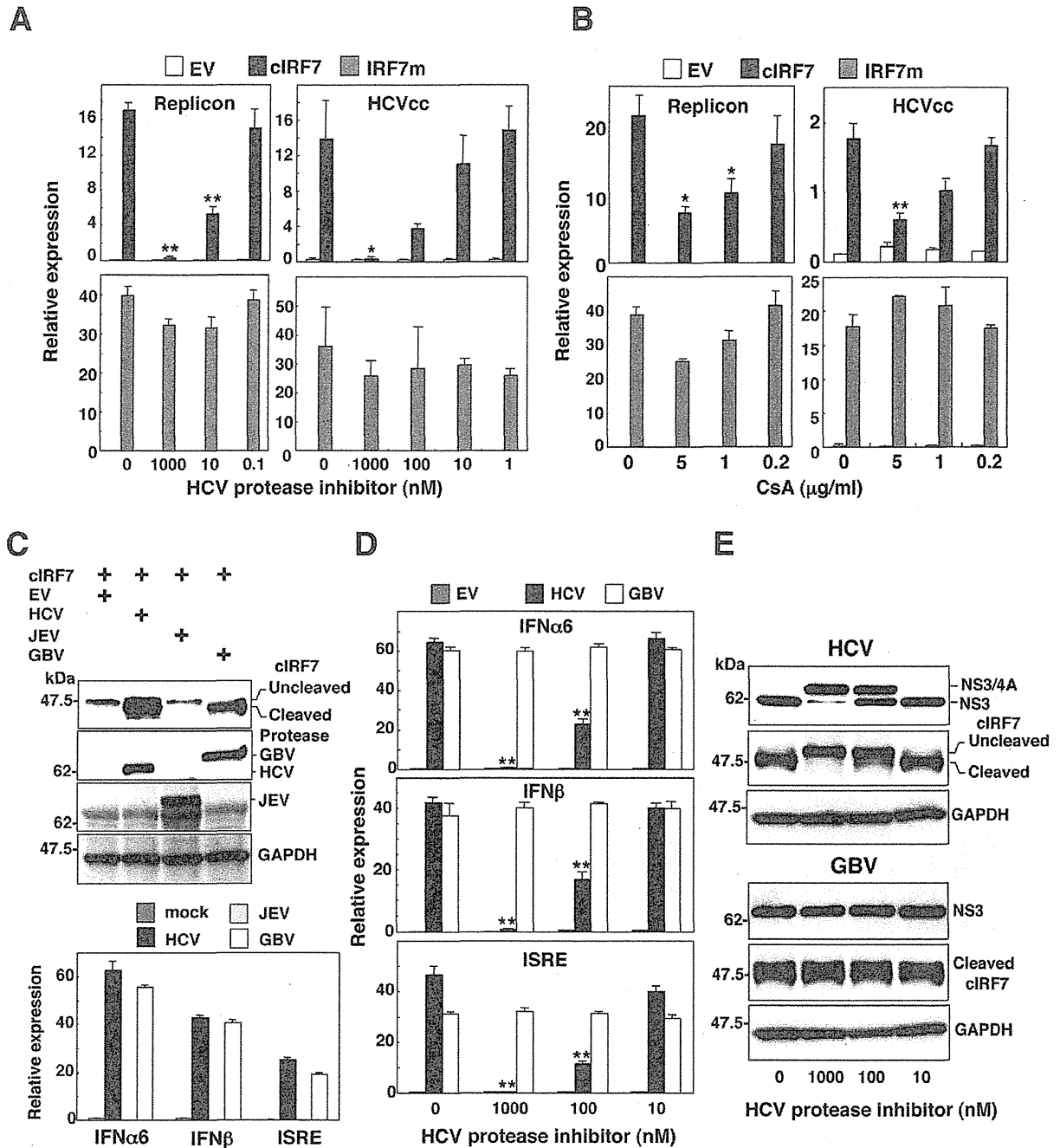


Figure 4. Specificity of activation of the IFN promoters by the expression of cIRF7. (A) HCV replicon cells (1.5×10^5 cells/well) or Huh70K1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were treated with various concentrations of HCV protease inhibitor (A) or cyclosporine A (CsA) (B), transfected with an empty vector (EV) (white bars) or plasmids encoding cIRF7 (black bars) or IRF7m (gray bars) together with 100 ng of a reporter plasmid encoding the luciferase gene under the control of the ISRE promoter, and luciferase activity was determined at 24 h post-transfection. (C top) A plasmid encoding cIRF7 was co-transfected with a plasmid encoding either FLAG-tagged HCVNS3/4A, FLAG-tagged GBVNS3/4A, or HA-tagged JEVNS2b/3 into 293T cells, and the expressions of cIRF7, viral proteases and GAPDH were determined by immunoblotting. (C bottom) 293T cells (2×10^5 cells/well) transfected with a plasmid encoding either EV (dark gray bars), FLAG-tagged HCVNS3/4A (black bars), FLAG-tagged GBVNS3/4A (white bars), or HA-tagged JEVNS2b/3 (gray bars) together with 100 ng of the plasmid encoding the luciferase gene under the control of the promoter of either IFN α 6, IFN β or ISRE, and luciferase activity was determined at 24 h post-transfection. (D) 293T cells (2×10^5 cells/well) were transfected with 100 ng of the reporter plasmids together with plasmids encoding EV (gray bars), FLAG-tagged HCVNS3/4A (black bars) or FLAG-tagged GBVNS3/4A (white bars) in the presence or absence of the HCV protease inhibitor, and luciferase activity was determined at 24 h post-transfection. (E) cIRF7 was co-expressed with FLAG-tagged HCVNS3/4A or FLAG-tagged GBVNS3/4A in 293T cells in the presence or absence of the HCV protease inhibitor, and the expressions of cIRF7, viral proteases and GAPDH were determined by immunoblotting. 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.g004

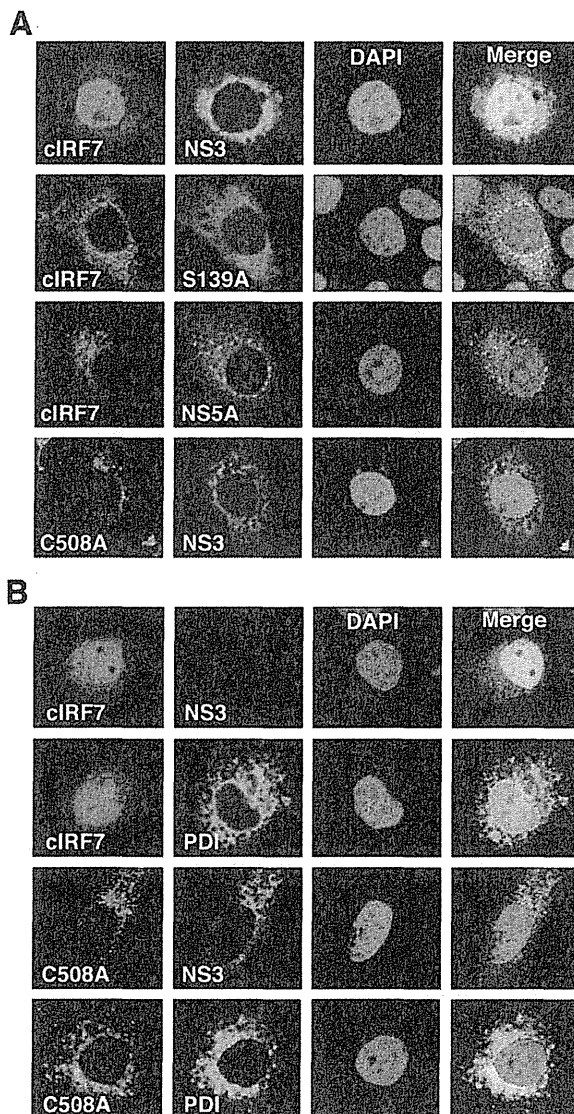


Figure 5. Activation of cIRF7 in cells expressing HCV protease.

(A) Huh7OK1 cells (5×10^4 cells/well) were co-transfected with plasmids encoding either EGFP-cIRF7 or EGFP-cIRF7(C508A) and plasmids encoding either HCVNS3/4A, HCVNS3/4A(S139A) or NS5A, harvested at 24 h post-transfection, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.25% saponin. HCV NS3 and NS5A were stained with the appropriate antibodies, followed by staining with AF594-conjugated second antibodies. (B) HCV replicon cells (5×10^4 cells/well) were transfected with plasmids encoding either EGFP-cIRF7 or EGFP-cIRF7(C508A), and endogenous expression of HCV NS3 and an ER marker, PDI, was detected in cells treated and stained with the appropriate antibodies as described above. Subcellular localization of cIRF7s, HCV proteins and PDI was determined by confocal microscopy after staining of nuclei by DAPI. The data shown in this figure are representative of three independent experiments. doi:10.1371/journal.pone.0015967.g005

human hepatocytes through an induction of sufficient amounts of type I IFN.

It is well known that patients achieving a rapid viral clearance by the treatment with PEG-IFN α showed a significant up-regulation of ISG, whereas a high level expression of ISG is

observed in nonresponsive patients before IFN therapy, probably due to a rapid induction of negative regulators for the IFN signaling pathway, such as the suppressor of cytokine signaling proteins [35,36]. These results suggest that chronic hepatitis C patients with a pre-activated IFN signaling pathway respond poorly to IFN therapy. In this study we also demonstrated that activation of various IFN promoters by the expression of the dominant active mutants of IRFs was more accentuated in cells replicating HCV rather than naïve cells, probably due to an undetectable expression of ISG in cells replicating HCV RNA as described previously [37]. However, the precise mechanisms underlying the enhancement of IFN activity by the expression of a dominant active mutant of IRFs in cells replicating HCV remain unknown. Filipowicz *et al.* suggested the possibility of recovery of the sensitivity to IFN therapy by the restoration of the endogenous IFN system to a “naïve” state through a blockage of the IFN response in nonresponders before treatment [36]. However, modulation of ISG expression before IFN therapy may induce a flare of HCV propagation in the liver of chronic hepatitis C patients. Therefore, it might be interesting to examine whether an effectiveness of cIRF7 are sustained in a state of occurring a negative regulator for IFN signaling pathway and preactivated IFN signaling pathway in cells replicating HCV.

VAP-A and VAP-B are suggested to be 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 the HCV propagation and not expressed in human hepatocytes probably involves in the determination of tissue tropism of HCV [20]. Although further studies will be needed to elucidate the effectiveness of the molecules *in vivo* experiment using drug delivery systems including viral and non-viral vectors in more detail, therapeutic molecules consisting of host factors involved in IFN induction such as IRF7 and in the suppression of HCV replication such as VAP-C fused with the IPS-1 sequences specifically cleaved by the HCV protease might be a promising approach capable of eliminating HCV without induction of severe cellular toxicity.

Materials and Methods

Cells and viruses

Vero and 293T cell lines were purchased from American Type Culture Collection (Manassas, VA). Huh7 cell line was kindly provided by Ralf Bartenschlager. Huh7OK1 cell line was previously established from interferone-treated Huh7 cells including HCV replicon and exhibited high susceptibility to HCVcc propagation [38]. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). Huh-9-13 cells harboring an HCV subgenomic RNA replicon of genotype 1b [39] were cultured in DMEM supplemented with 10% FCS, 1 mg/ml G418 and nonessential amino acids. The infectious RNA of the JFH1 strain was introduced into Huh7OK1 cells and the infectious titers were expressed as focus-forming units (FFU) [4]. Huh7 cells harboring a JEV subgenomic RNA replicon (Nakayama strain) were cultured in DMEM supplemented with 10% FCS and 1 μ g/ml puromycin. Preparation of the HCV subgenomic replicon cells 4 β R exhibiting an IFN-resistant phenotype and their cured cells 4 β Rc were described previously [27,28]. All cells were cultured at 37°C in a humidified atmosphere with 5% CO $_2$.

Plasmids and reagents

The cDNA fragments encoding IRF3 and IRF7 were amplified by PCR from a total RNA from THP-1 cells and cloned into

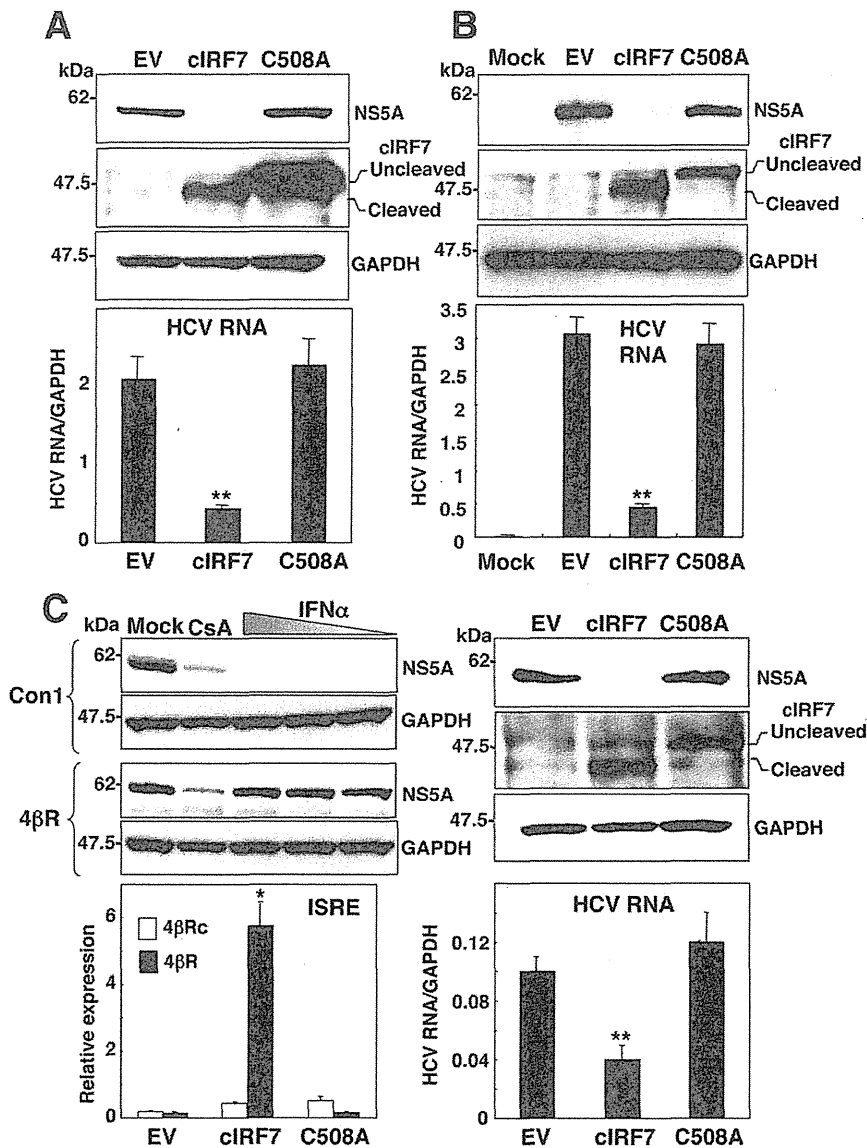


Figure 6. Suppression of HCV replication by the expression of cIRF7. (A) HCV replicon cells (3×10^5 cells/well) and (B) Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were transfected with a plasmid encoding either empty vector (EV), cIRF7 or cIRF7(C508A), and the expression of NS5A, cIRF7s and GAPDH (upper panels) and synthesis of viral RNA (lower panels) were determined at 72 h post-transfection by immunoblotting and real-time PCR, respectively. (C upper left) HCV Con1 replicon cells and 4βR replicon cells exhibiting an IFN-resistant phenotype (1.5×10^5 cells/well) were treated with the CsA ($5 \mu\text{g/ml}$) or 10^4 , 10^3 , and 10^2 units/ml of recombinant human IFN α and the expressions of NS5A and GAPDH were determined by immunoblotting. The 4βR replicon cells (3×10^5 cells/well) were transfected with EV or plasmid encoding either cIRF7 or cIRF7(C508A), and the expressions of NS5A, cIRF7s and GAPDH (C upper right) and synthesis of viral RNA (C lower right) were determined at 72 h post-transfection by immunoblotting and real-time PCR, respectively. The 4βR cells and their cured cells (4βRc) with the HCV genome eliminated (1×10^5 cells/well) were transfected with EV or plasmid encoding either cIRF7 or cIRF7(C508A) together with 100 ng of plasmid encoding the luciferase gene under the control of the ISRE promoter, and luciferase activity was determined at 24 h post-transfection (C lower left). 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.g006

pcDNA3.1-C-myc-His (Invitrogen, Carlsbad, CA). The mutants carrying a deletion in the auto-inhibitory domain (from amino acid residue 284 to 454) of IRF7 and the substitution of Ser³⁹⁶ with phosphomimetic Asp located in the carboxyl terminus of IRF3 were generated by the method of splicing by overlap extension and cloning into pcDNA3.1myc-His and designated as IRF7m and

IRF3m, respectively. N-terminally FLAG-tagged wild-type NS3/4A protease and its mutant substituted with Ser¹³⁹ to replaced with Ala (S139A) were prepared as described previously [33]. The cDNA fragment encoding a JEV protease was amplified from a total RNA of Vero cells infected with JEV (AT31 strain) and cloned into pcDNA3.1Flag/HA [40]. The cDNA fragment

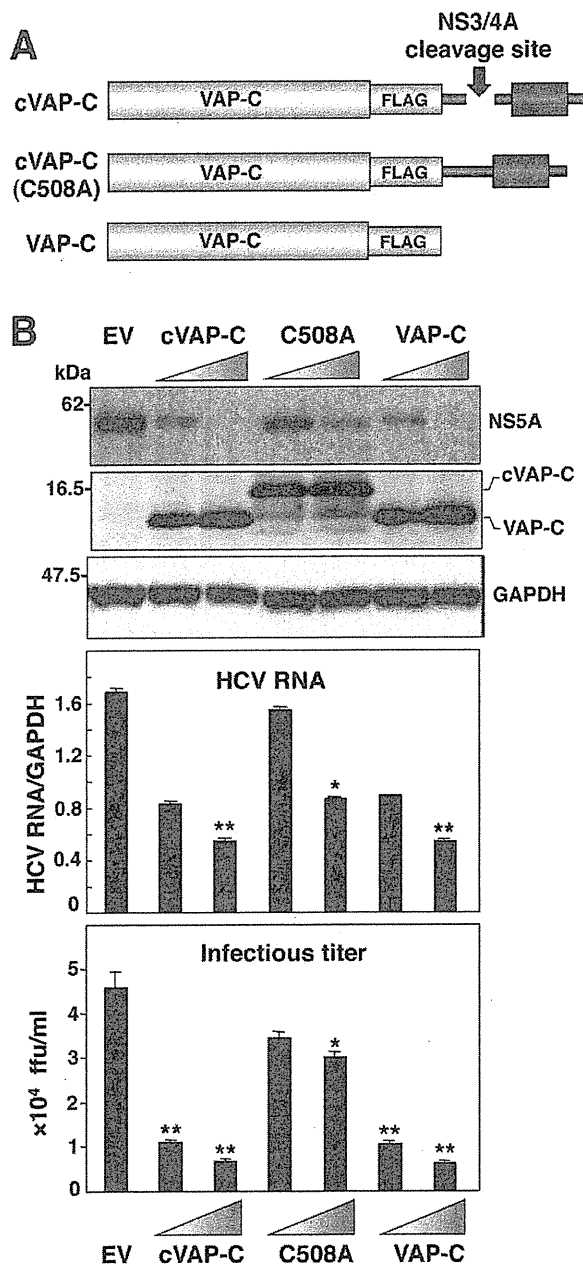


Figure 7. Suppression of HCV replication by the expression of cVAP-C. (A) Schematic representation of cVAP-C, cVAP-C(C508A) and VAP-C. Like cIRF7, cVAP-C is composed of the sequences of VAP-C, FLAG-tag, and the C-terminus domain of IPS-1. (B) Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were transfected with EV, or plasmid encoding either cIRF7 or cIRF7(C508A), and the expressions of NSSA, VAP-Cs and GAPDH (top panel), synthesis of viral RNA (middle panel) and infectious titers in the culture supernatants were determined at 72 h post-transfection by immunoblotting, real-time PCR, and focus forming assay, respectively. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$) versus the control cells or mock-infected cells.
doi:10.1371/journal.pone.0015967.g007

encoding a GBV-B protease was amplified from pGGB (kindly provided by Dr. H. Akari) [41] by PCR and cloned into pcDNA3.1Flag/HA. The chimeric IRF7 (cIRF7) composed of the IRF7m fused with FLAG-tag and the C-terminus of human IPS-1 (from amino acid residues 503 to 540 amino acid residues) containing a cleavage site of HCV NS3/4A, transmembrane domain and the ER retention signal [21] (Fig. 2A) was cloned into pcDNA3.1-c-myc-His. A cIRF7 mutant, C508A, was generated to be resistant to HCV NS3/4A protease by substitution of Cys⁵⁰⁸ of cIRF7 to Ala. The reporter constructs of IFN α 6, IFN β , and ISRE were kindly provided by Drs. T. Kawai and S. Akira. All PCR products were confirmed by sequencing by an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan). The HCV NS3/4A protease inhibitor, BILN2061 was purchased from Acme Bioscience (Belmont, CA). Human recombinant IFN α and cyclosporine A (CsA) were purchased from PBL Biomedical Laboratories (New Brunswick, NJ) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Reporter assay

Huh7 cells, HCV replicon cells, and Huh7OK1 cells infected with HCVcc were seeded onto 12-well plates at the concentration of 1.5×10^5 cells/well and transfected with 100 ng of each of the plasmids encoding the luciferase gene under the control of the IFN α 6, IFN β and ISRE promoter together with the various constructs by using FuGeneTM6 (Roche Molecular Biochemicals, Mannheim, Germany). Luciferase activity was determined by the Dual-luciferase reporter assay system (Promega Inc., Madison, WI) and the *Renilla* luciferase reporter gene was simultaneously transfected as an internal control.

Immunoblotting

HCV replicon cells and Huh7OK1 cells infected with HCVcc were transfected with the plasmids encoding each of the wild-type and the dominant active mutants of IRFs and harvested at 72 h post-transfection. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), suspended in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail tablets (Roche Molecular Biochemicals) and centrifuged at $14,000 \times g$ for 15 min at 4°C after incubation for 30 min at 4°C. Cell lysates were subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) after boiling in sample buffer and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). The membranes were blocked with PBS containing 0.05% Tween 20 and 5% skim milk at room temperature for 1 h, incubated with mouse monoclonal anti-FLAG M2 (Sigma), anti-hemagglutinin (HA) 16B12 (HA.11; BabCO, Richmond, CA), anti-NS5A mouse monoclonal antibody (Austral Biologicals, San Ramon, CA), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-hexahistidine monoclonal antibody (Santa Cruz) at room temperature for 1 h, and then with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody at room temperature for 1 h. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

A total RNA was prepared from HCV replicon cells and Huh7OK1 cells infected with HCVcc transfected with the plasmids encoding each of the IRF constructs using an RNeasy

mini kit (QIAGEN, Valencia, CA) and first-strand cDNA was synthesized by using ReverTra Acc (TOYOBO, Osaka, Japan) and oligo (dT)₂₀ primer. The expression of each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems). The HCV and GAPDH genes were amplified using the primer pairs of 5'-GAGTGTCTGCAGCCTCCA-3' and 5'-CACTCGCAAGCACCCTATCA-3', and 5'-ACCACAGTC-CATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3', respectively. The expression of each of mRNA was normalized with that of GAPDH.

Subcellular localization of cIrf7 in HCV- replicating cells

Cells transfected with the plasmids were harvested at 24 h post transfection, washed twice with PBS, fixed with PBS containing 4% paraformaldehyde, and permeabilized by incubation with PBS containing 0.25% saponin for 10 min. Cells were incubated for 1 h at 4°C with 1 μg/ml of anti-NS3 (251) mouse monoclonal antibody (Santa Cruz), anti-NS5A mouse monoclonal antibody (Austral Biologicals), or mouse monoclonal antibody to protein disulfide isomerase (PDI) (Affinity Bioreagents, Golden, CO) in

PBS containing 10% FCS (PBSF), and then incubated at room temperature for 1 h with 0.5 μg/ml of Alexa Flour 594-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) after three time washes with PBSF. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). After an extensive wash with PBSF, the samples were examined with a Fluoview FV1000 laser scanning confocal microscope (OLYMPUS, Tokyo, Japan).

Statistical analysis. Results were expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t* test.

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Author Contributions

Conceived and designed the experiments: TA Y. Matsuura. Performed the experiments: XW TA HK ST Y. Mori HT KM. Analyzed the data: NK TS MT. Contributed reagents/materials/analysis tools: NK MT. Wrote the paper: TA Y. Matsuura.

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Original article

Intracellular delivery of serum-derived hepatitis C virus

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Abstract

A robust and reliable cell culture system for serum-derived HCV (HCVser) has not been established yet because of the presence of neutralizing antibody and tropism for infection. To overcome this obstacle, we employed a lipid-mediated protein intracellular delivery reagent (PIDR) that permits internalization of proteins into cells. Although entry of HCVcc was not enhanced by the treatment with PIDR, entry of HCVser into hepatoma cell lines (Huh7 and HepG2) and immortalized primary hepatocytes (Hc and HuS/E2) was significantly enhanced by the PIDR treatment. The entry of HCVser into Huh7 cells in the presence of PIDR was resistant to the neutralization by an anti-hCD81 antibody, suggesting that PIDR is capable of internalizing HCVser in a receptor-independent manner. Interestingly, the PIDR-mediated entry of HCVser and HCVcc was enhanced by the addition of sera from chronic hepatitis C patients but not from healthy donors. In addition, neutralization of HCVcc infection by anti-E2 antibody was canceled by the treatment with PIDR. In conclusion, the PIDR is a valuable tool to get over the obstacle of neutralizing antibodies to internalize HCV into cells and might be useful for the establishment of *in vitro* propagation HCVser. © 2011 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.

Keywords: Hepatitis C virus; Protein intracellular delivery; Serum-derived virus

1. Introduction

More than 170 million individuals worldwide are infected with hepatitis C virus (HCV), and hepatic steatosis, cirrhosis and hepatocellular carcinoma (HCC) induced by HCV infection are life-threatening [1]. Although combined-therapy with peg-interferon (IFN) and ribavirin has achieved a sustained virological response in 50% of individuals infected with HCV genotype 1 [2], a more effective therapeutic modality for HCV infection is needed [3]. To this end, further detailed analyses of HCV are needed in order to clarify not only the viral life cycle but also the pathogenesis. Although cell culture systems for HCV (HCVcc) have been established based on the JFH-1

strain isolated from a fulminant hepatitis C patient [4], such systems were unable to establish chronic infection in chimpanzees [4] or to induce cell damage and inflammation in chimeric mice xenotransplanted with human hepatocytes [5], and therefore establishment of a robust cell culture system capable of propagating serum-derived HCV (HCVser) from hepatitis C patients is required.

Although previous reports suggested a partial replication of HCVser in the primary hepatocytes (PHH) freshly isolated from human liver [6], the level of viral RNA replication was low and reconfirmation of the viral propagation was not achieved due to the difficulty of providing a stable supply of the PHH. Recently, it was shown that a three-dimensional culture system of immortalized PHH was capable of propagating the HCVser from chronic hepatitis C patients [7,8]. HCVser in the patients was slightly amplified in these culture systems, but the levels of viral RNA replication were far lower than those of HCVcc in Huh7-derived adaptive cell lines. Part of the difficulty in establishing a cell culture system for HCVser might be attributable to: i) the

Abbreviations: HCV, hepatitis C virus; IFN, interferon; PHH, primary human hepatocyte; PIDR, protein intracellular delivery reagent; PCR, polymerase chain reaction; VSV, vesicular stomatitis virus.

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existence of high titers of neutralizing antibodies in the sera of hepatitis C patients [9]; ii) the heterogeneity of HCV particles (quasispecies), which exhibit different cell tropisms for infection and replication [10]; and iii) the inconsistent expression of the putative receptors for HCV entry, including CD81, SR-BI, claudin-1 and occludin [11]. It may be necessary to overcome these obstacles before a robust and reliable *in vitro* cell culture system can be established for HCVser.

Polybrene has been used for the efficient infection of retrovirus [12], and spinoculation has also been employed to accelerate the entry of various viruses, including retrovirus [13] and murine coronavirus [14]. Entry of HCVcc into not only the permissive cell line Huh7.5.1 but also the non-permissive cell line PLC/PRF/5 has been shown to be enhanced by spinoculation [15,16]. In this study, we examined the effects of these accelerating procedures for entry of HCVser and found that a cationic amphiphilic-based lipid-mediated protein intracellular delivery reagent (PIDR) [17] exhibited a potent enhancement of entry of HCVser. Our data suggest that PIDR allows complex formation with viral particles via both electrostatic and hydrophobic interactions and enhances internalization of the HCVser into cells in a receptor-independent manner.

2. Materials and methods

2.1. Sera

Sera from chronic hepatitis C patients and a cured patient possessing the anti-HCV antibodies were obtained at the Kyushu University Hospital after obtaining full informed consent from all patients. Seven serum samples from hepatitis C patients, including two window-period serum samples without any detectable anti-HCV antibodies, were obtained from the Benesis Corporation (Osaka, Japan). Human sera from healthy donors were obtained from Sigma–Aldrich Inc. (St. Louis, MO). Sera from healthy donors, chronic hepatitis patients and acute hepatitis patients were designated HDS, CHS, and AHS, respectively. The HCV-RNA titers of CHS and AHS were 7.15 ± 0.34 (range: 6.6–7.5) and 8.20 ± 0.14 (range: 8.1–8.3), respectively. The genotypes of HCV in these sera were 1a (7 patients) and 1b (11 patients).

2.2. Human liver cell lines and preparation of HCVcc

HepG2 and HEK-293T cell lines were obtained from the American Type Culture Collection (Rockville, MD). The Huh7OK1 cell line exhibits an efficient propagation of HCVcc as described previously [18]. The HepCD81 cell line stably expressing human CD81 was established as described previously [19]. HuS-E/2 was kindly provided by M. Hijikata, Kyoto University [20]. Hc (an immortalized human liver cell line) was purchased from the Applied Cell Biology Research Institute (Kirkland, WA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS). The *in vitro* transcribed RNA of the JFH-1 strain of HCV was introduced into Huh7OK1 cells [21] and culture supernatants were collected at 7 days post-

transfection and used as HCVcc. The infectivity of HCVcc was determined by focus forming assay as previously described [19].

2.3. Transfection of plasmids and intracellular delivery of proteins

The plasmids were transfected into cells by liposome-mediated transfection using *TransIT-LT1* (Mirus, Madison, WI). The proteins were introduced into cells by PIDR (PUL-Sin; Polyplus-transfection Inc., New York, NY) according to the manufacturer's protocol. FITC-conjugated mouse IgG antibody (Invitrogen Molecular Probes, Eugene, OR) or recombinant phycoerythrin (PE; Polyplus-transfection Inc.) was introduced into cells by the PIDR as a positive control.

2.4. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was prepared from cells using an RNeasy mini kit (Qiagen, Tokyo, Japan). The synthesis of first-strand cDNA and qRT-PCR was performed using TaqMan EZ RT-PCR Core Reagents and ABI Prism 7000 system (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's protocol. The primers for Taqman PCR were designed in a non-coding region as previously reported [22].

2.5. Infection of HCVser and HCVcc by spinoculation, polybrene and PIDR

Cells were seeded at 1×10^5 cells/well in a 48-well plate and cultured for 24 h. For spinoculation, 2 μ l of HCV-positive serum or HCVcc at a multiplicity of infection (MOI) of 0.05 were inoculated into cells and immediately centrifuged at $500 \times g$ for 120 min at room temperature. For infection of HCV by polybrene and PIDR, 2 μ g of polybrene or 1.5 μ l of PIDR were incubated with HCV-positive serum or HCVcc diluted in 20 μ l of phosphate-buffered saline (PBS) for 15 min at room temperature to allow complex formation [12]. Cells were trypsinized at 24 h post-inoculation, seeded in a 48-well plate to remove non-specific binding of HCV, and cultured for several days.

2.6. Production and infection of pseudotype vesicular stomatitis virus (VSV)

Pseudotype VSVs were generated as described previously [19]. The pseudotype VSVs, VSVpv/GFP and VSVpv/luc, bore the VSVG protein on the virion surface and replaced the G envelope gene with the green fluorescent protein (GFP) and luciferase genes, respectively. Pseudotype VSV bearing HCV E1 and E2 glycoproteins (HCVpv) was prepared as described previously [19]. These pseudotype viruses were inoculated into Huh7OK1 cells in the presence or absence of PIDR together with or without anti-VSVG polyclonal antibody (ab34774; Abcam Inc., Cambridge, MA) or CHS, and infectivity was determined at 24 h post-infection by the expression of GFP or luciferase activity after treatment with a passive lysis buffer (Promega Co., Madison, WI).

2.7. Inhibition of HCVcc and HCVser infection by the treatment with antibody against human CD81 and anti-E2 antibody (AP-33)

To determine the involvement of human CD81 in the intracellular delivery of HCV by PIDR, Huh7OK1 cells were pre-treated with 5 μ g/ml of anti-human CD81 monoclonal antibody (JS-81; BD Biosciences Pharmingen, Mountain View, CA) for 1 h at 37 °C and then inoculated with HCVcc or HCVser in the presence of PIDR. Anti-E2 monoclonal antibody (AP-33) was kindly provided by A.H. Patel, University of Glasgow [23]. AP-33 was pre-mixed with HCVcc for 1 h with or without PIDR and then cells were incubated with this mixture and cultured for several days.

3. Results

3.1. Effect of spinoculation and polybrene on the entry of HCVser and HCVcc

First, we examined the effect of spinoculation on the entry of HCVser or HCVcc. Intracellular HCV-RNA titers of Huh7OK1 cells upon infection of HCVser and HCVcc with or without spinoculation at 24 h post-infection were determined (Data not shown). Although entry of HCVcc into Huh7OK1 cells was 10-fold increased by the spinoculation, no effect was observed in the entry of HCVser. Next, we examined the effect of polybrene on the entry of HCVser and HCVcc into Huh7OK1 cells. Although polybrene induced a slight increase of the entry of HCVcc, no significant effect on the entry of HCVser was observed intracellularly at 24 h post-infection (Data not shown). These results indicated that neither spinoculation nor polybrene induced an enhancement of the entry of HCVser.

3.2. Internalization of viral particles by PIDR

To determine the efficacy of intracellular delivery of proteins by PIDR, FITC-conjugated mouse IgG and recombinant PE were introduced into Huh7OK1 cells by PIDR. Both FITC-conjugated IgG and PE were efficiently internalized into Huh7OK1 cells by the treatment with PIDR but not by the lipofection (Fig. 1A). Next, to determine the receptor-independent entry of viral particles into cells by the PIDR, the expression of GFP upon infection of a pseudotype VSV lacking VSVG (VSV Δ Gpv/GFP) into Huh7OK1 cells was examined. Although VSV Δ Gpv/GFP lost infectivity due to a lack of the G glycoprotein, the addition of PIDR facilitated entry of the particles (Fig. 1A). To further examine the effect of the presence of neutralization antibody on the delivery of viral particles by PIDR, the expression of GFP upon transduction of VSVpv/GFP into Huh7OK1 cells in the presence of neutralization antibody and PIDR was examined. Although VSVpv/GFP exhibited a high infectivity to Huh7OK1 cells and the infection was completely neutralized by the anti-VSVG antibody, treatment with PIDR partially recovered the infectivity of VSVpv/GFP neutralized by the antibody (Fig. 1B). Similar results were confirmed by using VSV Δ Gpv/luc and VSVpv/luc carrying the

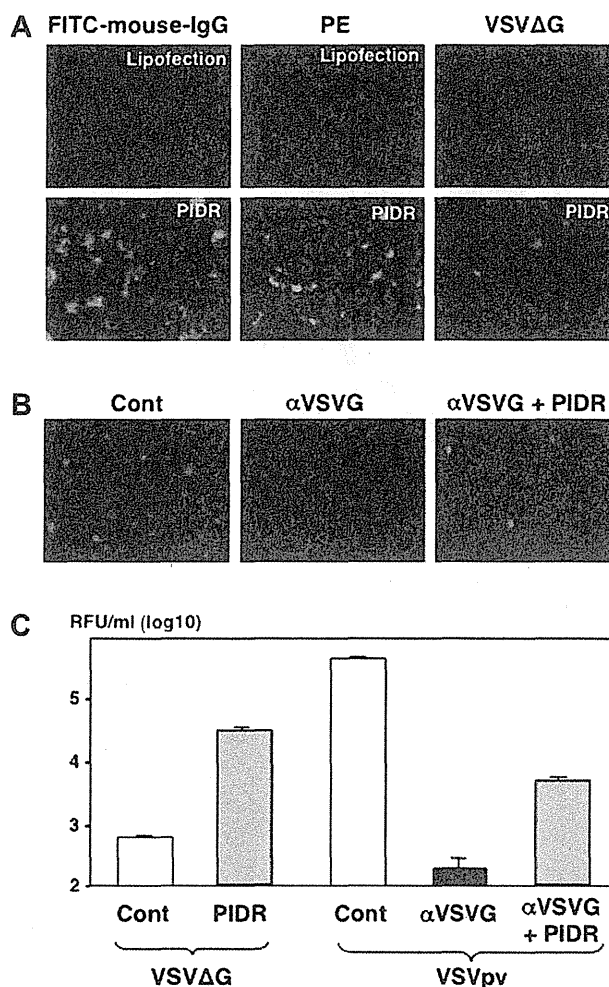


Fig. 1. Characterization of the intracellular delivery of proteins and viral particles by PIDR. (A) FITC-conjugated mouse IgG (left panels) or recombinant PE (center panels) was introduced into Huh7OK1 cells by the treatment with PIDR or a lipofection reagent. The expression of GFP upon infection of a pseudotype VSV lacking VSVG (VSV Δ Gpv/GFP) into Huh7OK1 cells in the presence (lower panel) and absence (upper panel) of PIDR was examined (right panels). (B) The effect of the presence of neutralization antibody on the delivery of viral particles by PIDR. Expression of GFP upon transduction of a VSVpv/GFP into Huh7OK1 cells in the presence of neutralization antibody and PIDR was examined. (C) The receptor-independent entry of viral particles was confirmed by using VSV Δ Gpv/luc and VSVpv/luc carrying the luciferase gene as a reporter.

luciferase gene as a reporter (Fig. 1C). These results indicate that PIDR is a useful tool to facilitate the entry of viral particles into target cells, irrespective of the authenticity of the envelope proteins of the particles or the presence of the neutralizing antibodies.

3.3. Effect of PIDR on the infection with HCVcc

To determine the effect of PIDR on the infection of HCV, HCVcc was inoculated into Huh7OK1 cells at an MOI of 0.05 in the presence or absence of PIDR and intracellular viral

RNA was measured every 24 h. No significant difference in the infection of HCVcc was observed by the addition of PIDR (Fig. 2A). Next, to mimic the infection of HCV in the presence of neutralization antibodies, HCVcc was mixed with CHS and

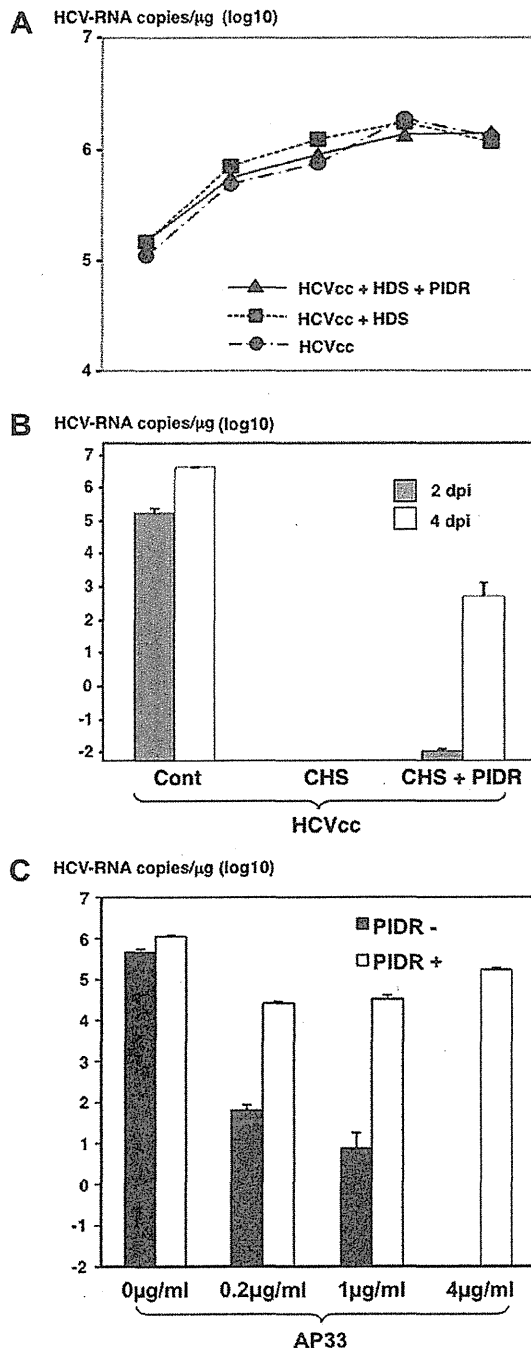


Fig. 2. Effect of PIDR on the infection with HCVcc. (A) HCVcc was inoculated into Huh7OK1 cells at an MOI of 0.05 in the presence or absence of healthy donor sera (HDS) and PIDR and intracellular viral RNA was measured every 24 h (B) HCVcc was mixed with sera from chronic hepatitis C patients (CHS) or/and PIDR and inoculated into Huh7OK1 cells. (C) HCVcc was pre-mixed with anti-E2 monoclonal antibody (AP-33) and inoculated into Huh7OK1 cells in the presence or absence of PIDR.

inoculated into Huh7OK1 cells. Although infection of HCVcc into Huh7OK1 was completely neutralized by the incubation with CHS, addition of PIDR recovered the infectivity of HCVcc (Fig. 2B). Furthermore, to confirm the effect of PIDR on the internalization of HCVcc interacting with neutralizing antibody, HCVcc was pre-incubated with AP-33 and inoculated into Huh7OK1 cells. Although infection of HCVcc was neutralized by the treatment with AP-33 in a dose-dependent manner, the neutralization by AP-33 was canceled by the treatment with PIDR (Fig. 2C). These results suggest that PIDR has the ability to internalize HCVcc even in the presence of neutralizing antibodies.

3.4. Effect of PIDR on the infection of HCVser

The efficient neutralizing activities of HCV infection in CHS were confirmed by a neutralization assay using pseudotype viruses. Infection of HCVpv bearing HCV E1 and E2 proteins but not of VSVpv bearing VSVG protein was significantly neutralized by the CHS (Fig. 3A). Next, to determine the effect of PIDR on the entry of HCVser in the presence of neutralizing antibodies, HCVser and CHS possessing the neutralizing antibodies against HCV were inoculated into Huh7OK1 cells with or without incubation with PIDR. Huh7OK1 cells inoculated with CHS pre-incubated with PIDR exhibited significantly higher HCV-RNA titers at 24 h post-infection than those without the treatment (Fig. 3B). Furthermore, to determine the amount of HCV internalized into cells, Huh7OK1 cells inoculated with CHS treated with PIDR were trypsinized and reseeded into a new culture plate at 24 h post-infection. HCV-RNA was detected in cells inoculated CHS pre-incubated with PIDR but not in those without PIDR treatment at 24 h after reseeding (Fig. 3C). These results indicate that treatment with PIDR permits HCVser to internalize into target cells even in the presence of neutralizing antibodies.

3.5. Neutralizing antibodies in sera from chronic hepatitis C patients enhance PIDR-mediated entry of HCV

No reduction of infectivity of HCVpv and VSVpv was observed by the incubation with AHS, suggesting that AHS possesses no detectable neutralizing antibodies to HCV (Fig. 4A). To examine the effect of neutralizing antibody on the intracellular delivery of HCVser by PIDR, AHS was incubated with the CHS carrying neutralization antibodies but no infectious HCV obtained from patients cured by the interferon therapy in the presence or absence of PIDR and inoculated into Huh7OK1 cells. Internalization of HCV in AHS was increased two-fold by the treatment with PIDR. However, intracellular viral RNA titer was slightly decreased by the incubation with CHS in the absence of PIDR, probably due to the neutralization by the antibodies, and addition of PIDR resulted in a three-fold enhancement of the entry of HCV in AHS in the presence of CHS compared with that in the absence of CHS (Fig. 4B). Next, Huh7OK1 cells were inoculated with HCVcc at an MOI of 0.05 after incubation with 0.4–40 μl/ml of HCV-negative CHS in the presence or absence of PIDR. Although infection of HCVcc was

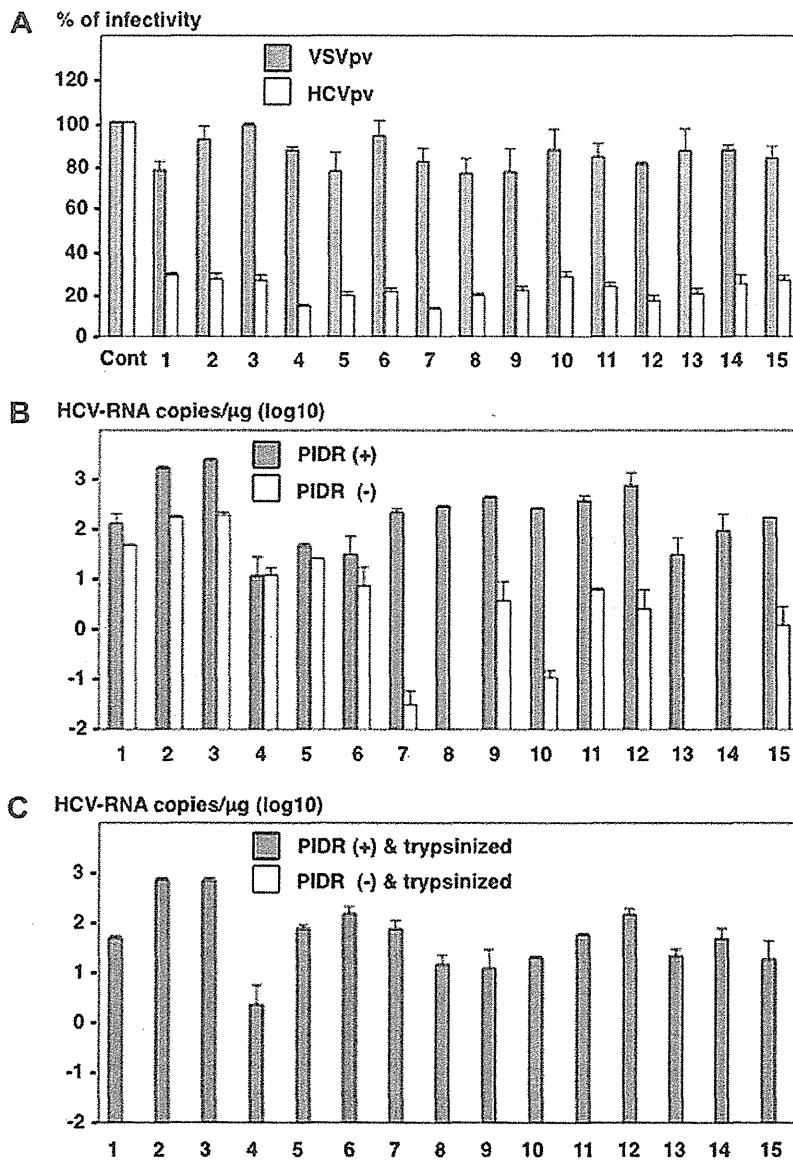


Fig. 3. Effect of PIDR on the infection with HCVser. (A) The neutralizing effect of antibodies in the CHS was determined by a neutralization assay using the pseudotype viruses. White and gray bars indicated VSVpv and HCVpv, respectively. (B) The effect of PIDR on the entry of HCVser in the CHS into Huh7OK1 cells. HCV-RNA titers in cells 24 h post-inoculation with HCVser in the presence and absence of PIDR are indicated by gray and white bars, respectively. (C) To determine the internalization of HCVser into Huh7OK1 cells, cells inoculated with the CHS in the presence (gray bar) or absence (white bars) of PIDR were trypsinized and reseeded into a new culture plate at 24 h post-infection, and HCV-RNA titers in the cells were determined at 24 h post-inoculation.

neutralized by CHS in a dose-dependent manner, addition of PIDR enhanced the infection of HCVcc in the presence of CHS in a dose-dependent manner (Fig. 4C). These results indicate that PIDR facilitates entry of HCVser in the presence of neutralizing antibody.

3.6. Human CD81-independent entry of HCVser by PIDR

Next, to determine the involvement of human CD81 (hCD81), a major receptor candidate for HCV [24], on the PIDR-mediated entry of HCVser, Huh7OK1 cells were pre-treated with anti-hCD81 antibody and inoculated with HCVser treated

with PIDR. Although pretreatment with anti-hCD81 antibody resulted in a significant reduction in the entry of HCVser, treatment with PIDR enhanced the entry of HCVser irrespective of the presence of the anti-human CD81 antibody (Fig. 5A). In addition, although entry of HCVser into HepG2 and HepCD81 cells was low and independent of the expression of hCD81, treatment with PIDR enhanced the entry of HCVser irrespective of the expression of human CD81 (Fig. 5B). These results suggest that the PIDR-mediated entry of HCVser is independent of the expression of hCD81 and is effective for the entry of HCVser into various cell lines other than Huh7-derived cell lines.