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Microbes and Infection 13 (2011) 405–412

www.elsevier.com/locate/micinf

Original article

# Intracellular delivery of serum-derived hepatitis C virus

Takasuke Fukuhara [a,b], Hideki Tani [a], Mai Shiokawa [a], Yukinori Goto [a], Takayuki Abe [a], Akinobu Taketomi [b], Ken Shirabe [b], Yoshihiko Maehara [b], Yoshiharu Matsuura [a,*]

[a] *Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan*
[b] *Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Received 7 September 2010; accepted 11 January 2011
Available online 22 January 2011

**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.


*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.

* Corresponding author. Fax: +81 6 6879 8269.
*E-mail address:* matsuura@biken.osaka-u.ac.jp (Y. Matsuura).

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doi:10.1016/j.micinf.2011.01.005

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 *Trans*IT-LT1 (Mirus, Madison, WI). The proteins were introduced into cells by PIDR (PULSin; 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 $1 \times 10^5$ cells/well in a 48-well plate and cultured for 24 h. For spinoculation, 2 μl of HCV-positive serum or HCVcc at a multiplicity of infection (MOI) of 0.05 were inoculated into cells and immediately centrifuged at 500× *g* for 120 min at room temperature. For infection of HCV by polybrene and PIDR, 2 μg of polybrene or 1.5 μl of PIDR were incubated with HCV-positive serum or HCVcc diluted in 20 μl 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 μg/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 (VSVΔGpv/GFP) into Huh7OK1 cells was examined. Although VSVΔGpv/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 VSVΔGpv/luc and VSVpv/luc carrying the 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.

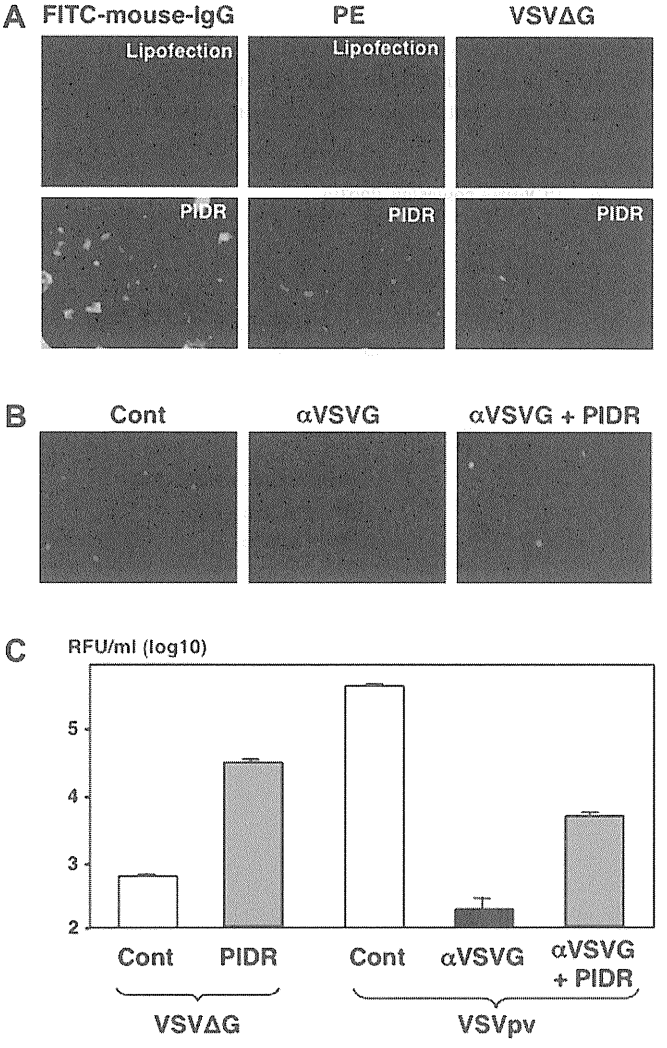


Fig. 1. Characterization of the intracellular delivery of proteins and viral particles by PIDR. (A) FITC-conjugated mouse IgG (left panels) or recombinant PE (center panels) was introduced into Huh7OK1 cells by the treatment with PIDR or a lipofection reagent. The expression of GFP upon infection of a pseudotype VSV lacking VSVG (VSVΔGpv/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 VSVΔGpv/luc and VSVpv/luc carrying the luciferase gene as a reporter.

### 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 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.

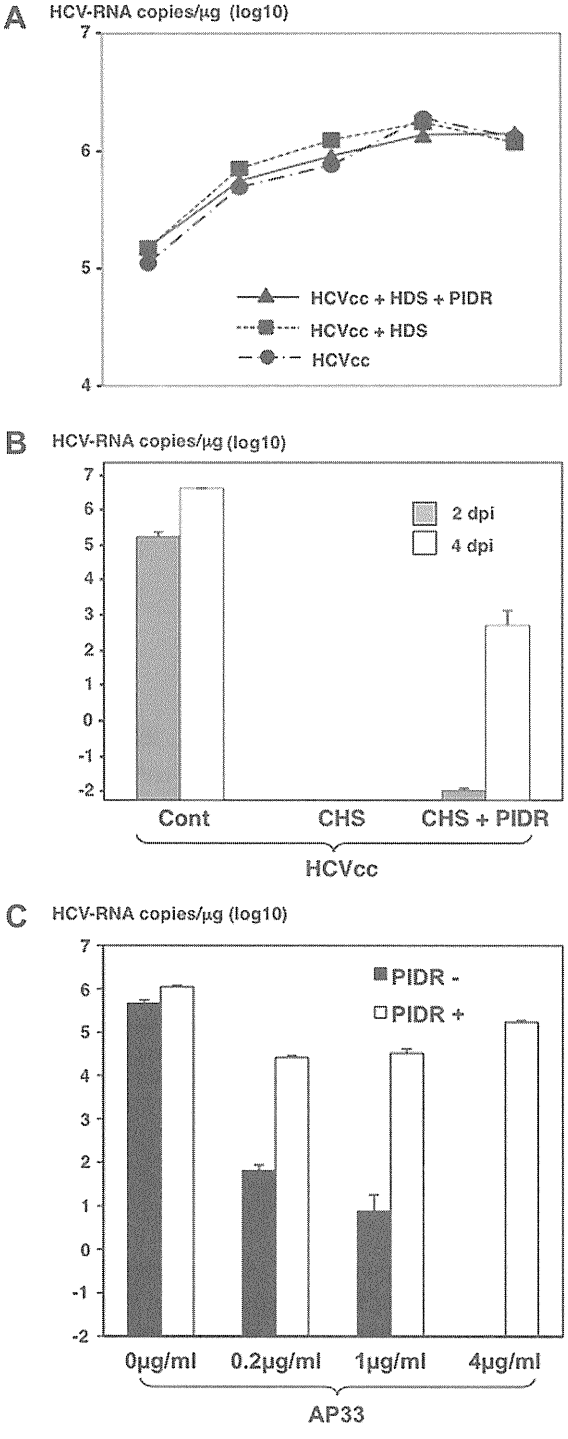


Fig. 2. Effect of PIDR on the infection with HCVcc. (A) HCVcc was inoculated into Huh7OK1 cells at an MOI of 0.05 in the presence or absence of healthy donor sera (HDS) and PIDR and intracellular viral RNA was measured every 24 h (B) HCVcc was mixed with sera from chronic hepatitis C patients (CHS) or/and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

### *3.4. Effect of PIDR on the infection of HCVser*

The efficient neutralizing activities of HCV infection in CHS were comfirmed 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

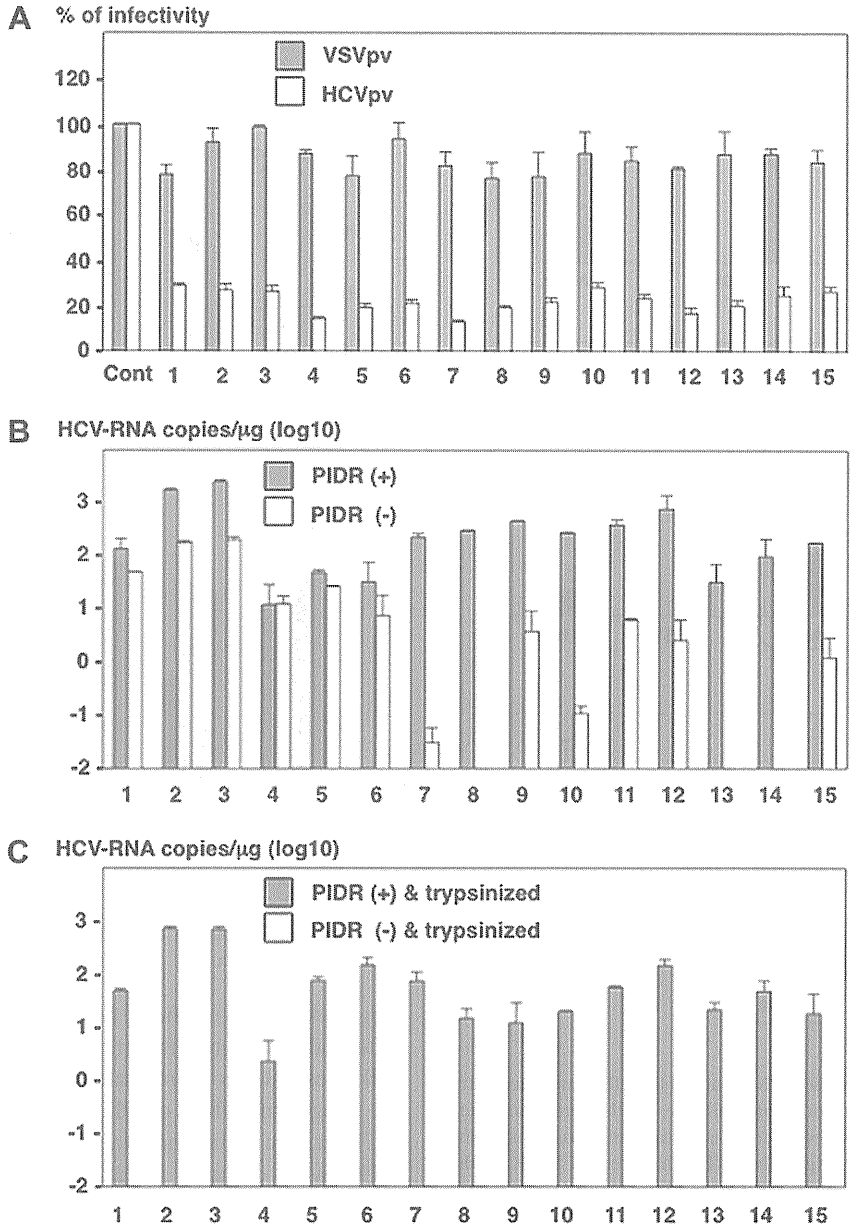


Fig. 3. Effect of PIDR on the infection with HCVser. (A) The neutralizing effect of antibodies in the CHS was determined by a neutralization assay using the pseudotype viruses. White and gray bars indicated VSVpv and HCVpv, respectively. (B) The effect of PIDR on the entry of HCVser in the CHS into Huh7OK1 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 CD81antibody (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.

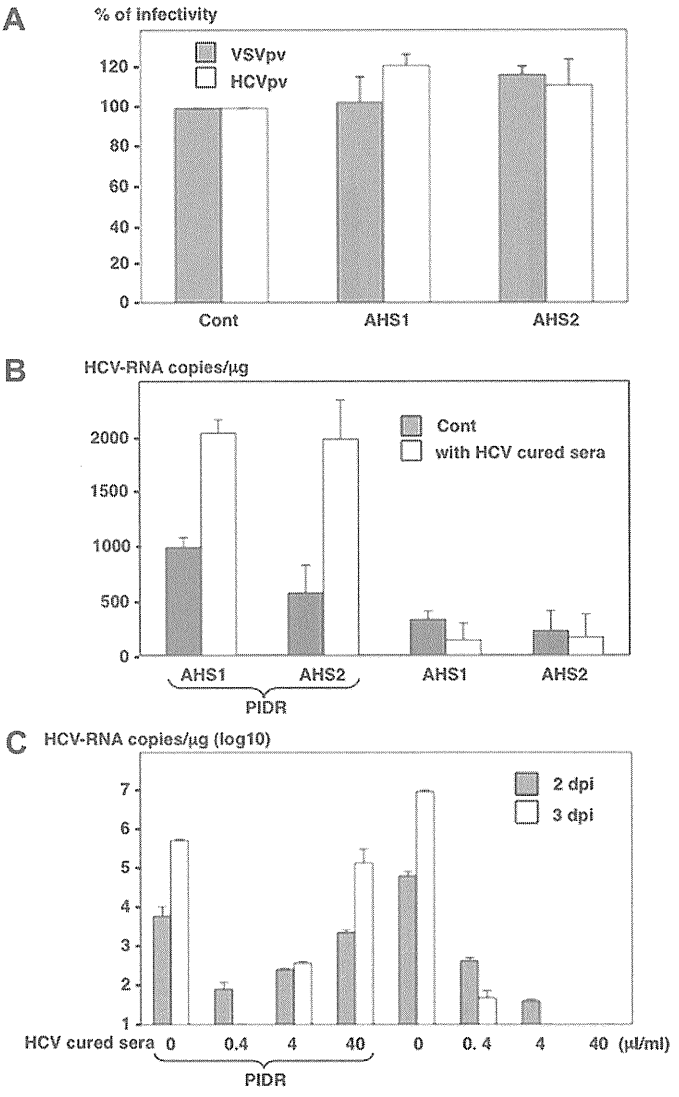


Fig. 4. Neutralizing antibodies in the CHS enhanced the PIDR-mediated entry of HCV. (A) The absence of neutralizing antibodies in the sera from acute hepatitis C patients (AHS) was determined by a neutralization assay using the HCVpv (white bars) and VSVpv (gray bar). (B) The effect of PIDR on the entry of HCVser into Huh7OK1 cells in the presence of neutralizing antibodies. The AHS were incubated with the CHS carrying neutralization antibodies but no infectious HCV obtained from patients cured by the IFN therapy (white bars) or HDS (gray bar) in the presence (left) or absence (right) of PIDR and inoculated into Huh7OK1 cells. The HCV-RNA titers in cells were determined at 24 h post-inoculation. (C). The effect of neutralizing antibodies on the PIDR-mediated infection of HCVcc. 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 (left) or absence (right) of PIDR. Gray and white bars indicate the HCV-RNA titers at 2 and 3 days after infection, respectively.

### *3.7. Effect of PIDR on the entry of HCVser into immortalized human hepatocytes*

Recently, Aly et al. reported that immortalized human hepatocytes, HuS/E2 cells, exhibited a high susceptibility to the infection with HCVser [7]. Therefore, we examined the effect of PIDR on the entry of HCVser into immortalized human hepatocytes, including Hc and HuS/E2 cells. The addition of PIDR enhanced the entry of HCVser into both Hc and HuS/E2 cells

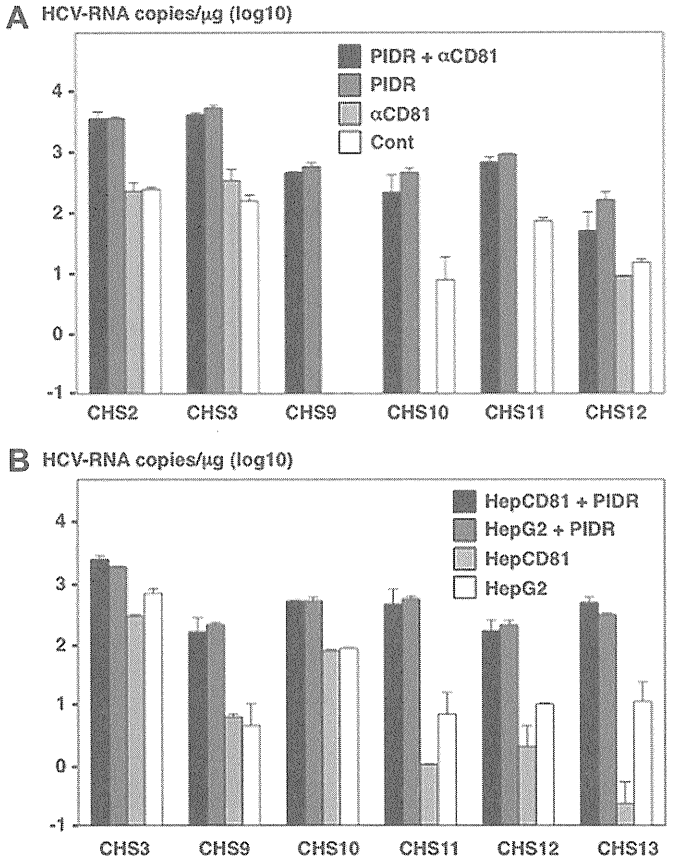


Fig. 5. Human CD81-independent entry of HCVser by PIDR. (A) The effect of anti-hCD81 antibody on the entry of HCVser into Huh7OK1 cells in the presence or absence of PIDR. (B) The effect of PIDR on the entry of HCVser into HepG2 and HepCD81 cells. HCV-RNA titers in cells were determined at 24 h post-inoculation.

(Fig. 6A and B). These results indicate that PIDR has the potential to enhance the entry of HCVser into not only cancer cell lines but also immortalized hepatocytes. Next, to evaluate the long-term effect of PIDR treatment on the infectivity of HCVser, Hc cells inoculated with CHS pre-incubated with PIDR were cultured for a long period. HCV-RNA could be detected at 10, 15 and 20 days after PIDR-mediated infection (Fig. 6C). However, significant elevations of HCV-RNA titers were not seen (Data not shown).

## 4. Discussion

In this study, we examined the efficiency of intracellular deliveries of HCVser by using spinoculation, polybrene and PIDR and found that the PIDR exhibited the highest efficacy on the entry of HCVser into target cells. Especially, trypsinization and reseeding of cells dramatically reduced HCV-RNA levels in groups that were not treated with PIDR as compared to those were treated with PIDR (Fig. 3B and C), and PIDR treatment dramatically increased the internalization of HCVcc treated with CHS or AP-33 at 2 and 4 days after infection (Fig. 2B and C). These results suggest that PIDR is feasible to deliver HCV/CHS complexes into target cells that allow productive infection. In addition, PIDR facilitated the entry of

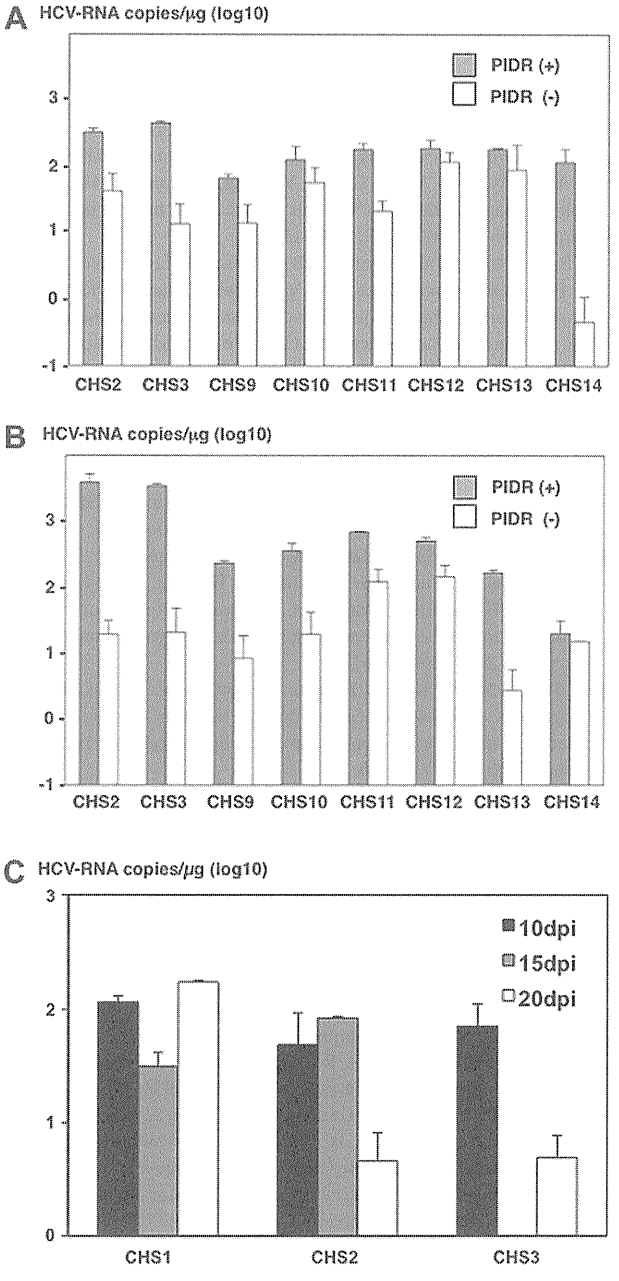


Fig. 6. Effect of PIDR on the entry of HCVser into immortalized human hepatocytes. The effect of PIDR on the entry of HCVser into immortalized human hepatocytes, such as Hc (A) and HuS/E2 (B) cells. The HCV-RNA titers in cells were determined at 24 h post-inoculation. (C) HCV-RNA titers in Hc cells inoculated with HCVser were evaluated at 10, 15 and 20 days after PIDR-mediated infection.

HCVser into the hepatoma cell lines and immortalized human hepatocytes in an hCD81-independent manner. Furthermore, we demonstrated that the intracellular delivery of HCVser by PIDR was enhanced by the addition of anti-HCV antibodies in sera from chronic hepatitis C patients, suggesting that PIDR is an effective reagent for the intracellular delivery of HCVser into the target cells in a receptor-independent manner.

Although direct evidence of enhancement of the adsorption and penetration by the application of spinoculation and polybrene has not been demonstrated yet, sedimentation of the virus particles to the cell surface by the spinoculation and electrostatic interactions between viral particles and cells by the charged polybrene are suggested to overcome the first barrier between virus particles and cells [12–14]. PIDR is a cationic amphiphilic-based protein delivery reagent that forms a complex with proteins through electrostatic and hydrophobic interactions [17]. The complexes of protein molecules and PIDR have been shown to interact with heparan sulfate proteoglycans on the cell surface, and then to be internalized through endocytosis, after which the protein molecules are released from the complexes into the cytoplasm [17], suggesting that PIDR is capable of enhancing not only adsorption but also penetration of HCVser.

Although HCVser are composed of heterogenous viral populations and a large fraction of the viral particles was associated with lipoproteins or neutralizing antibodies [25], these particles are capable of invading into human hepatocytes and establishing a persistent infection *in vivo* [1]. Therefore, it is feasible to speculate that some host factors are involved in the entry of HCVser into hepatocytes *in vivo*. Recently, Stamataki et al. [26] suggested that peripheral blood B lymphocytes participate as a reservoir for HCV for persistent infection and as a vehicle for transinfection to hepatocytes. Although the precise mechanisms of the entry of HCV have not been clarified yet, PIDR is an efficient modality to overcome the obstacles to the entry of HCV.

Recent studies have revealed that at least four cellular molecules play crucial roles in the infection of HCV into hepatocytes *in vitro*: hCD81, scavenger receptor class B type I (SR-BI) [27], and tight junction proteins claudin-1 [28] and occludin [11]. In this study, the entry of HCVser by the treatment with PIDR was shown to be independent from hCD81. Although the involvement of receptor candidates other than hCD81 was not examined in this study, PIDR was shown to enhance the entry of HCVser in cell lines including Huh7, HepG2, HepCD81, Hc and HuS/E2, suggesting that PIDR is capable of enhancing the entry of HCV through a pathway independent from the expression of these receptor candidates.

Previous studies have indicated that HCV infects not only hepatocytes but also lymphoid tissues and peripheral blood mononuclear cells [29], and that the quasispecies nature of viral particles was different among tissues infected with HCV [10]. Furthermore, it was shown that the *in vitro* transcribed JFH-1 RNA used for the recovery of infectious particles contained $2.21 \times 10^{11}$ copies/μg [30], which is much higher than the amount of viral RNA detected in the patient's sera. The variety of cell tropisms depending on the quasispecies of HCV particles, a low viral load in sera co-existing with neutralization antibodies, and the lack of identified co-factors including functional environment of the liver might be the major obstacles to establishing cell culture systems for the propagation of HCVser. Several approaches have been taken for the establishment of an *in vitro* cell culture system of HCV, including the culture of human liver cells in a three-dimensional radial-flow bioreactor [31], the three-dimensional culture of immortalized primary hepatocytes [7], and the micropatterned culture of primary hepatocytes [8]. These innovative approaches to the cell culture of liver cells, in combination with PIDR which is able to overcome the first barrier of HCV propagation might

contribute to a breakthrough in the establishment of a robust cell culture system of HCVser.

In this study, we demonstrated that PIDR is able to internalize HCV in a receptor-independent manner and provides a clue toward the development of a cell culture system of HCVser in the presence of neutralization antibodies. PIDR may also be useful for the study of viruses that are difficult to internalize into cells due to their low viral titers or the presence of neutralizing antibodies.

## Acknowledgments

We thank H. Murase for secretarial work.

This research was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare, and the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

## References

[1] L.-B. Seeff, Natural history of chronic hepatitis C, Hepatology 36 (2002) S35–S46.

[2] M.-P. Manns, J.-G. McHutchison, S.-C. Gordon, V.-K. Rustgi, M. Shiffman, R. Reindollar, Z.-D. Goodman, K. Koury, M. Ling, J.-K. Albrecht, Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial, Lancet 358 (2001) 958–965.

[3] J.-M. Pawlotsky, S. Chevaliez, J.-G. McHutchison, The hepatitis C virus life cycle as a target for new antiviral therapies, Gastroenterology 132 (2007) 1979–1998.

[4] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.-G. Krausslich, M. Mizokami, R. Bartenschlager, T. Jake-Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[5] N. Hiraga, M. Imamura, M. Tsuge, C. Noguchi, S. Takahashi, E. Iwao, Y. Fujimoto, H. Abe, T. Maekawa, H. Ochi, C. Tateno, K. Yoshizawa, A. Sakai, Y. Sakai, M. Honda, S. Kaneko, T. Wakita, K. Chayama, Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon, FEBS Lett. 581 (2007) 1983–1987.

[6] S. Molina, V. Castet, L. Pichard-Garcia, C. Wychowski, E. Meurs, J.-M. Pascussi, C. Sureau, J.-M. Fabre, A. SaCunha, D. Larrey, J. Dubuisson, J. Coste, J. McKeating, P. Maurel, C. Fournier-Wirth, Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent, J. Virol. 82 (2008) 569–574.

[7] H.-H. Aly, Y. Qi, K. Atsuzawa, N. Usuda, Y. Takada, M. Mizokami, K. Shimotohno, M. Hijikata, Strain-dependent viral dynamics and virus-cell interactions in a novel in vitro system supporting the life cycle of blood-borne hepatitis C virus, Hepatology 50 (2009) 689–696.

[8] A. Ploss, S.-R. Khetani, C.-T. Jones, A.-J. Syder, K. Trehan, V.-A. Gaysinskaya, K. Mu, K. Ritola, C.-M. Rice, S.-N. Bhatia, Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures, Proc. Natl. Acad. Sci. U. S. A 107 (2010) 3141–3145.

[9] D.-R. Burton, Antibodies, viruses and vaccines, Nat. Rev. Immunol. 2 (2002) 706–713.

[10] R. Sobesky, C. Feray, F. Rimlinger, N. Derian, A. Dos Santos, A.-M. Roque-Afonso, D. Samuel, C. Brechot, V. Thiers, Distinct hepatitis C virus core and F protein quasispecies in tumoral and nontumoral hepatocytes isolated via microdissection, Hepatology 46 (2007) 1704–1712.

[11] A. Ploss, M.-J. Evans, V.-A. Gaysinskaya, M. Panis, H. You, Y.-P. de Jong, C.-M. Rice, Human occludin is a hepatitis C virus entry factor required for infection of mouse cells, Nature 457 (2009) 882–886.

[12] N. Landazuri, J.-M. Le Doux, Complexation of retroviruses with charged polymers enhances gene transfer by increasing the rate that viruses are delivered to cells, J. Gene Med. 6 (2004) 1304–1319.

[13] U. O'Doherty, W.-J. Swiggard, M.-H. Malim, Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding, J. Virol. 74 (2000) 10074–10080.

[14] R. Watanabe, S. Matsuyama, F. Taguchi, Receptor-independent infection of murine coronavirus: analysis by spinoculation, J. Virol. 80 (2006) 4901–4908.

[15] L. Ye, X. Wang, S. Wang, G. Luo, Y. Wang, H. Liang, W. Ho, Centrifugal enhancement of hepatitis C virus infection of human hepatocytes, J. Virol. Methods 148 (2008) 161–165.

[16] I. Benedicto, F. Molina-Jimenez, B. Bartosch, F.-L. Cosset, D. Lavillette, J. Prieto, R. Moreno-Otero, A. Valenzuela-Fernandez, R. Aldabe, M. Lopez-Cabrera, The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection, J. Virol. 83 (2009) 8012–8020.

[17] C.-O. Weill, S. Biri, A. Adib, P. Erbacher, A practical approach for intracellular protein delivery, Cytotechnology 56 (2008) 41–48.

[18] T. Okamoto, H. Omori, Y. Kaname, T. Abe, Y. Nishimura, T. Suzuki, T. Miyamura, T. Yoshimori, K. Moriishi, Y. Matsuura, A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication, J. Virol. 82 (2008) 3480–3489.

[19] H. Tani, Y. Komoda, E. Matsuo, K. Suzuki, I. Hamamoto, T. Yamashita, K. Moriishi, K. Fujiyama, T. Kanto, N. Hayashi, A. Owsianka, A.-H. Patel, M.-H. Whitt, Y. Matsuura, Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins, J. Virol. 81 (2007) 8601–8612.

[20] H.-H. Aly, K. Watashi, M. Hijikata, H. Kaneko, Y. Takada, H. Egawa, S. Uemoto, K. Shimotohno, Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes, J. Hepatol. 46 (2007) 26–36.

[21] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.-R. Burton, S.-F. Wieland, S.-L. Uprichard, T. Wakita, F.-V. Chisari, Robust hepatitis C virus infection in vitro, Proc. Natl. Acad. Sci. U. S. A 102 (2005) 9294–9299.

[22] T. Morris, B. Robertson, M. Gallagher, Rapid reverse transcription-PCR detection of hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system, J. Clin. Microbiol. 34 (1996) 2933–2936.

[23] R.F. Clayton, A. Owsianska, J. Aitken, S. Graham, D. Bhella, A.H. Patel, Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles, J. Virol. 76 (2002) 7672–7682.

[24] P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A.-J. Weiner, M. Houghton, D. Rosa, G. Grandi, S. Abrignani, Binding of hepatitis C virus to CD81, Science 282 (1998) 938–941.

[25] D. Lavillette, Y. Morice, G. Germanidis, P. Donot, A. Soulier, E. Pagkalos, G. Sakellariou, L. Intrator, B. Bartosch, J.-M. Pawlotsky, F.-L. Cosset, Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection, J. Virol. 79 (2005) 6023–6034.

[26] Z. Stamataki, C. Shannon-Lowe, J. Shaw, D. Mutimer, A.-B. Rickinson, J. Gordon, D.-H. Adams, P. Balfe, J.-A. McKeating, Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells, Blood 113 (2009) 585–593.

[27] E. Scarselli, H. Ansuini, R. Cerino, R.-M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, A. Vitelli, The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus, EMBO. J. 21 (2002) 5017–5025.

[28] M.-J. Evans, T. von Hahn, D.-M. Tscherne, A.-J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J.-A. McKeating, P.-D. Bieniasz, C.-M. Rice, Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry, Nature 446 (2007) 801–805.

[29] J.-T. Blackard, N. Kemmer, K.-E. Sherman, Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences, Hepatology 44 (2006) 15–22.

[30] T. Kato, T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, T. Wakita, Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon, Gastroenterology 125 (2003) 1808–1817.

[31] H. Aizaki, S. Nagamori, M. Matsuda, H. Kawakami, O. Hashimoto, H. Ishiko, M. Kawada, T. Matsuura, S. Hasumura, Y. Matsuura, T. Suzuki, T. Miyamura, Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor, Virology 314 (2003) 16–25.

Virus Research xxx (2011) xxx–xxx

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Review

# Structure of hepatitis E viral particle

Yoshio Mori[a,b], Yoshiharu Matsuura[a,*]

[a] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan
[b] Department of Virology III, National Institute of Infectious Diseases, Tokyo, Japan

ARTICLE INFO

*Article history:*
Available online xxx

*Keywords:*
Hepatitis E virus
Capsid protein
Crystal structure
Particle assembly

ABSTRACT

Hepatitis E is acute hepatitis caused by infection of hepatitis E virus (HEV) via a fecal-to-oral or zoonotic route. HEV is a small, non-enveloped virus containing positive strand RNA as a genome. Recently, the three-dimensional structures of the HEV-like particles and spike domain protruded from the surface of the particle expressed by recombinant baculovirus or bacteria have been revealed. Based on these reports, the structural features of the HEV capsid subunit and viral particle are reviewed to give insights to the mechanisms underlying the particle assembly, antigenicity, host cell attachment and native virion packaging.



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## 1. Introduction

Earlier studies using immuno-electron microscopy revealed that hepatitis E virus (HEV) obtained from human fecal specimens was a nonenveloped icosahedral particle with indentations on the surface (Balayan et al., 1983; Bradley et al., 1988; Sreenivasan et al., 1984). Particles purified by sucrose gradient centrifugation had a diameter of 320–340 Å (Bradley et al., 1988). Based on observation by electron microscopy, the morphology of HEV was similar to those of "small round viruses" in feces samples, such as Norwalk virus (the family *caliciviridae*) and hepatitis A virus (the family *picornaviridae*). Like other hepatitis viruses, HEV could not efficiently replicate in cell culture until recently. Therefore, almost of detailed structural analyses regarding HEV particles relied upon recombinant proteins by baculoviral or bacterial expression system.

HEV is the sole member of the genus *hepevirus* within the family *hepeviridae* (Panda et al., 2007). This virus has a single, positive-stranded RNA genome of 7.2 kb in length, which is capped with m7G and polyadenylated at the 5′- and 3′-termini, respectively (Okamoto, 2007). The genome contains three open reading frames (ORF), ORF1, ORF2 and ORF3. The viral capsid protein encoded by ORF2 works for particle assembly, binding to host cells, and eliciting of neutralizing antibodies. Expression of truncated capsid protein in insect cells by baculovirus expression system resulted in self-assembly of the capsid protein and production of two types of HEV-like particle (HEV-LP) with different diameters (Li et al., 1997, 2005, 2007; Xing et al., 2010). In this review, the small and large HEV-LPs were designated as HEV-LP/$T=1$ and HEV-LP/$T=3$, respectively, based on difference of packaging of the capsid protein

*Abbreviations:* HEV, hepatitis E virus; HEV-LP, HEV-like particle; HSPGs, heparan sulfate proteoglycans; NOB, neutralizing-of-binding; ORF, open reading frame.

* Corresponding author. Tel.: +81 6 6879 8340; fax: +81 6 6879 8269.
*E-mail address:* matsuura@biken.osaka-u.ac.jp (Y. Matsuura).

0168-1702/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.virusres.2011.03.015

**Table 1**
Structural properties of HEV-LPs and HEV virion.

| | HEV-LP/$T=1$ | HEV-LP/$T=3$ | HEV virion |
|---|---|---|---|
| Diameter | 270 Å[a] | 410 Å[b] | 320–340 Å[c] |
| Amino acid residues forming particle | ORF2 aa126–601 (as a minimum requirement)[d] | ORF2 aa112–608?[b] | ORF2 aa1–660? |
| Number of capsid subunit | 60[a] | 180[b] | 180? |
| Triangulation number | $T=1$[a] | $T=3$[b] | $T=3$? |
| RNA packaging | No[a] | Yes[b] | Yes |

[a] Xing et al. (1999).
[b] Xing et al. (2010).
[c] Bradley et al. (1988).
[d] Li et al. (2005).

(Table 1 and see below). The structural analyses of HEV-LP/$T=1$ preceded those of HEV/$T=3$ because of simplicity of purification of the former from the cell supernatant. In the first structural study using a low-resolution (22 Å) cryoelectron microscopy, it was shown that the genotype 1 HEV-LP/$T=1$ formed $T=1$ icosahedral particle composed of 60 copies of the truncated capsid protein (Xing et al., 1999). HEV-LP/$T=1$ appeared to be empty due to no significant density of RNA inside and exhibited 270 Å in diameter, which is less than the diameter of partially purified native virions. However, HEV-LP/$T=1$ displayed similar properties to the native HEV particles in terms of antigenicity and surface substructure (Li et al., 2004; Xing et al., 1999). Thus, HEV-LP/$T=1$ is thought to be a good material to approach a three-dimensional structure of the native HEV. Until now, three laboratories, including us, succeeded to resolve the crystal structures of HEV-LP/$T=1$ of genotypes 1 (Xing et al., 2010), 3 (Yamashita et al., 2009) and 4 (Guu et al., 2009). Meanwhile, a cryoelectron microscope structure of HEV-LP/$T=3$ has been reported very recently (Xing et al., 2010), suggesting the more plausible packaging of the HEV virion. In addition, another study using a bacteria expression system illustrated the more detailed structure of the protruding spike domain of HEV (Li et al., 2009). Here, the accumulating information from mainly these reports is reviewed to understand the structural basis regarding the particle assembly, antigenicity, host cell binding and the native virion packaging.

## 2. Primary structure of the HEV capsid protein

ORF2 encodes the major capsid protein composed of 660 amino acid residues (Fig. 1). Among four major mammalian HEV genotypes, genetic homology of amino acid residues of the capsid

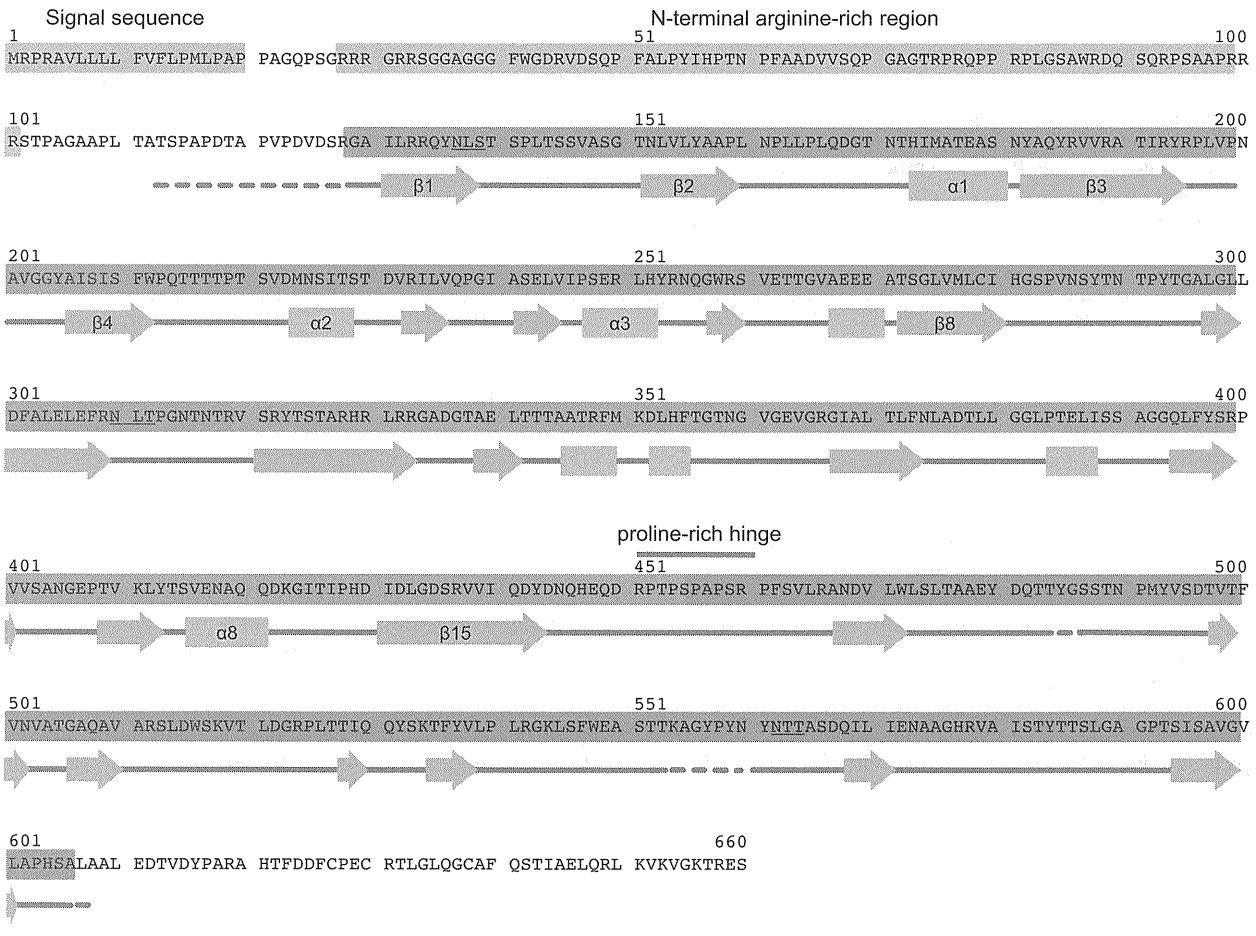


**Fig. 1.** Secondary structure assignment of the capsid protein of HEV-LP. It is represented based on the data from the crystal structure of HEV-LP of the genotype 3 2712 strain (PDB ID, 2ZTN). The truncated form of the amino acid residues 112–608 is used for production of HEV-LP. The S, M and P domains are shown in pink, green and blue, respectively. α-Helices, β-sheets and loops are indicated as orange rectangles, purple arrows and thick lines, respectively. Disorder regions are shown by dotted lines.

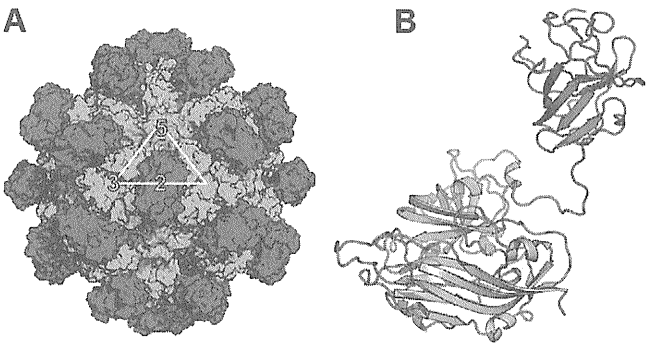


**Fig. 2.** Crystal structures of HEV-LP (A) and the capsid protein monomer (B). The S, M and P domains are shown in pink, green and blue, respectively. (A) The surface diagram of HEV-LP. Representatives of 2-, 3- and 5-fold icosahedral axes are indicated.

protein was over 85% (data not shown). Many of amino acid divergences were found in the N-terminal 111 amino acids residues. Compared to other nonenvelope viruses, it is notable that the HEV capsid protein has a property of general secreted proteins, which contain a signal sequence at the N-terminus and N-linked glycosylation sites (three asparagine residues 137, 310, 562) (Fig. 1). Although the capsid protein expressed in mammalian cells was detected as 74 kDa unglycosylated and 88 kDa glycosylated forms (Jameel et al., 1996), it is controversial which forms (or both) build the virion. The N-terminal domain followed by the signal sequence (residues 28–101) is an arginine-rich domain resemble to an RNA-binding domain of the coat proteins of tombusviruses. In insect cells, the HEV capsid protein was proteolytically cleaved, resulting in yields of several polypeptides with various molecular weights (Li et al., 1997). The 53 kDa-truncated form, which lacked the signal sequence, N-terminal arginine-rich and C-terminal domains, was able to self-assemble to the HEV-LP/$T=1$ in the supernatant of insect Tn5 cells. Minimum requirement for assembly of HEV-LP/$T=1$ were amino acid residues 126–601(Li et al., 2005). Another truncated form of 64 kDa was highly associated with cells, but it was also self-assembled to HEV-LP/$T=3$ (Li et al., 2007; Xing et al., 2010). The precise amino acid residues consisting the 64 kDa capsid protein are not known. However, the N-terminal domain followed by the putative signal sequence was required for HEV-LP/$T=3$ formation because it was detected upon expression of the capsid protein including amino acids 14–608, but not 112–608 (data not shown).

## 3. Overall structure of HEV-LP/$T=1$

The crystal structures of HEV-LP/$T=1$ derived from the genotypes 1 (PDB ID; 2ZZQ), 3 (PDB ID; 2ZTN) and 4 (PDB ID; 3HAG) were resolved at the resolution of amino acid level (3.5–3.8 Å) (Guu et al., 2009; Xing et al., 2010; Yamashita et al., 2009). These structures were almost identical in spite of some divergence of amino acid residues (Xing et al., 2010). HEV-LP/$T=1$ showed a $T=1$ symmetry with an internal diameter of approximately 125 Å and an external diameter of approximately 270 Å. The particles are composed of 60 subunits of the truncated capsid proteins, and icosahedral 2-, 3- and 5-fold axes were found in the particle (Fig. 2A). The pronounced structural feature is 30 dimeric protruding spikes of 30 Å in length at the surface of the 2-fold axes. Board depressions are located around the 5-fold axis.

## 4. Tertiary structure of the HEV capsid protein

The crystal structures of the capsid monomer building HEV-LP/$T=1$ showed that they could be divided into three domains designated as S (shell), M (middle) and P (protruding) (Xing et al., 2010; Yamashita et al., 2009) (Figs. 1 and 2). In the report by Guu et al. (2009), the M and P domains were designated as the P1 and P2 domains, respectively, according to the finding of moderate protrusions formed by the M domain over the internal shell of the particle. These domains are composed of 128–319, 320–455 and 456–606, respectively, in the case of the genotype 3 HEV-LP/$T=1$ (Yamashita et al., 2009). All of the three domains fold to β-barrel structures by anti-parallel β-sheets (Figs. 1 and 2B). The S domain displays a typical jerry roll-like β-barrel fold as observed in various $T=3$ viral capsid proteins. Interestingly, a structural homolog search revealed that the HEV S domain is more related to the jerry-roll domains of coat proteins of members of the genera *tombusviruses* and *sobemoviruses*, which are plant small round viruses with a $T=3$ symmetry, than those of any animal viruses (Guu et al., 2009). Among animal viruses, the domain of the Norwalk virus capsid protein, which was thought to be originally related to that of HEV, exhibited much less homology than that of Seneca Valley virus, an oncolytic picornaviruses (Guu et al., 2009).

The other domains, M and P, represent very remarkable structure compared with viral capsid proteins that have been ever reported (Fig. 2B). The M domain is tightly associated with the S domain and locates at the surface around the 3-fold axis of the particle. The related domains were not found among the small round viruses with a $T=3$ symmetry with the structural similarity (Guu et al., 2009). The P domain dimer forms the protruding spike around the 2-fold axis of HEV-LP/$T=1$ as well as those of the caliciviruses and the plant tombusviruses. However, the HEV P domain also does not share significant structural homology with any other viral proteins (Guu et al., 2009). The P domain of the caliciviruses could divide into two subdomains, P1 and P2 (Chen et al., 2006; Prasad et al., 1999), while the HEV P domain is composed of a single domain. The size of HEV P domain is roughly half of those of the caliciviruses. Furthermore, the P dimer of HEV indicates a crossing topology of the P versus M and S domains while those of the caliciviruses do a parallel topology (Yamashita et al., 2009) (Fig. 3A). The flexibility of a long proline-rich hinge region linked between the M and P domains (amino acid residues 445–467) allows this unique topology of HEV-LP/$T=1$. In addition, the hinge also contributes to topological change of the protruding spike in HEV-LP/$T=3$ (see below) (Xing et al., 2010).

Approximately 10 amino acid residues at the N-terminus of the truncated capsid proteins building HEV-LP/$T=1$ (amino acid residues 118–127) of the genotypes 1 and 4 were resolved to be as the extended arm domain (Guu et al., 2009; Xing et al., 2010), of which related domain was found in the coat protein of tombusvirus. The extended loop interacts the adjacent capsid subunit, contributing formation of the capsid protein dimer of HEV-LP/$T=1$.

## 5. Packaging of HEV-LP/$T=1$

Three different interactions of capsid subunits are required for packaging of HEV-LP/$T=1$; briefly dimeric, trimeric and pentameric interactions around 2-, 3- and 5-fold icosahedral axes, respectively. The three domains, S, M and P, play different roles of the packaging of HEV-LP/$T=1$. The S domain is involved in all the interactions and builds the integral shell by itself. The inner surface of the shell is rich in basic amino acids (6 arginine residues per one subunit) (Guu et al., 2009). This cluster of arginine residues may contribute to neutralize negative charges of the genomic RNA. The M domain located around the 3-fold axis and is involved in trimeric interaction (Fig. 3B). The domain also interacts with the P domain of counterpart, contributing to the dimeric interaction to some extent (Yamashita et al., 2009). The P domain works only dimeric interaction. It was reported that the P domain expressed alone formed a strong dimer (Li et al., 2009). The tight interaction is

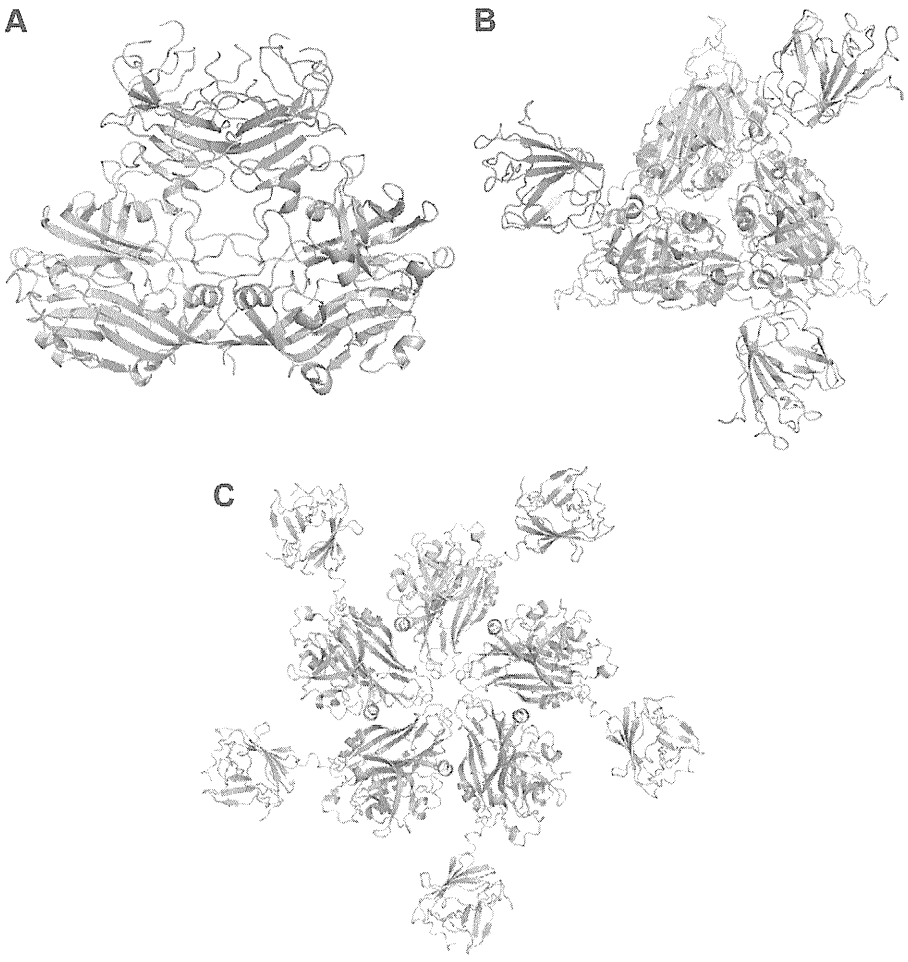


**Fig. 3.** Packagings of dimer, trimer and pentamer at 2-, 3- and 5-fold icosahedral axes, respectively. The side view of the dimer (A) and the views from outside of the trimer (B) and pentamer (C) are illustrated. The S, M and P domains of one monomer are shown in pink, green and blue, respectively.

mediated by a hydrophobic cluster of the dimer interface (Li et al., 2009).

The surface areas buried by the dimeric, trimeric and pentameric interactions were approximately 5300–5900 Å$^2$, 3000–3400 Å$^2$ and 1400–1600 Å$^2$, respectively, suggesting that the pentameric formation could limit rate of particle assembly (Guu et al., 2009; Xing et al., 2010). Notably, the center of the pentamer structure is thinnest in the particle (Yamashita et al., 2009) (Fig. 3C). The mutagenesis analysis revealed that Tyr288 in the center of the pentamer was crucial for the particle formation (Yamashita et al., 2009). Aromatic amino acids (phenylalanine and tryptophan) were replaceable to Tyr288 in terms of the particle formation. This residue is completely conserved in major mammalian and avian genotypes of HEV (Yamashita et al., 2009). Meanwhile, the other aromatic residues were found in the corresponding position of the novel HEV found in wild rats (Johne et al., 2010) and the structurally related caliciviruses and tombusviruses (Yamashita et al., 2009). This suggests that the aromatic amino acid residues in the center of 5-fold axis are functionally conserved during the evolutional process.

Based on the width of the interaction area, it is hypothesized that the packaging of HEV proceeds in the sequence of dimeric, trimeric, and pentameric formation. Xing et al. (2010) attempted to evaluate the packaging sequence using an *in vitro* reassembly assay of the capsid proteins disassembled from HEV-LP/$T$ = 3 by a combination of chelating and reduced reagents. As a result, decamer complexes (pentamers of dimer), but not hexamers (trimers of dimer), were observed in the assay, suggesting that the pentamer formation is more preferentially occurred than the trimer formation via not only simple protein–protein interactions.

## 6. Cell-attachment region and antigenic sites of neutralizing antibodies

It was reported that HEV-LP/$T$ = 1 bond to cell surface via heparan sulfate proteoglycans (HSPGs), and HSPGs were required for HEV infection in culture cells (Kalia et al., 2009). In the cases of Norwalk virus and feline calicivirus, the apical surface of the protruding spike was involved in binding to the receptor molecules, histo-blood antigens (Bu et al., 2008; Choi et al., 2008) and feline junctional adhesion molecule (Bhella et al., 2008), respectively. In order to determine crucial amino acid residues for cell-binding, we examined the cell-attachment activity of a series of HEV-LP/$T$ = 1 with double or single substitutions in amino acid residues at the surface of the P domain in the previous study (Yamashita et al., 2009). The result showed that the double substitutions, T489A and P491A, N560A and Y561A, N562A and T564A, or T585A and T586A, resulted in lack of attachment activity of HEV-LP/$T$ = 1 to Huh7 and A549 cells, which were known to be susceptive to HEV infection (Fig. 4A). These residues were mapped in the exposed loops of the apical center region of the protruding spike, suggesting that this region is involved in binding to receptor molecules such as HSPGs. The data suggesting that the dimer formation of HEV capsid protein was prerequisite for the receptor-binding (He et al., 2008) would support the involvement of the apical surface near the dimeric interacting domain of the P dimer in cell-attachment.

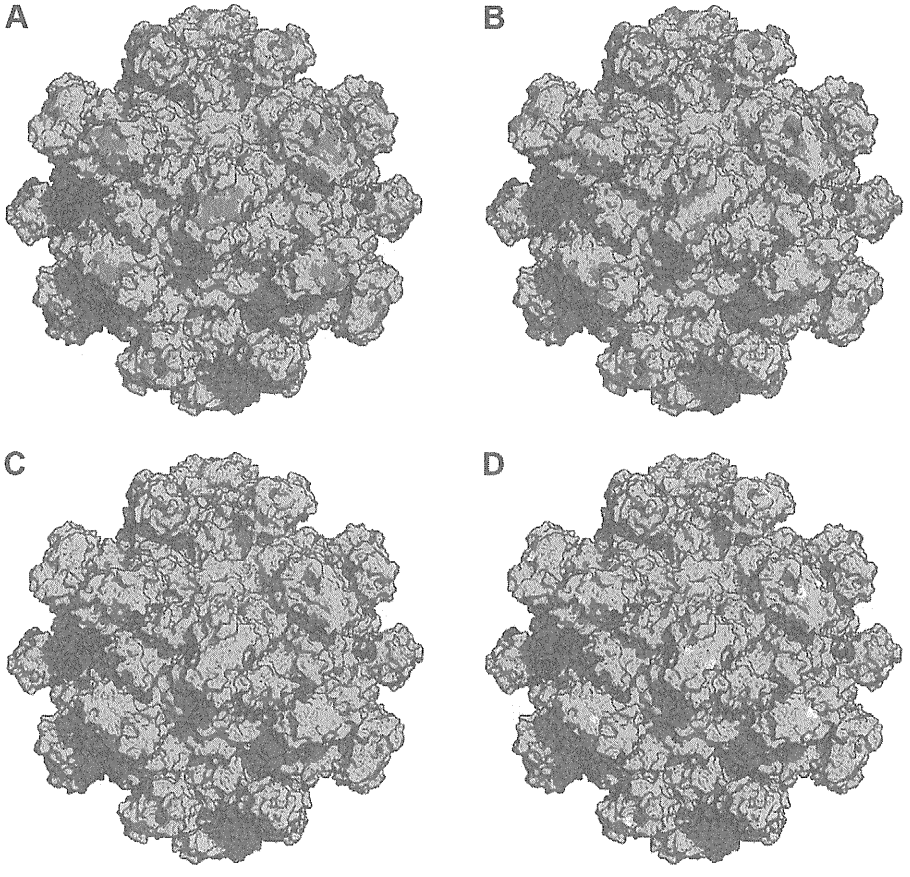


**Fig. 4.** Functional domains mapped in the surface diagrams of HEV-LP. Amino acid residues involved in the cell-attachment (A; red) and in the recognition by NOB antibodies, MAB1323 (B; blue) and MAB272 (C; green), were determined in the previous study (Yamashita et al., 2009). (D) One of the potential N-glycosylation sites, Asn562 (yellow), was located near the cell-attachment region and the epitope of MAB1323.

Several neutralizing or neutralizing-of-binding (NOB) antibodies against the HEV capsid protein were reported (Emerson et al., 2006; He et al., 2008; Schofield et al., 2000, 2003; Takahashi et al., 2008a; Yamashita et al., 2009). The NOB antibodies inhibit cell-attachment of HEV-LP/$T=1$. The linear epitopes were found in the M and P domains (He et al., 2008; Schofield et al., 2000) while the conformational epitopes were in the P domain (Yamashita et al., 2009), suggesting that the M and P domains play a role on entry steps. Especially, the epitopes of the neutralizing antibody reported by Schofield et al. (2000) and the NOB antibody MAB1323 by us (Yamashita et al., 2009) were mapped at the apical region of the protruding spike, overlapping with the cell-attachment region (Fig. 4B). Thus, these antibodies could physically hamper the attachment of HEV-LP/$T=1$. Another NOB antibody MAB272 in our study recognized the side surface of the P domain just over the M domain (Yamashita et al., 2009) (Fig. 4C). The result might support an involvement of the M domain in entry steps although it is unclear the mechanism. Guu et al. (2009) found the structural similarity of the HEV M domain to the endosialidase of bacteriophage K1F, which binds to sialic acid molecules. Therefore, the M domain might be involved in binding to another cell receptors.

## 7. Glycosylation of the HEV capsid protein

Because of a lack of the signal peptide-like sequence of the HEV-LP capsid protein, it was not glycosylated in spite of containing the three potential N-glycosylation sites, Asn137, Asn310 and Asn562. In the 3D structure of HEV-LP/$T=1$, Asn137 is mapped adjacent to the interface of the capsid pentamer and Asn310 is completely hidden by the interface of the capsid subunit trimer. Therefore, if it occurs at all, this modification at these sites will interfere the assembly of at least HEV-LP/$T=1$. Interestingly, Asn562 was located in the apical center of the protruding spike as well as the cell-attachment region and antigenic sites of some neutralizing antibodies (Fig. 4D). Sugar chain at Asn562 probably masks these regions. Therefore, it is supposed that non-glycosylated form of the capsid protein assembles into fully functional virus particle while the glycosylated form works unknown functions other than particle assembly.

## 8. A packaging model of a native $T=3$ virion

Viruses with a $T=3$ symmetry often produces $T=1$ small particles. Both particles are generally composed of identical capsid subunit but of different copy numbers ($T=3$; 180 subunits, $T=1$; 60 subunits). Previous studies on plant tombusviruses indicated that the N-terminal domain played an important role in switching of transition from $T=3$ to $T=1$ symmetry (Hsu et al., 2006; Kakani et al., 2008). In this context, it is thought that HEV-LP/$T=1$ production is caused by deletion of the N-terminal basic domain and the native virion has a $T=3$ symmetry. A recent report by Xing et al. (2010) strongly supported this concept. Upon infection in insect cells with the recombinant baculovirus harboring the HEV capsid protein with deletion of the only N-terminal 13 amino acids, two kinds of particles, HEV-LP/$T=1$ and HEV-LP/$T=3$, which were composed of approximately 53 kDa and 64 kDa capsid proteins, respectively, were yielded. They successfully illustrated cryoelectron microscopy image of HEV-LP/$T=3$. The HEV-LP/$T=3$ had an overall diameter of 410 Å. The HEV-LP/$T=3$ had a $T=3$ symmetry and was composed of 180 copies of the capsid protein, which

were grouped into three unique monomers (A, B and C monomers) according to their geometric environments. Similar to many other $T=3$ viruses, A and B subunits formed the dimer with bent conformation around the 5-fold icosahedral axis, while C monomers formed the dimer with flat conformation at the 2-fold icosahedral axis. Interestingly, the orientation of the P domain of the C–C dimer of HEV-LP/$T=3$ relative to its M and S domains was approximately 90° different to those of the A–B dimer of HEV-LP/$T=3$ and the dimer of HEV-LP/$T=1$. The proline-rich hinge linking the P and M domains was likely to contribute to this transition of the P domain orientation.

## 9. Conclusion and subjects

HEV capsid polypeptides are currently undergoing clinical trials as vaccine candidates (Shrestha et al., 2007; Zhu et al., 2010). Furthermore, HEV-LP/$T=1$ may be available as a carrier for foreign DNA (Takamura et al., 2004) or epitopes (Niikura et al., 2002). Recent progresses in the structural studies on HEV particles will provide useful information not only for evaluation of HEV life cycles such as assembly, entry to cells and disassembly but also for the development of such monovalent or polyvalent vaccines. However, several subjects still remain in the structural study. First, the structure of the whole capsid protein has not been resolved. Particularly, it is possible that the C-terminal amino acids deleted in the capsid protein of HEV-LPs might integrates folding and functions of the protruding spike. Another subject is that the existences of two types of virions, nonenveloped virions found in fecal samples and "enveloped" virions found in serum samples, were suggested (Takahashi et al., 2008b; Yamada et al., 2009). It was reported that the envelope virus associated with the ORF3 protein and lipids, but the structure is largely unclear. Further studies are required to evaluate these subjects.

## Acknowledgements

We thank H. Murase for her secretarial work. This work was supported in part by grants-in-aid from the Research and Development Program for New Bioindustry Initiatives of Bio-oriented Technology Research Advancement Institution (BRAIN) and the Foundation for Research Collaboration Center on Emerging and Re-emerging infections.

## References

Balayan, M.S., Andjaparidze, A.G., Savinskaya, S.S., Ketiladze, E.S., Braginsky, D.M., Savinov, A.P., Poleschuk, V.F., 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal–oral route. Intervirology 20, 23–31.

Bhella, D., Gatherer, D., Chaudhry, Y., Pink, R., Goodfellow, I.G., 2008. Structural insights into calicivirus attachment and uncoating. J. Virol. 82, 8051–8058.

Bradley, D., Andjaparidze, A., Cook Jr., E.H., McCaustland, K., Balayan, M., Stetler, H., Velazquez, O., Robertson, B., Humphrey, C., Kane, M., et al., 1988. Aetiological agent of enterically transmitted non-A, non-B hepatitis. J. Gen. Virol. 69 (Pt 3), 731–738.

Bu, W., Mamedova, A., Tan, M., Xia, M., Jiang, X., Hegde, R.S., 2008. Structural basis for the receptor binding specificity of Norwalk virus. J. Virol. 82, 5340–5347.

Chen, R., Neill, J.D., Estes, M.K., Prasad, B.V., 2006. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. Proc. Natl. Acad. Sci. U. S. A. 103, 8048–8053.

Choi, J.M., Hutson, A.M., Estes, M.K., Prasad, B.V., 2008. Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus. Proc. Natl. Acad. Sci. U. S. A. 105, 9175–9180.

Emerson, S.U., Clemente-Casares, P., Moiduddin, N., Arankalle, V.A., Torian, U., Purcell, R.H., 2006. Putative neutralization epitopes and broad cross-genotype neutralization of hepatitis E virus confirmed by a quantitative cell-culture assay. J. Gen. Virol. 87, 697–704.

Guu, T.S., Liu, Z., Ye, Q., Mata, D.A., Li, K., Yin, C., Zhang, J., Tao, Y.J., 2009. Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. Proc. Natl. Acad. Sci. U. S. A. 106, 12992–12997.

He, S., Miao, J., Zheng, Z., Wu, T., Xie, M., Tang, M., Zhang, J., Ng, M.H., Xia, N., 2008. Putative receptor-binding sites of hepatitis E virus. J. Gen. Virol. 89, 245–249.

Hsu, C., Singh, P., Ochoa, W., Manayani, D.J., Manchester, M., Schneemann, A., Reddy, V.S., 2006. Characterization of polymorphism displayed by the coat protein mutants of tomato bushy stunt virus. Virology 349, 222–229.

Jameel, S., Zafrullah, M., Ozdener, M.H., Panda, S.K., 1996. Expression in animal cells and characterization of the hepatitis E virus structural proteins. J. Virol. 70, 207–216.

Johne, R., Plenge-Bonig, A., Hess, M., Ulrich, R.G., Reetz, J., Schielke, A., 2010. Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. J. Gen. Virol. 91, 750–758.

Kakani, K., Reade, R., Katpally, U., Smith, T., Rochon, D., 2008. Induction of particle polymorphism by cucumber necrosis virus coat protein mutants in vivo. J. Virol. 82, 1547–1557.

Kalia, M., Chandra, V., Rahman, S.A., Sehgal, D., Jameel, S., 2009. Heparan sulfate proteoglycans are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral infection. J. Virol. 83, 12714–12724.

Li, S., Tang, X., Seetharaman, J., Yang, C., Gu, Y., Zhang, J., Du, H., Shih, J.W., Hew, C.L., Sivaraman, J., Xia, N., 2009. Dimerization of hepatitis E virus capsid protein E2s domain is essential for virus–host interaction. PLoS Pathog. 5, e1000537.

Li, T.C., Scotti, P.D., Miyamura, T., Takeda, N., 2007. Latent infection of a new alphanodavirus in an insect cell line. J. Virol. 81, 10890–10896.

Li, T.C., Suzaki, Y., Ami, Y., Dhole, T.N., Miyamura, T., Takeda, N., 2004. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. Vaccine 22, 370–377.

Li, T.C., Takeda, N., Miyamura, T., Matsuura, Y., Wang, J.C., Engvall, H., Hammar, L., Xing, L., Cheng, R.H., 2005. Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. J. Virol. 79, 12999–13006.

Li, T.C., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M.A., Uchida, T., Takeda, N., Miyamura, T., 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J. Virol. 71, 7207–7213.

Niikura, M., Takamura, S., Kim, G., Kawai, S., Saijo, M., Morikawa, S., Kurane, I., Li, T.C., Takeda, N., Yasutomi, Y., 2002. Chimeric recombinant hepatitis E virus-like particles as an oral vaccine vehicle presenting foreign epitopes. Virology 293, 273–280.

Okamoto, H., 2007. Genetic variability and evolution of hepatitis E virus. Virus Res. 127, 216–228.

Panda, S.K., Thakral, D., Rehman, S., 2007. Hepatitis E virus. Rev. Med. Virol. 17, 151–180.

Prasad, B.V., Hardy, M.E., Dokland, T., Bella, J., Rossmann, M.G., Estes, M.K., 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science 286, 287–290.

Schofield, D.J., Glamann, J., Emerson, S.U., Purcell, R.H., 2000. Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. J. Virol. 74, 5548–5555.

Schofield, D.J., Purcell, R.H., Nguyen, H.T., Emerson, S.U., 2003. Monoclonal antibodies that neutralize HEV recognize an antigenic site at the carboxyterminus of an ORF2 protein vaccine. Vaccine 22, 257–267.

Shrestha, M.P., Scott, R.M., Joshi, D.M., Mammen Jr., M.P., Thapa, G.B., Thapa, N., Myint, K.S., Fourneau, M., Kuschner, R.A., Shrestha, S.K., David, M.P., Seriwatana, J., Vaughn, D.W., Safary, A., Endy, T.P., Innis, B.L., 2007. Safety and efficacy of a recombinant hepatitis E vaccine. N. Engl. J. Med. 356, 895–903.

Sreenivasan, M.A., Arankalle, V.A., Sehgal, A., Pavri, K.M., 1984. Non-A, non-B epidemic hepatitis: visualization of virus-like particles in the stool by immune electron microscopy. J. Gen. Virol. 65 (Pt 5), 1005–1007.

Takahashi, M., Hoshino, Y., Tanaka, T., Takahashi, H., Nishizawa, T., Okamoto, H., 2008a. Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system. Arch. Virol. 153, 657–666.

Takahashi, M., Yamada, K., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T., Okamoto, H., 2008b. Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces. Arch. Virol. 153, 1703–1713.

Takamura, S., Niikura, M., Li, T.C., Takeda, N., Kusagawa, S., Takebe, Y., Miyamura, T., Yasutomi, Y., 2004. DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration. Gene Ther. 11, 628–635.

Xing, L., Kato, K., Li, T., Takeda, N., Miyamura, T., Hammar, L., Cheng, R.H., 1999. Recombinant hepatitis E capsid protein self-assembles into a dual-domain $T=1$ particle presenting native virus epitopes. Virology 265, 35–45.

Xing, L., Li, T.C., Miyazaki, N., Simon, M.N., Wall, J.S., Moore, M., Wang, C.Y., Takeda, N., Wakita, T., Miyamura, T., Cheng, R.H., 2010. Structure of hepatitis E virion-sized particle reveals an RNA-dependent viral assembly pathway. J. Biol. Chem..

Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Nagashima, S., Tanaka, T., Okamoto, H., 2009. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. J. Gen. Virol. 90, 1880–1891.

Yamashita, T., Mori, Y., Miyazaki, N., Cheng, R.H., Yoshimura, M., Unno, H., Shima, R., Moriishi, K., Tsukihara, T., Li, T.C., Takeda, N., Miyamura, T., Matsuura, Y., 2009. Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. Proc. Natl. Acad. Sci. U. S. A. 106, 12986–12991.

Zhu, F.C., Zhang, J., Zhang, X.F., Zhou, C., Wang, Z.Z., Huang, S.J., Wang, H., Yang, C.L., Jiang, H.M., Cai, J.P., Wang, Y.J., Ai, X., Hu, Y.M., Tang, Q., Yao, X., Yan, Q., Xian, Y.L., Wu, T., Li, Y.M., Miao, J., Ng, M.H., Shih, J.W., Xia, N.S., 2010. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet 376, 895–902.

Virus Research 163 (2012) 405–409

Contents lists available at SciVerse ScienceDirect

Virus Research

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Short communication

# Translocase of outer mitochondrial membrane 70 induces interferon response and is impaired by hepatitis C virus NS3

Yuri Kasama[a], Makoto Saito[a], Takashi Takano[b], Tomohiro Nishimura[c], Masaaki Satoh[a,d], Zhongzhi Wang[a], Salem Nagla Elwy Salem Ali[a,e], Shinji Harada[e], Michinori Kohara[f], Kyoko Tsukiyama-Kohara[a,*]

[a] Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo Kumamoto City, Kumamoto 860-8556, Japan
[b] Division of Veterinary Public Health, Nippon Veterinary and Life Science University, 1-7-1 Kyonan, Musashino, Tokyo 180-8602, Japan
[c] KAKETSUKEN, Kyokushi, Kikuchi, Kumamoto 869-1298, Japan
[d] Department of Virology I, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[e] Department of Medical Virology, Faculty of Life Sciences, Kumamoto University, Japan
[f] Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan

ARTICLE INFO

*Article history:*
Received 5 September 2011
Received in revised form 13 October 2011
Accepted 13 October 2011
Available online 20 October 2011

*Keywords:*
HCV
Tom70
MAVS
IFN
IRF-3
NS3

ABSTRACT

Hepatitis C virus (HCV) elevated expression of the translocase of outer mitochondrial membrane 70 (Tom70). Interestingly, overexpression of Tom70 induces interferon (IFN) synthesis in hepatocytes, and it was impaired by HCV. Here, we addressed the mechanism of this impairment. The HCV NS3/4A protein induced Tom70 expression. The HCV NS3 protein interacted in cells, and cleaved the adapter protein mitochondrial anti-viral signaling (MAVS). Ectopic overexpression of Tom70 could not inhibit this cleavage. As a result, IRF-3 phosphorylation was impaired and IFN-β induction was suppressed. These results indicate that MAVS works upstream of Tom70 and the cleavage of MAVS by HCV NS3 protease suppresses signaling of IFN induction.



Type I interferon (IFN) induction is the front line of host defense against viral infection. Intracellular double-stranded RNA is a viral replication intermediate and contains pathogen-associated molecular patterns (PAMPS) (Saito et al., 2008) that are recognized by pathogen-recognition receptors (PRRs) to induce IFN. One PRR family includes the Toll-like receptors (TLRs), which are predominantly expressed in the endosome (Heil et al., 2004). Another route of IFN induction takes place in the cytosol through activation of specific RNA helicases, such as retinoic acid-inducible (RIG)-I and melanoma differentiation associated gene 5 (MDA5). The ligand for RIG-I is an uncapped 5′ triphosphate RNA, which is found in viral RNAs of the *Flaviviridae* family, including hepatitis C virus (HCV), paramyxovirus, and rhabdoviruses (Kato et al., 2006). MDA5 recognizes viruses with protected 5′ RNA ends, for example, picornaviruses (Hornung et al., 2006). The adapter protein that links the RNA helicase to the downstream MAPK, NF-κB, and IRF-3 signaling pathways is referred to as the mitochondrial anti-viral signaling (MAVS) protein (Seth et al., 2005); alternative names include IPS-1, interferon-promoter stimulator 1; VISA, virus-induced signaling adaptor; and CARDIF, CARD adapter inducing IFN. HCV nonstructural protein 3 (NS3) possesses a serine protease domain at the N terminus (amino acids (aa) 1–180) and has been found to cleave adaptor proteins, MAVS at aa 508 (Meylan et al., 2005) and Toll/IL-1R domain-containing adapter inducing IFN-β-deficient (TRIF at aa 372; Ferreon et al., 2005). These cleavages provoke abrogation of the induction of the IFN pathway.

The translocase of the outer membrane (TOM) is responsible for initial recognition of mitochondrial preproteins in the cytosol (Baker et al., 2007; Neupert and Herrmann, 2007). The TOM machinery consists of 2 import receptors, Tom20 and Tom70, and, along with several other subunits, comprises the general import pore (Abe et al., 2000). Recently, Tom70 was found to interact with MAVS (Liu et al., 2010). Ectopic expression or silencing of Tom70, respectively, enhanced or impaired IRF3-mediated gene expression and IFN-β production. Sendai virus infection accelerated the

* Corresponding author. Present address: Transboundary Animal Diseases Center, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto Kagoshima-shi, Kagoshima 890-0065, Japan. Tel.: +81 99 285 3589/96 373 5560; fax: +81 99 285 3589/96 373 5562.
*E-mail address:* kkohara@kumamoto-u.ac.jp (K. Tsukiyama-Kohara).

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doi:10.1016/j.virusres.2011.10.009

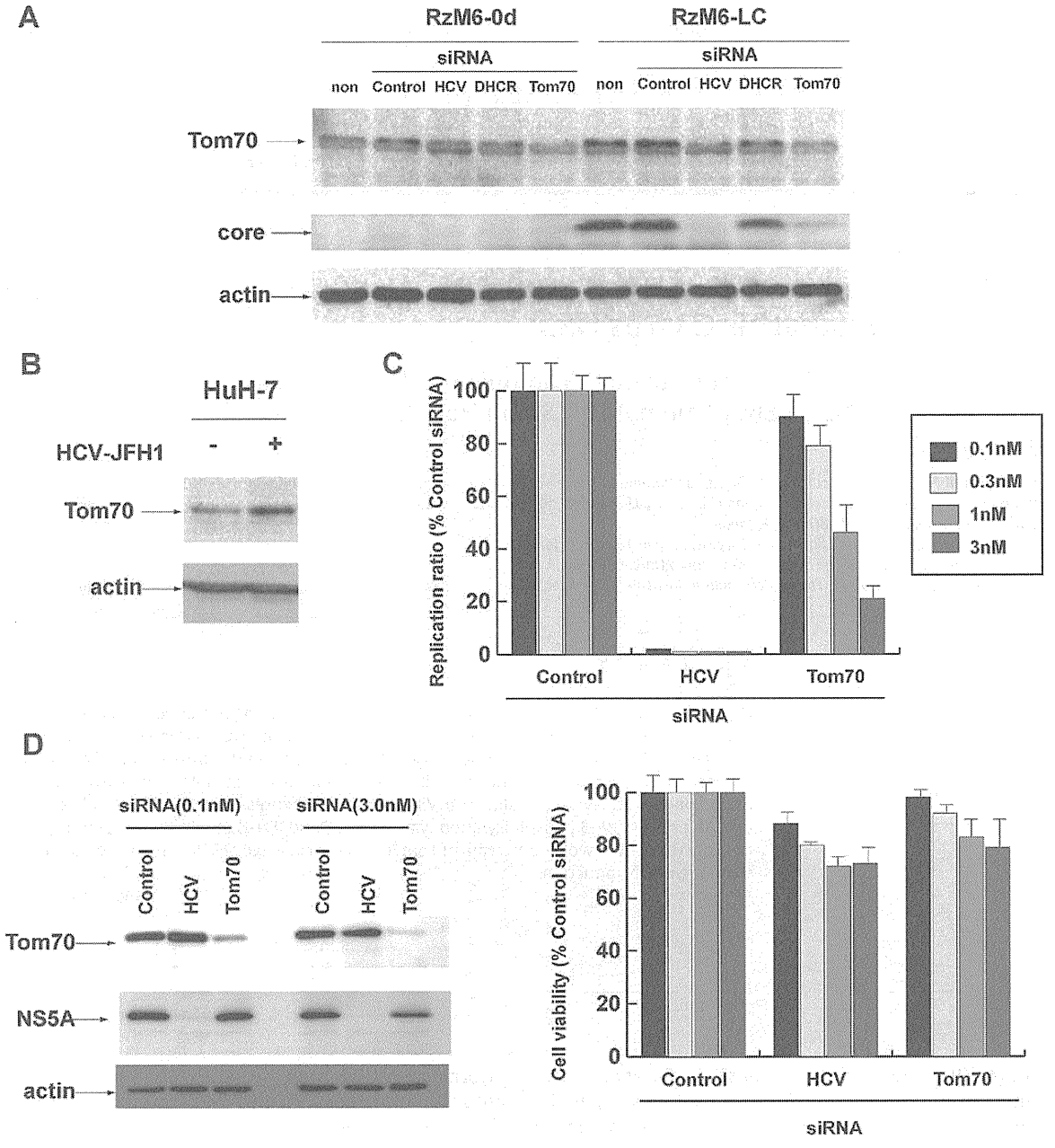


**Fig. 1.** HCV induces overexpression of Tom70 but impairs Tom70-induced IFN synthesis. (A) RzM6 cells (HCV-) and RzM6-LC cells (HCV+) were transfected with siRNAs of control (non-target siRNA#3: Thermo Fisher Scientific), HCV (R5: 5′-GUCUCGUAGACCGUGCAUCAuu-3′), DHCR24 (Nishimura et al., 2009), and Tom70 (Takano et al., 2011a). Control cells were mock-transfected. Tom70 protein was detected with MAb2-243a (Takano et al., 2011a) and actin protein was detected as an internal control (lower column). (B) HuH-7 cells were infected with HCV JFH-1 strain; Tom70 protein and actin protein were detected. (C) The HCV replicon cells (FLR3-1; Takano et al., 2011b) were transfected with siRNAs (control, HCV (R7: 5′-GUCUCGUAGACCGUGCACCAuu-3′), Tom70; 0.1, 0.3, 1, 3 nM) and HCV replication activity was measured with luciferase activity using the Bright-Glo luciferase assay kit (Promega). Cell viability was measured using WST-8 (Dojindo) reagent. Ratio with those of control siRNA treatment was calculated. Vertical bars were S.D. (D) HCV replicon cells (FLR3-1) were transfected with control, HCV (R7) and Tom70 siRNAs (0.1, 0.3 nM) and Tom70, NS5A and actin proteins were detected.

Tom70-mediated IFN induction and the interaction of Tom70 with MAVS. These recent findings indicated that Tom70 might be a critical mediator during IFN induction (Liu et al., 2010).

We previously observed that HCV induces Tom70 and is related to the apoptotic response (Takano et al., 2011a). However, no synergistic effect was observed for IFN induction by Tom70 and HCV. Therefore, in the present study, we have investigated the mechanism of modification of the Tom70-induced IFN synthesis pathway by HCV and clarified a finely balanced system regulated by viral protein.

The expression of Tom70 protein was examined using western blotting and modification by HCV was characterized (Fig. 1A). The level of Tom70 protein was increased in RzM6-LC cells compared with that in RzM6-0d cells (Tsukiyama-Kohara et al., 2004). The full-length HCV-RNA expression was induced by 4-hydroxytamoxifen (100 nM) and passaged for more than 44 days in RzM6-LC cells, and HCV expression was not induced in RzM6-0d cells. Silencing of HCV expression by siRNA (R5; Thermo Scientific) abolished core protein expression, and decreased the level of Tom70 protein expression in RzM6-LC cells (Fig. 1A). Silencing of Tom70 by siRNA significantly decreased the level of HCV core protein expression in RzM6-LC cells (Fig. 1A). The siRNA against 3-beta-hydroxysterol-delta24 reductase (DHCR24) slightly decreased the level of Tom70 protein. In contrast, the

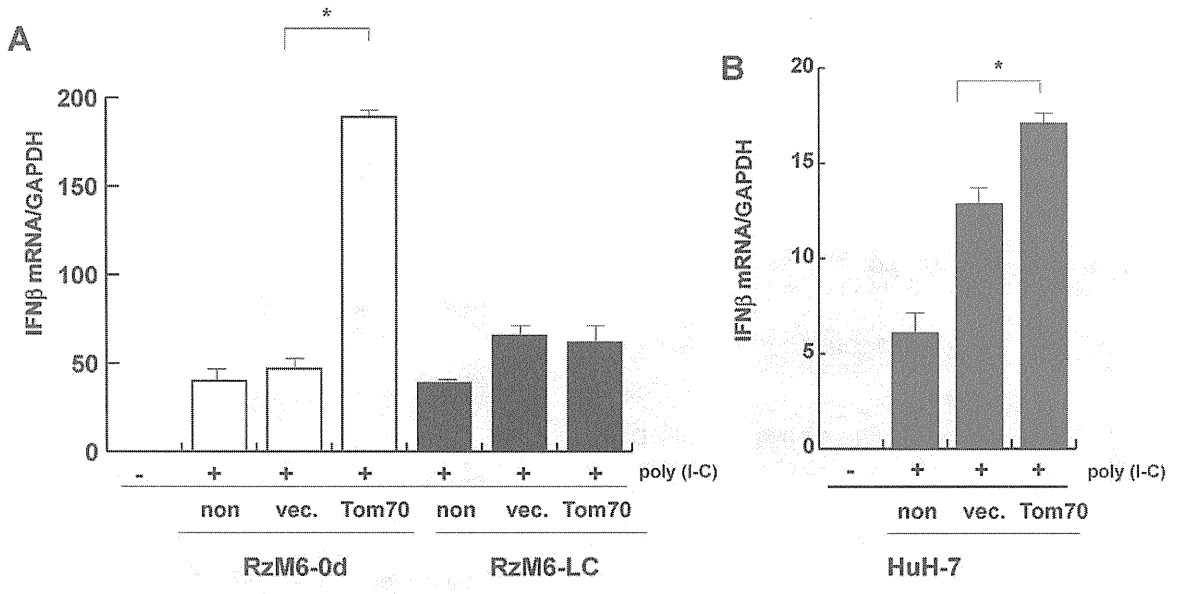


**Fig. 2.** Tom70-induced IFN synthesis was impaired by HCV. (A) RzM6-0d cells and LC cells were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly(I–C) (GE Healthcare) (5 μg) was transfected with RNAi Max reagent (Invitrogen) and IFN-βmRNA was measured after 6 h of poly(I–C) treatment. Vertical bars indicate S.D. $^*p < 0.05$. (B) HuH-7 cells were transfected with mock-vector, control vector, or Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA. Vertical bars indicate S.D. $^*p < 0.05$.

control siRNA did not have a significant effect on Tom70 protein expression.

We next examined the effects of HCV JFH-1 (Wakita et al., 2005) infection on Tom70 expression (Fig. 1B). Infection with HCV significantly increased the level of Tom70 protein expression. We also examine the role of Tom70 in HCV replication (Fig. 1C and D). Silencing of Tom70 by siRNA decreased the HCV replication in a dose dependent manner.

Thus, HCV induces Tom70 expression, and Tom70 is involved in viral replication.

It was recently shown that Tom70 recruits TBK1/IRF3 to mitochondria by binding to Hsp90 and inducing IFN-β synthesis (Liu et al., 2010). Therefore, we examined the effects of Tom70 overexpression on IFN synthesis and modification by HCV (Fig. 2). Level of IFN-β mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN-β mRNA synthesis in the absence of HCV after poly(I–C) treatment (RzM6-0d cells). However, the Tom70-mediated induction of IFN-β mRNA transcription was impaired in the presence of HCV (RzM6-LC cells) (Fig. 2A). Overexpression of Tom70 induced IFN-β mRNA synthesis in HuH-7 cells (Fig. 2B). Induction of IFN-β mRNA was lower in HuH-7 cells than HepG2 based RzM6 cells, which might be due to the defect in IFN induction system in HuH-7 cells (Preiss et al., 2008).

We have further addressed the mechanism of impairment of IFN-β mRNA transcription by HCV.

To identify the viral protein that was responsible for the induction of Tom70, we examined the Tom70 protein expression levels in HCV core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B protein-expressing cells (data not shown), and Tom70 protein expression level was highest in the NS3/4A-expressing cells than was observed in cells expressing other proteins (Fig. 3A, data not shown), indicating an effect of HCV NS3/4A protein on Tom70 expression.

The expression vector of Myc- and His-tagged Tom70 was transfected into the empty control or NS3/4A-expressing cells and immunoprecipitated with anti-Myc antibody (Suppl. Fig. 1A). Results showed that Myc-Tom70 was precipitated in both cells (right panel) and NS3 protein was specifically precipitated by anti-Myc antibody in the NS3/4A-expressing cells (left panel). NS4A protein could not be detected (data not shown).

We next stained the NS3/4A-expressing cells with anti-NS3 and -Tom70 antibodies, and observed with confocal microscopy (Suppl. Fig. 1B). The signal of NS3 protein was clearly merged with that of Tom70, strongly supporting the possibility that the NS3 protein co-localizes with the Tom70 protein.

To clarify the effect of Tom70 on NS3, we transfected NS3/4A-expressing cells with the siRNA of Tom70 (Fig. 3A). Silencing of Tom70 decreased the level of NS3 protein in cells, but did not influence the levels of the MAVS and NF-κB proteins. These results suggest the possibility that Tom70 may increase the stability of NS3 protein in cells.

Tom70 reportedly interacts with MAVS during viral infection (Liu et al., 2010). Therefore, we examined the MAVS protein in cells expressing either the control empty or NS3/4A lenti-virus vector (Fig. 3B). Cleavage of MAVS (indicated as ΔMAVS) was observed in NS3/4A protein-expressing cells, as was reported previously (Meylan et al., 2005). Overexpression of Tom70 did not have a significant effect on the MAVS expression level and did not prevent MAVS cleavage by NS3. IRF-3 phosphorylation was suppressed in NS3/4A-expressing cells and was not influenced by Tom70 overexpression. The induction of IFN-β was impaired in NS3/4A-expressing cells, even in the presence of Tom70 overexpression (Fig. 3C). These data may indicate that MAVS exists upstream of Tom70 and that cleavage of MAVS by NS3/4A impaired the downstream signaling activation of IRF-3 phosphorylation (Suppl. Fig. 2).

Mitochondria provide a substantial platform for the regulation of IFN signaling. The MAVS adapter protein is a member of the family of RIG-I like receptors (RLRs), which links the mitochondria to the mammalian antiviral defense system (Seth et al., 2005). Proteomic studies have demonstrated that MAVS interacts with Tom70 (Liu et al., 2010). This interaction was accelerated by Sendai virus infection and synergized with ectopic expression of Tom70 to significantly increase the production of IFN-β (Liu et al., 2010). The results of the present study revealed that infection with HCV induced Tom70 expression, but the presence of HCV impaired IFN

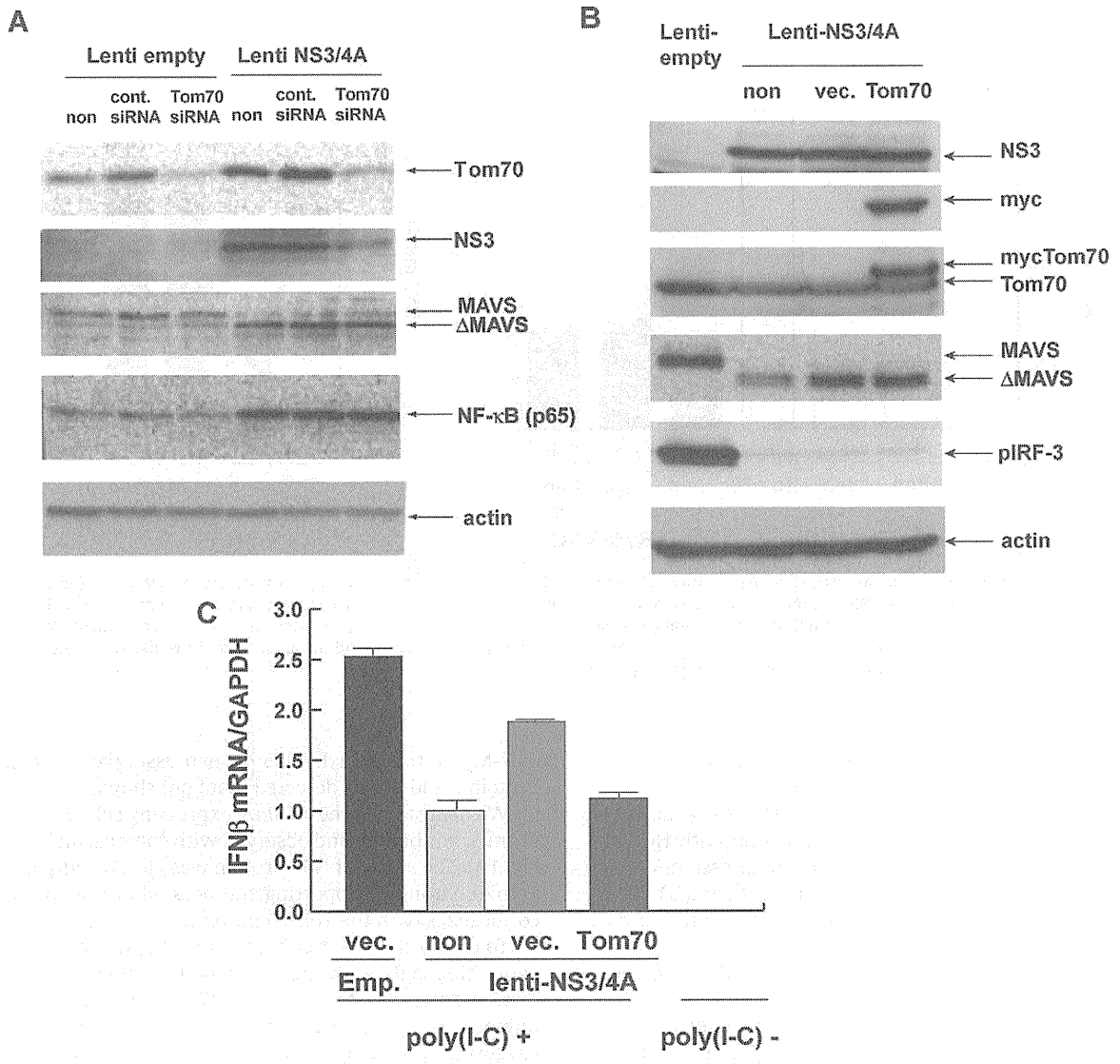


**Fig. 3.** Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A impaired IRF-3 phosphorylation even in the presence of Tom70. (A) Empty or NS3/4A-lenti virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or mock-transfected (non) as a control. MAVS, NS3, Tom70, and actin proteins were detected by western blot. (B) Empty or NS3/4A-expressing HepG2 cells were transfected with control pcDNA vector (vec.) and pcDNA6 (Invitrogen)-Tom70 or mock-transfected (non) as a control. NS3, Tom70, phosphorylated IRF-3, MAVS, and actin proteins were examined by western blot. (C) IFN-β mRNA was measured by RTD-PCR and normalized with GAPDH mRNA amount in empty or NS3/4A expressing cells with transfection of mock (non), pcDNA-vector (vec.) or pcDNA-Tom70 (Tom70). Poly(I–C) was treated, as described in the legend of Fig. 2.

induction. It has been reported that the C-terminal transmembrane domain (TM) of MAVS interacts with the N-terminal transmembrane domain of Tom70 (Liu et al., 2010). The HCV NS3 protein cleaves MAVS at residue 508 (Meylan et al., 2005), which should impair the interaction of MAVS and Tom70. This may attenuate the downstream signaling pathway (TBK-IRF3) and the induction of IFN synthesis (Suppl. Fig. 2). In our study, the level of NF-κB protein was not significantly influenced by Tom70 in the presence or absence of NS3. This may indicate that other pathways, such as TLR3 and downstream pathways, might compensate to maintain the NF-κB protein expression level in the absence of the MAVS-Tom70 signaling pathway.

Infection with HCV induced expression of Tom70, but the activation of the IFN signaling pathway was abrogated by the HCV NS3 protease. These findings indicate that recovery of the MAVS-Tom70 pathway may be a means to increase the efficacy of IFN therapy against HCV infection.

Recently, we observed that overexpression of Tom70 increased the resistance to the TNFα-induced apoptotic response (Takano et al., 2011a), indicating that Tom70 overexpression might contribute to the apoptotic resistance of HCV-infected cells and the establishment of persistent HCV infection. Thus, Tom70 might be a novel target for the regulation of HCV infection.

## Acknowledgements

Authors thank Professor Yoshiharu Matsuura for providing the rabbit polyclonal NS5A antibody. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.10.009.

## References


Abe, Y., Shodai, T., Muto, T., Mihara, K., Torii, H., Nishikawa, S., Endo, T., Kohda, D., 2000. Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. Cell 100 (5), 551–560.

Baker, M.J., Frazier, A.E., Gulbis, J.M., Ryan, M.T., 2007. Mitochondrial protein-import machinery: correlating structure with function. Trends Cell Biol. 17 (9), 456–464.

Ferreon, J.C., Ferreon, A.C., Li, K., Lemon, S.M., 2005. Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. J. Biol. Chem. 280 (21), 20483–20492.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303 (5663), 1526–1529.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., Endres, S., Hartmann, G., 2006. 5′-Triphosphate RNA is the ligand for RIG-I. Science 314 (5801), 994–997.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.S., Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441 (7089), 101–105.

Liu, X.Y., Wei, B., Shi, H.X., Shan, Y.F., Wang, C., 2010. Tom70 mediates activation of interferon regulatory factor 3 on mitochondria. Cell Res. 20 (9), 994–1011.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., Tschopp, J., 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437 (7062), 1167–1172.

Neupert, W., Herrmann, J.M., 2007. Translocation of proteins into mitochondria. Annu. Rev. Biochem. 76, 723–749.

Nishimura, T., Kohara, M., Izumi, K., Kasama, Y., Hirata, Y., Huang, Y., Shuda, M., Mukaidani, C., Takano, T., Tokunaga, Y., Nuriya, H., Satoh, M., Saito, M., Kai, C., Tsukiyama-Kohara, K., 2009. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. J. Biol. Chem. 284 (52), 36442–36452.

Preiss, S., Thompson, A., Chen, X., Rodgers, S., Markovska, V., Desmond, P., Visvanathan, K., Li, K., Locarnini, S., Revill, P., 2008. Characterization of the innate immune signalling pathways in hepatocyte cell lines. J. Viral Hepat. 15 (12), 888–900.

Saito, T., Owen, D.M., Jiang, F., Marcotrigiano, J., Gale Jr., M., 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 454 (7203), 523–527.

Seth, R.B., Sun, L., Ea, C.K., Chen, Z.J., 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 122 (5), 669–682.

Takano, T., Kohara, M., Kasama, Y., Nishimura, T., Saito, M., Kai, C., Tsukiyama-Kohara, K., 2011a. Translocase of outer mitochondrial membrane 70 expression is induced by hepatitis C virus and is related to the apoptotic response. J. Med. Virol. 83 (5), 801–809.

Takano, T., Tsukiyama-Kohara, K., Hayashi, M., Hirata, Y., Satoh, M., Tokunaga, Y., Tateno, C., Hayashi, Y., Hishima, T., Funata, N., Sudo, M., Kohara, M., 2011b. Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. J. Hepatol. 55 (3), 512–521.

Tsukiyama-Kohara, K., Tone, S., Maruyama, I., Inoue, K., Katsume, A., Nuriya, H., Ohmori, H., Ohkawa, J., Taira, K., Hoshikawa, Y., Shibasaki, F., Reth, M., Minatogawa, Y., Kohara, M., 2004. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J. Biol. Chem. 279 (15), 14531–14541.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11 (7), 791–796.

MAJOR ARTICLE

# Monoclonal Antibody 2-152a Suppresses Hepatitis C Virus Infection Through Betaine/GABA Transporter-1

**Masaaki Satoh,[1] Makoto Saito,[1] Takashi Takano,[1,2] Yuri Kasama,[1] Tomohiro Nishimura,[1,3] Yasumasa Nishito,[4] Yuichi Hirata,[2] Masaaki Arai,[2] Masayuki Sudoh,[5] Chieko Kai,[6] Michinori Kohara,[2] and Kyoko Tsukiyama-Kohara[1]**

[1]Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, Honjo Kumamoto City; [2]Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Kamikitazawa, Setagaya-ku; [3]KAKETSUKEN, Kyokushi, Kikuchi, Kumamoto; [4]Center for Microarray Analysis, Tokyo Metropolitan Institute of Medical Science; [5]Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd., Kajiwara, Kamakura-City, Kanagawa; and [6]Laboratory of Animal Research Center, Institute of Medical Science, University of Tokyo, Shirokane-dai Minato-Ku, Japan

***Background.*** We recently established a monoclonal antibody (2-152a MAb) that binds to 3β-hydroxysterol-Δ24-reductase (DHCR24) by immunizing mice with cells (RzM6-LC) persistently expressing hepatitis C virus (HCV). Here, we aimed to analyze the activity of 2-152a MAb against HCV replication and explore the molecular mechanism underlying the antiviral activity.

***Methods.*** We characterized the effects of 2-152a MAb on HCV replication and performed a microarray analysis of antibody-treated HCV replicon cells. The molecules showing a significant change after the antibody treatment were screened to examine their relationship with HCV replication.

***Results.*** The antibody had antiviral activity both in vitro and in vivo (chimeric mice). In the microarray analysis, 2-152a MAb significantly suppressed the expression of betaine/GABA transporter-1 (BGT-1) in 2 HCV replicon cell lines but not in HCV-cured cells. Silencing of BGT-1 expression by small interfering RNA (siRNA) revealed significant suppression of HCV replication and infection without cytotoxicity. Further, BGT-1 expression was significantly increased in the presence of HCV ($P < .05$).

***Conclusions.*** Our results suggest that 2-152a MAb suppresses HCV replication and infection through BGT-1. These findings highlight important roles of BGT-1 in HCV replication and reveal a possible target for anti-HCV therapy.

Hepatitis C virus (HCV) causes chronic hepatitis and hepatocellular carcinoma (HCC) [1–3]. Chronic HCV infection is a major global public health concern because it affects at least 170 million people worldwide [2]. The most effective treatment against HCV currently comprises a combination therapy of PEGylated α-interferon (IFN-α) and ribavirin [4, 5]. However, considering that sustained virological responses develop in only approximately half of the patients infected with HCV genotype 1, the clinical efficacy of this therapy is limited [6, 7]. Efforts to develop therapies against HCV are further hindered by the high level of viral variation and capacity of the virus to cause chronic infection. Therefore, there is an urgent need to develop effective treatments against chronic HCV infection.

In a previous study, we established a cell line expressing HCV (RzM6-LC) to investigate the effects of persistent HCV expression on cell growth [8]. We also established a monoclonal antibody (2-152a MAb) against the RzM6-LC cell line to produce clones that recognize both cell surface and intracellular molecules. Using this method, we identified 3b-hydroxysterol-D24-reductase (DHCR24) as the recognition molecule of this antibody.

Received 30 November 2010; accepted 10 May 2011.

Correspondence: Kyoko Tsukiyama-Kohara, PhD, DVM, Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto-shi, Kumamoto 860-8556, Japan (kkohara@kumamoto-u.ac.jp).

**The Journal of Infectious Diseases 2011;204:1172–80**



0022-1899 (print)/1537-6613 (online)/2011/2048-0005$14.00
DOI: 10.1093/infdis/jir501