TABLE 2 Effects of derivative compounds of 1 on HCV replication[a]

| Compound | $EC_{50}$ ($\mu$M) | $CC_{50}$ ($\mu$M) | SI |
|---|---|---|---|
| 5 | 1.82 (0.58–5.68) | 45.1 (14.3–52.5) | 24.8 |
| 6 | 2.29 (1.57–3.34) | >100 | >43.7 |
| 7 | 2.83 (1.43–5.78) | 17.0 (5.25–38.7) | 6.01 |
| 8 | 4.03 (3.51–4.63) | 87.8 (59.1–172) | 21.8 |

[a] The $EC_{50}$ and $CC_{50}$ values are reported, with 95% confidence intervals in parentheses, from a representative experiment performed in triplicate.

vation of NF-κB involves degradation of a suppressor protein, IκB-α. As shown in Fig. 7D, IκB-α protein levels were strongly decreased in cells treated with compound 1. Additionally, activation of the NF-κB pathway by TNF-α treatment significantly suppressed HCV replication (Fig. 7E). These results indicated that the antiviral action of compound 1 partially involved activation of the NF-κB signaling pathway.

## DISCUSSION

Using a chimeric luciferase reporter-based subgenomic HCV-Feo replicon assay and an HCV-JFH1 cell culture, we discovered 4 novel anti-HCV compounds from cell-based screening of a library of 4,004 chemicals (Fig. 1 and Table 1). These compounds displayed anti-HCV activity at noncytotoxic concentrations. The most potent of the leading hit compounds was MCNA, and SAR analyses revealed that 4 compounds with structures similar to that of MCNA also had antiviral activity (Fig. 5). Furthermore, we showed that the *N*-(morpholine-4-carbonyloxy) amidine moiety within the structure of MCNA is essential for antiviral activity (Fig. 6).

After the development of the HCV replicon system, the study of HCV replication and discovery of novel anti-HCV agents have shown great progress. To date, our group and others have already made several successful attempts to discover novel inhibitors of HCV replication. Using Huh7/Rep-Feo cells, we previously identified novel anti-HCV substances, including cyclosporins (18, 19), short interfering RNA (siRNA) (31), hydroxyl-methyl-glutaryl coenzyme A reductase inhibitors (10), and epoxide compounds (20). Huh7/Rep-Feo cells were also used for screening a whole-genome siRNA library and successfully identified human genes that support HCV replication (27). Although the HCV replicon system cannot screen for inhibitors of viral entry and release, it is still a useful tool for rapid, reliable, and reproducible high-throughput screening. In our study, we used library sets of compounds that had probably not been used for anti-HCV drug screening before.

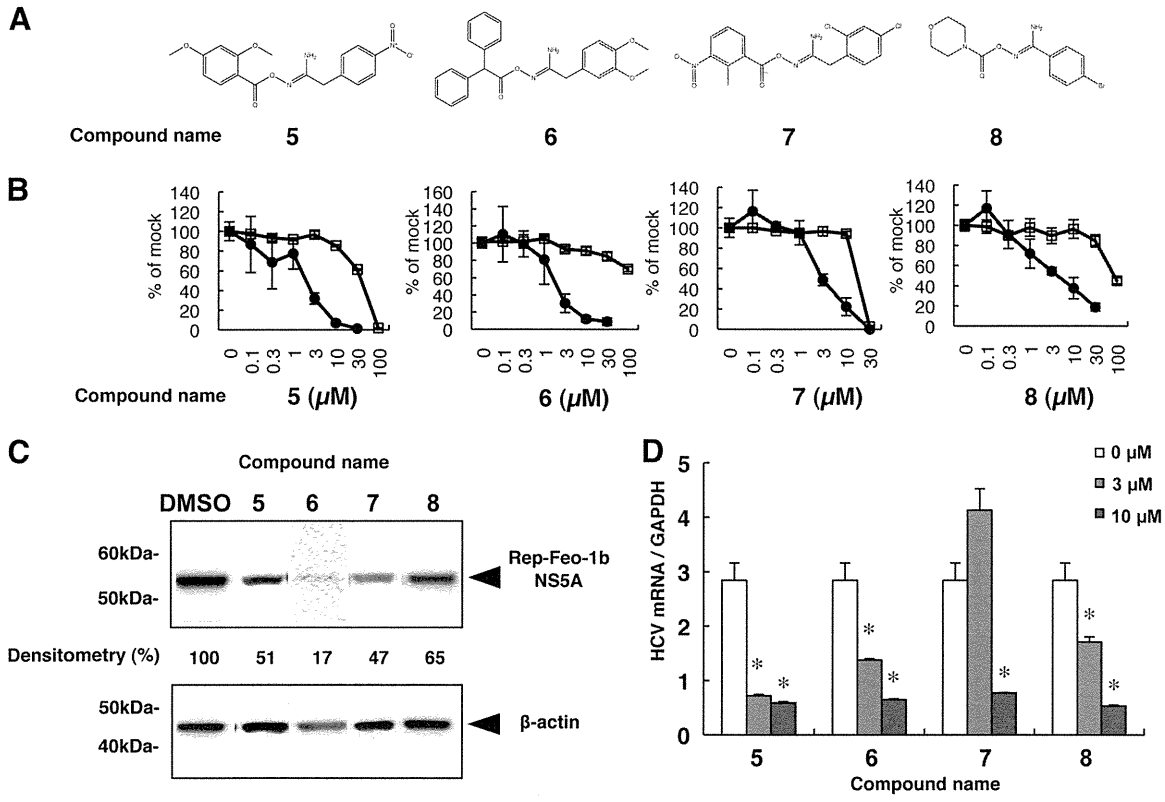


FIG 5 Effects of derivatives of compound 1 on HCV replication. (A) Chemical structures of screening hits of compound 1 derivatives. (B) Huh7/Rep-Feo cells were treated with various concentrations of each compound for 48 h. Luciferase activity for HCV RNA replication is shown as a percentage of the DMSO-treated control luciferase activity (solid circles). Cell viability is also shown as a percentage of control viability (open squares). Each point represents the mean of triplicate data points, with standard deviations represented as error bars. (C) HCV NS5A protein expression levels in Huh7/Rep-Feo cells after treatment with the hit compounds. Huh7/Rep-Feo cells were treated with DMSO and derivative compounds at 5 μM for 48 h, and Western blotting was performed using anti-HCV NS5A and anti-β-actin antibodies. Densitometry of the NS5A protein was performed, and the results are indicated as percentages of the DMSO-treated control. The assay was repeated three times, and a representative result is shown. (D) Huh7.5.1 cells were transfected with HCV-JFH1 RNA and cultured in the presence of the indicated compounds at a concentration of 3 μM or 10 μM. At 72 h after transfection, total cellular RNA was extracted, followed by real-time RT-PCR. The bars indicate means and SD. The asterisks indicate *P* values of less than 0.01. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

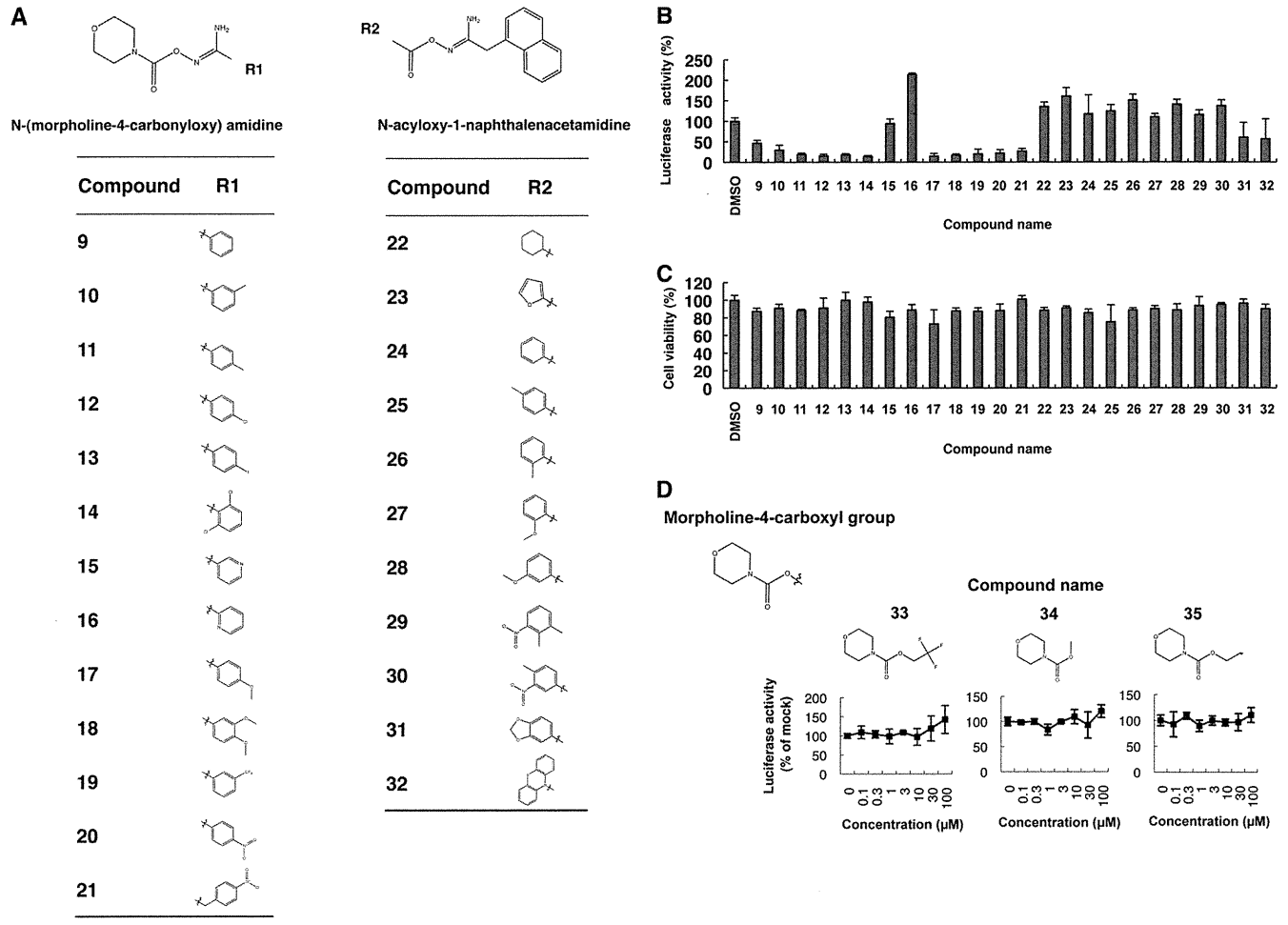


FIG 6 SARs of derivatives of compound 1 that contain *N*-(morpholine-4-carbonyloxy) amidine or *N*-acyloxy-1-naphthalenacetamidine moieties. (A) Chemical structures of compounds with *N*-(morpholine-4-carbonyloxy) amidine or *N*-acyloxy-1-naphthalenacetamidine analyzed for SARs. (B) Huh7/Rep-Feo cells were cultured in the presence of 13 compounds with *N*-(morpholine-4-carbonyloxy) amidine and 11 compounds with *N*-acyloxy-1-naphthalenacetamidine at a fixed concentration of 5 $\mu$M. The internal luciferase activities were measured after 48 h of culture. Luciferase activity for HCV RNA replication levels is shown as a percentage of the drug-negative (DMSO) control. Assays were performed in triplicate. The bars indicate means and SDs. (C) Cell viability is shown as a percentage of control viability. Assays were performed in triplicate. The bars indicate means and SD. (D) Effects of morpholine-4-carboxyl compounds on HCV replication. Huh7/Rep-Feo cells were treated with various concentrations of compound 33, 34, or 35 for 48 h. Luciferase activity for HCV RNA replication levels is shown as a percentage of the drug-negative (DMSO) control. Each point represents the mean of triplicate data points, with standard deviations represented as error bars.

TABLE 3 Effects of *N*-(morpholine-4-carbonyloxy) amidine compounds on HCV replication[a]

| Compound | $EC_{50}$ ($\mu$M) | $CC_{50}$ ($\mu$M) | SI |
|---|---|---|---|
| 9 | 8.62 (7.03–10.6) | >100 | >11.1 |
| 10 | 3.32 (2.28–4.84) | 47.0 (15.7–76.4) | 14.2 |
| 11 | 1.55 (1.04–2.30) | 48.8 (12.8–95.6) | 31.5 |
| 12 | 1.52 (1.14–2.02) | 51.0 (16.2–96.7) | 33.6 |
| 13 | 1.60 (1.36–1.88) | 38.6 (29.4–50.7) | 24.1 |
| 14 | 1.63 (1.34–2.00) | >100 | >61.4 |
| 15 | ND | ND | ND |
| 16 | ND | ND | ND |
| 17 | 1.77 (1.39–2.26) | 63.3 (21.8–128) | 35.8 |
| 18 | 3.80 (2.48–5.83) | 100 (86.8–138) | 26.3 |
| 19 | 1.99 (1.59–2.48) | 55.1 (14.8–105) | 27.7 |
| 20 | 2.61 (1.68–4.05) | >100 | >38.3 |
| 21 | 1.55 (1.45–1.67) | 94.6 (93.0–98.0) | 61.0 |

[a] The $EC_{50}$ and $CC_{50}$ values are reported, with 95% confidence intervals in parentheses, from a representative experiment performed in triplicate. ND, not determined.

The morpholine moiety is a common pharmacophore present in many biosynthetic compounds, such as antimycotic agents (4, 21), and inhibitors of phosphoinositide 3-kinases (5, 16, 30), TNF-$\alpha$-converting enzymes (14), and matrix metalloproteinases (1). Among the antimycotic morpholine derivatives, amorolfine has been widely used to treat onychomycosis (4, 21). The morpholino oxygen in a synthetic phosphoinositide 3-kinase inhibitor, LY294002, participated directly in a key hydrogen-bonding interaction at the ATP-binding site of phosphoinositide 3-kinase $\gamma$ (30). Although the morpholine moieties are key components of many inhibitors, anti-HCV morpholine compounds have not yet been reported. In this report, we demonstrated for the first time that a morpholine-bearing compound, *N*-(morpholine-4-carbonyloxy) amidine, had a potent antiviral effect on HCV replication.

Among the 4 hit compounds, MCNA activated the NF-$\kappa$B pathway (Fig. 7A, B, C, and D). NF-$\kappa$B is a central regulator of innate and adaptive immune responses. NF-$\kappa$B-induced tran-

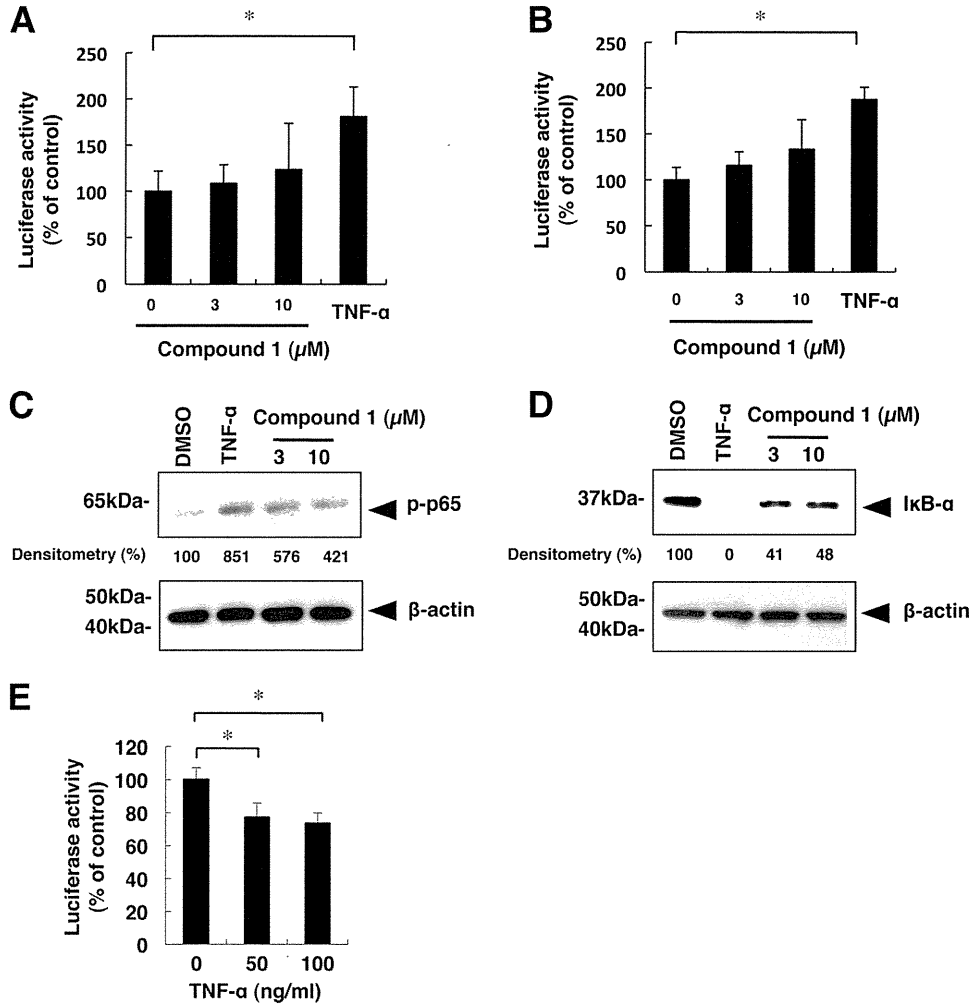


FIG 7 Effects of compound 1 on the NF-κB signaling pathway. (A and B) NF-κB-responsive luciferase reporter assays. The plasmids pNF-κB-TA-Fluc and pRL-CMV were cotransfected into Huh7 cells (A) or HCV replicon-expressing cells (B). At 24 h after transfection, cells were treated with compound 1 at a concentration of 0, 3, or 10 μM. After 6 h, luciferase activities were measured, and the values were normalized against *Renilla* luciferase activities. As a positive control, cells were treated with TNF-α (50 ng/ml). Assays were performed in triplicate. The bars indicate means and SD. (C and D) Huh7 cells were treated for 30 min with compound 1 at concentrations of 3 and 10 μM, and Western blot analyses of phosphorylated p65 and IκBα were conducted. As a positive control, cells were treated with TNF-α (50 ng/ml). β-Actin served as a loading control. Densitometry of phosphorylated p65 protein and IκB-α protein was performed, and the results are indicated as percentages of the DMSO-treated control. The assay was repeated three times, and a representative result is shown. (E) Effect of activation of NF-κB signaling on HCV RNA replication. Huh7/Rep-Feo cells were treated with TNF-α at a concentration of 0, 50, or 100 ng/ml for 48 h. Luciferase activity for HCV RNA replication levels is shown as a percentage of untreated negative-control luciferase activity. Assays were performed in replicates of 6. The asterisks indicate *P* values of less than 0.01.

scription is induced in response to a variety of signals, including proinflammatory cytokines, stress induction, and by-products of microbial and viral infection (9). In HCV-infected cells, activation of transcription factors, such as NF-κB and many interferon regulatory factors, proceeds mainly through Toll-like receptors and RIG-I-dependent host signaling pathways triggered by double-stranded RNA products (3). NF-κB, interferon regulatory factor 1, and interferon regulatory factor 3 bind to the positive regulatory domains of the IFN-β promoter to induce IFN-β expression and elicit antiviral states in host cells (23). Therefore, we hypothesized that the augmentation of host antiviral response through NF-κB activation is an important strategy for anti-HCV treatment. Our demonstration that the activation of NF-κB signaling suppressed HCV replication appears to follow this strategy (Fig. 7E). In support of the idea, Toll-like receptor 7 agonist has shown anti-HCV effects in a preclinical study (7). The anti-HCV activities of MCNA cannot be explained solely by NF-κB activation, because its antiviral activity was much more potent than selective NF-κB activation by TNF-α treatment (Fig. 7E). There remains the possibility that MCNA has a direct viral target. It will be important to assess whether long-term exposure to the compounds could select resistant variants. Although other mechanisms may underlie the antiviral activity, we hypothesize that one of the antiviral mechanisms of MCNA is NF-κB activation that is independent of IFN signaling.

Although several DAAs are currently in advanced clinical trials and the recently approved telaprevir and boceprevir combination therapies achieved high sustained virologic response rates, the frequent emergence of drug-resistant viruses is a major weakness of such agents. An ongoing search for more potent

and less toxic antiviral agents to improve anti-HCV chemotherapeutics is necessary. Our results indicate that MCNA and related *N*-(morpholine-4-carbonyloxy) amidine compounds constitute a new class of anti-HCV agents. Additional investigations elucidating their mechanism of action, and future modifications to improve anti-HCV activity, may open a new anti-HCV therapeutic window.

## ACKNOWLEDGMENTS

We thank Frank Chisari for providing Huh7.5.1 cells, Takaji Wakita for providing the plasmid pJFH1full, and the Chemical Biology Screening Center of Tokyo Medical and Dental University for their assistance in this work.

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Japan Society for the Promotion of Science, Ministry of Health, Labor and Welfare; the Japan Health Sciences Foundation; and the National Institute of Biomedical Innovation.

We declare that we have nothing to disclose regarding funding from industries or conflicts of interest with respect to the manuscript.

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Virus Genes (2012) 44:374–381
DOI 10.1007/s11262-012-0712-2

# Development of a drug assay system with hepatitis C virus genome derived from a patient with acute hepatitis C

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Received: 5 October 2011 / Accepted: 1 January 2012 / Published online: 18 January 2012


**Abstract** We developed a new cell culture drug assay system (AH1R), in which genome-length hepatitis C virus (HCV) RNA (AH1 strain of genotype 1b derived from a patient with acute hepatitis C) efficiently replicates. By comparing the AH1R system with the OR6 assay system that we developed previously (O strain of genotype 1b derived from an HCV-positive blood donor), we demonstrated that the anti-HCV profiles of reagents including interferon-$\gamma$ and cyclosporine A significantly differed between these assay systems. Furthermore, we found unexpectedly that rolipram, an anti-inflammatory drug, showed anti-HCV activity in the AH1R assay but not in the OR6 assay, suggesting that the anti-HCV activity of rolipram differs depending on the HCV strain. Taken together, these results suggest that the AH1R assay system is useful for the objective evaluation of anti-HCV reagents and for the discovery of different classes of anti-HCV reagents.

**Keywords** HCV · Acute hepatitis C · Anti-HCV drug assay system · Anti-HCV activity of rolipram

## Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [1, 2]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1].

Human hepatoma HuH-7 cell culture-based HCV replicon systems derived from a number of HCV strains have been widely used for various studies on HCV RNA replication [3, 4] since the first replicon system (based on the Con1 strain of genotype 1b) was developed in 1999 [5]. Genome-length HCV RNA replication systems (see Fig. 2 for details) derived from a limited number of HCV strains (H77, N, Con1, O, and JFH-1) are also sometimes used for such studies, as they are more useful than the replicon systems lacking the structural region of HCV, although the production of infectious HCV from the genome-length HCV RNA has not been demonstrated to date [3, 4]. Furthermore, these RNA replication systems have been improved enough to be suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [3, 4, 6]. We also developed an HuH-7-derived cell culture assay system (OR6) in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [7]. Such reporter assay systems could save time and facilitate the mass screening of anti-HCV reagents, since the values of luciferase correlated well with the level of HCV RNA after treatment with anti-HCV reagents. Furthermore, OR6 assay system became more useful as a drug assay system than the HCV subgenomic replicon-based reporter assay systems developed to date [3, 4], because the older systems lack the Core-NS2 regions containing structural proteins likely to be involved in the events that take place in the HCV-infected human liver.

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Indeed, by the screening of preexisting drugs using the OR6 assay system, we have identified mizoribine [8], statins [9], hydroxyurea [10], and teprenone [11] as new anti-HCV drug candidates, indicating that the OR6 assay system is useful for the discovery of anti-HCV reagents.

On the other hand, we previously established for the first time an HuH-7-derived cell line (AH1) that harbors genome-length HCV RNA (AH1 strain of genotype 1b) derived from a patient with acute hepatitis C [12]. In that study, we noticed different anti-HCV profiles of interferon (IFN)-$\gamma$ or cyclosporine A (CsA) between AH1 and O cells supporting genome-length HCV RNA (O strain) replication [7]. From these results, we supposed that the diverse effects of IFN-$\gamma$ or CsA were attributable to the difference in HCV strains [12].

To test this assumption in detail, we first developed an AH1 strain-derived assay system (AH1R) corresponding to the OR6 assay system, and then performed a comparative analysis using AH1R and OR6 assay systems. In this article, we report that the difference in HCV strains causes the diverse effects of anti-HCV reagents, and we found unexpectedly by AH1R assay that rolipram, an anti-inflammatory drug, is an anti-HCV drug candidate.

## Materials and methods

### Reagents

IFN-$\alpha$, IFN-$\gamma$, and CsA were purchased from Sigma-Aldrich (St. Louis, MO). Rolipram was purchased from Wako Pure Chemical Industries (Osaka, Japan).

### Plasmid construction

The plasmid pAH1RN/C-5B/PL,LS,TA,$(VA)_3$ was constructed from pAH1 N/C-5B/PL,LS,TA,$(VA)_3$ encoding genome-length HCV RNA clone 2 (See Fig. 2) obtained from AH1 cells [12], by introducing a fragment of the RL gene from pORN/C-5B into the *Asc*I site before the neomycin phosphotransferase ($Neo^R$) gene as previously described [7].

### RNA synthesis

The plasmid pAH1RN/C-5B/PL,LS,TA,$(VA)_3$ DNA was linearized by *Xba*I, and used for RNA synthesis with T7 MEGAscript (Ambion, Austin TX) as previously described [7].

### Cell cultures

AH1R and OR6 cells supporting genome-length HCV RNAs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.3 mg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA). AH1c-cured cells, which were created by eliminating HCV RNA from AH1 cells [12] by IFN-$\gamma$ treatment, were also cultured in DMEM supplemented with 10% FBS.

### RNA transfection and selection of G418-resistant cells

Genome-length HCV (AH1RN/C-5B/PL,LS,TA,$(VA)_3$) RNA synthesized in vitro was transfected into AH1c cells by electroporation, and the cells were selected in the presence of G418 (0.3 mg/mL) for 3 weeks as described previously [13].

### RL assay for anti-HCV reagents

To monitor the effects of anti-HCV reagents, RL assay was performed as described previously [14]. Briefly, the cells were plated onto 24-well plates ($2 \times 10^4$ cells per well) in triplicate and cultured with the medium in the absence of G418 for 24 h. The cells were then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to a luciferase assay using the RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration ($EC_{50}$) of each reagent was determined.

### Quantification of HCV RNA

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Applied Science, Indianapolis, IN, USA) as described previously [7]. The experiments were done in triplicate.

### IFN-$\alpha$ treatment to evaluate the assay systems

To monitor the anti-HCV effect of IFN-$\alpha$ on AH1R cells, $2 \times 10^4$ cells and $5 \times 10^5$ cells were plated onto 24-well plates (for luciferase assay) and 10 cm plates (for quantitative RT-PCR assay) in triplicate, respectively, and cultured for 24 h. The cells were then treated with IFN-$\alpha$ at final concentrations of 0, 1, 10, and 100 IU/mL for 24 h, and subjected to luciferase and quantitative RT-PCR assays as described above.

### Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane were performed as described previously [13]. The antibodies used in this study were those against HCV Core (CP11 monoclonal antibody;

Institute of Immunology, Tokyo), NS5B, and E2 (generous gifts from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-$\beta$-actin antibody (AC-15; Sigma, St. Louis, MO, USA) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

### WST-1 cell proliferation assay

The cells were plated onto 96-well plates ($1 \times 10^3$ cells per well) in triplicate and then treated with rolipram at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration ($CC_{50}$) of rolipram was estimated. The selective index (SI) value of rolipram was also estimated by dividing the $CC_{50}$ value by the $EC_{50}$ value.

### RT-PCR and sequencing

To amplify the genome-length HCV RNA, RT-PCR was performed separately in two fragments as described previously [7, 15]. Briefly, one fragment covered from 5′-untranslated region to NS3, with a final product of approximately 6.2 kb, and the other fragment covered from NS2 to NS5B, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis of the HCV open reading frame (ORF) after cloning into pBR322MC. PrimScript (Takara Bio) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively. The nucleotide sequences of each of the three independent clones obtained were determined using the Big Dye terminator cycle sequencing kit on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

### Statistical analysis

Differences between AH1R and OR6 cell lines were tested using Student's $t$ test. $P$ values $<0.05$ were considered statistically significant.

## Results

### Development of a luciferase reporter assay system that facilitates the quantitative monitoring of genome-length HCV-AH1 RNA replication

To develop an HCV AH1 strain-derived assay system corresponding to the OR6 assay system [7], a genome-length HCV RNA encoding RL (AH1RN/C-5B/PL,LS,TA,$(VA)_3$) was transfected into AH1c cells. Following 3 weeks of culturing in the presence of G418, more than 10 colonies were obtained, and then 8 colonies (#2, #3, #4, #5, #6, #8, #13, and #14) were successfully proliferated. We initially selected colonies #2, #3, and #14 because they had high levels of RL activity ($>4 \times 10^6$ U/$1.6 \times 10^5$ cells) (Fig. 1a). However, RT-PCR and the sequencing analyses revealed that the genome-length HCV-AH1 RNAs obtained from these colonies each had an approximately 1 kb deletion in the E2 region (data not shown). In this regard, we previously observed similar phenomenon and described the difficulty of the development of a luciferase reporter assay system using the genome-length HCV RNA of more than 12 kb [7], suggesting that the NS5B polymerase possesses the limited elongation ability (probably up to a total length of 12 kb). Indeed, in that study, we could overcome this obstacle by the selection of the colony harboring a complete genome-length HCV RNA among the obtained G418-resistant colonies [7]. Therefore, we next carried out the selection among the other colonies. Fortunately, we found that colony #4, showing a rather high level of RL activity ($2 \times 10^6$ U/$1.6 \times 10^5$ cells), possessed a complete genome-length HCV-AH1 RNA without any deleted forms, although most of the other colonies possessed some amounts of a deleted form in addition to a complete genome-length HCV-AH1 RNA (data not shown). We demonstrated that the HCV RNA sequence was not integrated into the genomic DNA in colony #4 (data not shown). From these results, we finally selected colony #4, and it was thereafter referred to as AH1R and used for the following studies.

We first demonstrated that AH1R cells expressed sufficient levels of HCV proteins (Core, E2, and NS5B) by Western blot analysis for the evaluation of anti-HCV reagents, and the expression levels were almost equivalent to those in OR6 cells (Fig. 1b). In this analysis, we confirmed that the size of the E2 protein in AH1R cells was 7 kDa larger than that in OR6 cells (Fig. 1b), as observed previously [12]. This result indicates that AH1R cells express AH1 strain-derived E2 protein possessing two extra N-glycosylation sites [12]. We next demonstrated good correlations between the levels of RL activity and HCV RNA in AH1R cells (Fig. 1c), as we previously demonstrated in OR6 cells treated with IFN-$\alpha$ for 24 h [7]. These correlations indicate that AH1R cells were as useful as OR6 cells as a luciferase assay system.

### Aa substitutions detected in genome-length HCV RNA in AH1R cells

To examine whether or not genome-length HCV RNA in AH1R cells possesses additional conserved mutations such as adaptive mutations, we performed a sequence analysis of HCV RNA in AH1R cells. The results (Fig. 2) revealed that

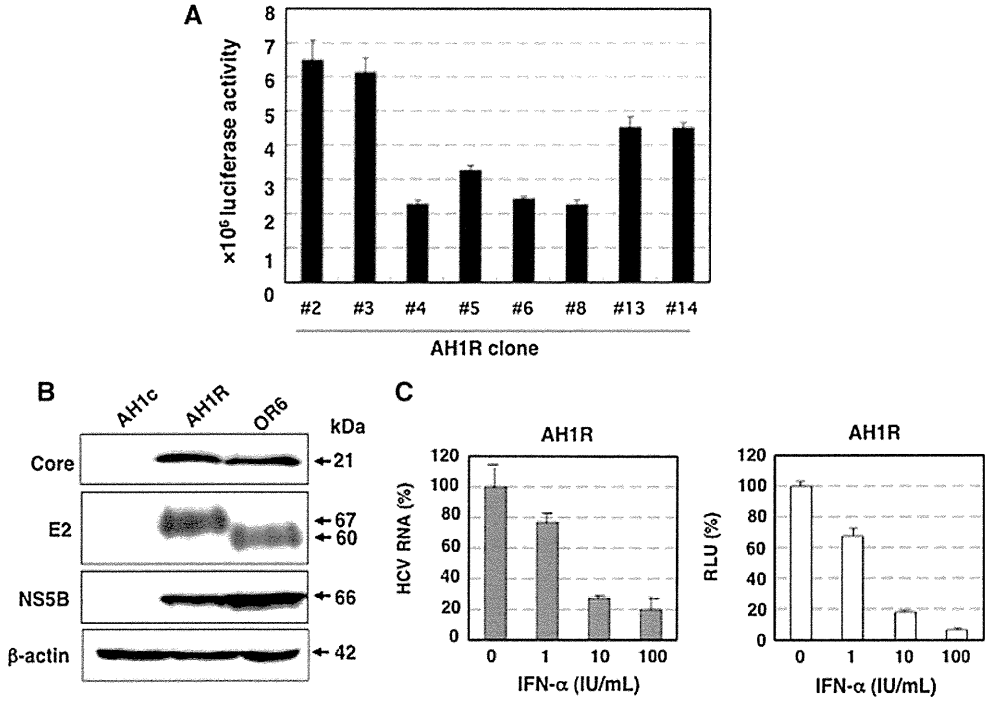


**Fig. 1** Characterization of AH1R cells harboring genome-length HCV RNA. **a** Selection of G418-resistant cell clones. The levels of HCV RNA in G418-resistant cells were monitored by RL assay. **b** Western blot analysis. AH1c, AH1R, and OR6 cells were used for the comparison. *Core, E2,* and *NS5B* were detected by Western blot analysis. *β*-actin was used as a control for the amount of protein loaded per lane. **c** RL activity is correlated with HCV RNA level. The AH1R cells were treated with IFN-α (0, 1, 10, and 100 IU/mL) for 24 h, and then a luciferase reporter assay (*right panel*) and quantitative RT-PCR (*left panel*) were performed. The relative luciferase activity (RLU) (%) or HCV RNA (%) calculated at each point, when the level of luciferase activity or HCV RNA in non-treated cells was assigned to be 100%, is presented here

two additional mutations accompanying aa substitutions (W860R (NS2) and A1218E (NS3)) were detected commonly among the three independent clones sequenced, suggesting that these additional mutations are required for the efficient replication or stability of genome-length HCV RNA. The P1115L (NS3), L1262S (NS3), V1897A (NS4B), and V2360A (NS5A) mutations derived from the sAH1 replicon [12] were conserved in AH1R cell-derived clones. However, AH1-clone-2-specific mutations (T1338A and V1880A) were almost reverted to the consensus sequences of AH1 RNA [12] except for V1880A in AH1R clone 2 (Fig. 2). In addition, the Q63R (Core) mutation was observed in two of three clones (Fig. 2).

## Comparison between the AH1R and OR6 assay systems regarding the sensitivities to IFN-α, IFN-γ, and CsA

Using quantitative RT-PCR analysis, we previously examined the anti-HCV activities of IFN-α, IFN-γ, and CsA in AH1 and O cells, and noticed different anti-HCV profiles of IFN-γ and CsA between AH1 and O cells [12]. In that study, AH1 cells seemed to be more sensitive than the O cells to CsA (significant difference was observed when 0.063, 0.12, or 0.25 μg/mL of CsA was used). Conversely, AH1 cells seemed to be less sensitive than the O cells to IFN-γ (significant difference was observed when 1 or 10 IU/mL of IFN-γ was used). However, we were not able to determine precisely the $EC_{50}$ values of these reagents, because of the unevenness of the data obtained by RT-PCR.

After developing the AH1R assay system in this study, we determined the $EC_{50}$ values of IFN-α, IFN-γ, and CsA using the AH1R assay and compared the values with those obtained by the OR6 assay. The results revealed that AH1R assay was more sensitive than OR6 assay to IFN-α ($EC_{50}$; 0.31 IU/mL for AH1R, 0.45 IU/mL for OR6) (Fig. 3a) and CsA ($EC_{50}$; 0.11 μg/mL for AH1R, 0.42 μg/mL for OR6) (Fig. 3b), and that the OR6 assay was more sensitive than the AH1R assay to IFN-γ ($EC_{50}$; 0.69 IU/mL for AH1R, 0.28 IU/mL for OR6) (Fig. 3c). Regarding these anti-HCV reagents, the anti-HCV activities observed between the AH1R and OR6 assays differed significantly in all of the concentrations examined (Fig. 3). In addition, regarding these anti-HCV reagents, cell growth was not suppressed within the concentrations used. Regarding IFN-γ and CsA, the present results clearly support those of our previous

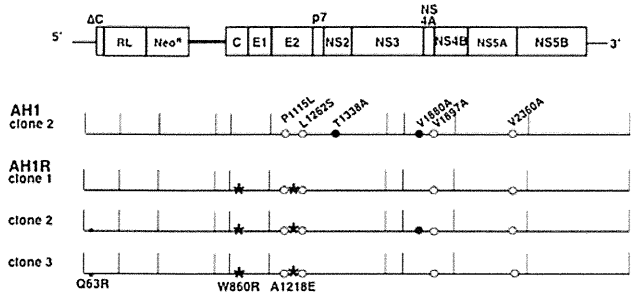


**Fig. 2** Aa substitutions detected in intracellular AH1R genome-length HCV RNA. The *upper portion* shows schematic gene organization of genome-length HCV RNA encoding the *RL* gene developed in this study. Genome-length HCV RNA consists of 2 cistrons. In the first cistron, RL is translated as a fusion protein with $Neo^R$ by HCV-IRES, and in the second cistron, all of HCV proteins (C-NS5B) are translated by encephalomyocarditis virus (EMCV)-IRES introduced in the region upstream of C-NS5B regions. Genome-length HCV RNA-replicating cells possess the G418-resistant phenotype because $Neo^R$ is produced by the efficient replication of genome-length HCV RNA. Therefore, when genome-length HCV RNA is excluded from the cells or when its level is decreased, the cells are killed in the presence of G418. In this system, anti-HCV activity is able to evaluate the value of the reporter (RL activity) instead of the quantification of HCV RNA or HCV proteins. In addition, it has been known that the infectious HCV is not produced from this RNA replication system [3, 4, 6]. Core to NS5B regions of three independent clones (*AH1R clones 1–3*) sequenced are presented. W860R and A1218E conserved substitutions are indicated by *asterisks*. Q63R substitutions detected in two of three clones are each indicated by a *small dot*. Core to NS5B regions of *AH1 clone 2*, used to establish the AH1R cell line, are also presented. AH1-specific conserved substitutions and *AH1-clone-2*-specific substitutions are indicated by *open circles* and *black circles*, respectively

study [12]. Therefore, we suggest that the diverse effects of these anti-HCV reagents are due to the difference in HCV strains, although we are not able to completely exclude the possibility that AH1R cells are compromised cells causing the different responses against anti-HCV reagents. In summary, the previous and present findings suggest that the AH1R assay system is also useful for the evaluation of anti-HCV reagents as an independent assay system.

## Anti-HCV activity of rolipram was clearly observed in the AH1R assay, but not in the OR6 assay

From the above findings, we supposed that the anti-HCV reagents reported to date might show diverse effects between the drug assay systems derived from the different HCV strains. To test this assumption, we used the AH1R and OR6 assay systems to evaluate the anti-HCV activity of more than 10 pre-existing drugs (6-Azauridine, bisindoly maleimide 1, carvedilol, cehalotaxine, clemizole, 2′-deoxy-5-fluorouridine, esomeprazole, guanazole, hemin, homoharringtonine, methotrexate, nitazoxanide, resveratrol, rolipram, silibinin A, Y27632, etc.), which other groups had evaluated using an assay system derived from the Con1 strain (genotype 1b) or JFH-1 strain (genotype 2a). The results revealed that most of these reagents in the AH1R assay showed similar levels of anti-HCV activities compared with those in the OR6 assay or those of the previous studies (data not shown). However, we found that only rolipram, a selective phosphodiesterase 4 (PDE4) inhibitor [16] that is used as an anti-inflammatory drug, showed moderate anti-HCV activity ($EC_{50}$ 31 μM; $CC_{50} > 200$ μM; $SI > 6$) in the AH1R assay, but no such activity in the OR6 assay (upper panel in Fig. 4a). This remarkable difference was confirmed by Western blot analysis (lower panel in Fig. 4a). It is unlikely that rolipram's anti-HCV activity is due to the inhibition of exogenous RL, $Neo^R$ or encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), all of which are encoded in the genome-length HCV RNA, because the AH1R and OR6 assay systems possess the same structure of genome-length HCV RNA except for HCV ORF. To demonstrate that rolipram's anti-HCV activity is not due to the clonal specificity of the cells or the specificity of genome-length HCV RNA, we examined the anti-HCV activity of rolipram using the monoclonal HCV replicon RNA-replicating cells (sAH1 cells for AH1 strain [12], and sO cells for O strain [13]). The results

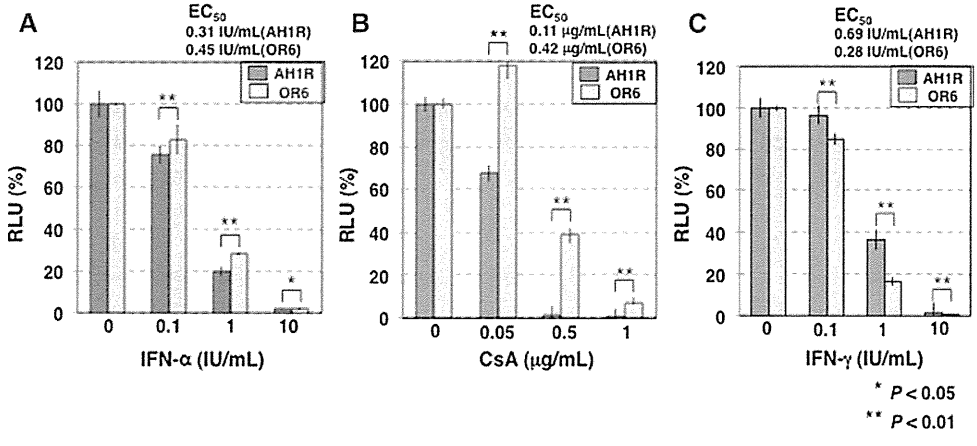


**Fig. 3** The diverse effects of anti-HCV reagents on AH1R and OR6 assay systems. AH1R and OR6 cells were treated with anti-HCV reagents for 72 h, and then the RL assay was performed as described in Fig. 1c. **a** Effect of IFN-α. **b** Effect of CsA. **c** Effect of IFN-γ

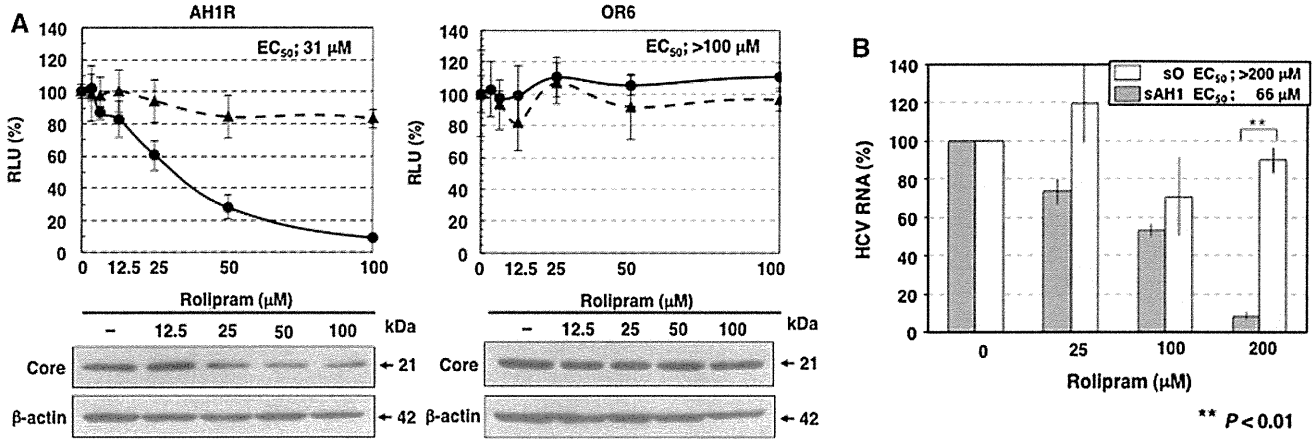


**Fig. 4** Anti-HCV activity of rolipram. **a** Rolipram sensitivities on genome-length HCV RNA replication in AH1R and OR6 assay systems. AH1R and OR6 cells were treated with rolipram for 72 h, followed by RL assay (*black circle* with *linear line* in the *upper panels*) and WST-1 assay (*black triangle* with *broken line* in the *upper panels*). The relative value (%) calculated at each point, when the level in non-treated cells was assigned to 100%, is presented here. Western blot analysis of the treated cells for the HCV Core was also performed (*lower panels*). **b** Rolipram sensitivities on HCV replicon RNA replication in sAH1 and sO cells. sAH1 and sO cells were treated with rolipram for 72 h, and extracted total RNAs were subjected to quantitative RT-PCR for HCV 5′ untranslated region as described previously [7]. The HCV RNA (%) calculated at each point, when the level of HCV RNA in non-treated cells was assigned to be 100%, is presented here

revealed by quantitative RT-PCR that rolipram showed moderate anti-HCV activity ($EC_{50}$ 66 μM) in sAH1 cells, but no such activity in sO cells (Fig. 4b). Anti-HCV activity of rolipram in sAH1 cells was a little weaker than that in AH1R cells (Fig. 4b). The similar phenomenon that the anti-HCV activity in genome-length HCV RNA-based reporter assay is stronger than that in HCV subgenomic replicon-based reporter assay was observed regarding other anti-HCV reagents in our previous studies [14, 17, 18]. This result suggests that the anti-HCV activity of rolipram is not either a clone-specific or genome-length HCV RNA-specific phenomenon. In our previous studies also [14, 18], we demonstrated that anti-HCV activities of several reagents including ribavirin and statins were not due to the clonal specificity of the cells. On the other hand, it was recently reported that rolipram did not show anti-HCV activity in the JFH-1 strain-derived assay [19]. Taken together, the previous and present results suggest that rolipram's anti-HCV activity differs depending on the HCV strain. In summary, rolipram was identified as a new anti-HCV candidate using the AH1R assay system.

## Discussion

In the present study, we developed for the first time a drug assay system (AH1R), derived from the HCV-AH1 strain (from a patient with acute hepatitis C), in which HCV-AH1 RNA is efficiently replicated. Using this system, we found that rolipram, an anti-inflammatory drug, had potential anti-HCV activity. This potential had not been detected by preexisting assay systems such as OR6, in which HCV-O RNA was derived from an HCV-positive blood donor. Since an HCV replicon harboring the sAH1 cell line, the parent of the AH1R cell line, was obtained from OR6-cured cells [12], the divergence in rolipram's effects between AH1R and OR6 cells is probably attributable to the difference in HCV strains rather than to the difference in cell clones. Indeed, rolipram's anti-HCV activity was not observed in another ORL8 assay system (O strain), which was recently developed using a new hepatoma Li23 cell line (data not shown) [15]. Therefore, we propose that multiple assay systems derived from different HCV strains are required for the discovery of anti-HCV reagents such as rolipram or for the objective evaluation of anti-HCV activity.

Comparative evaluation analysis of anti-HCV activities of IFN-α, IFN-γ, and CsA using AH1-strain-derived AH1R and O-strain-derived OR6 assay systems demonstrated that each of these anti-HCV reagents showed significantly diverse antiviral effects between the two systems. Regarding IFN-γ and CsA, the present results obtained using a luciferase reporter assay fully supported our previous findings [12] using quantitative RT-PCR analysis. However, in the present analysis, we noticed that IFN-α also showed significantly diverse effects (especially at less than 1 IU/mL) between the AH1R and OR6 assays.

The differences in IFN-α sensitivity may be attributable to the difference in aa sequences in the IFN sensitivity-determining region (ISDR; aa 2209-2248 in the HCV-1b genotype), in which aa substitutions correlate well with IFN sensitivity in patients with chronic hepatitis C [20], because the AH1 strain possesses three aa substitutions (T2217A, H2218R, and A2224 V) in ISDR, whereas the O strain possesses no aa substitutions. However, no report has demonstrated the correlation between IFN sensitivity and the substitution numbers in ISDR using the cell culture-based HCV RNA replication system.

Alternatively, Akuta et al. [21] reported that aa substitutions at position 70 and/or position 91 in the HCV Core region of patients infected with the HCV-1b genotype are pretreatment predictors of null virological response (NVR) to pegylated IFN/ribavirin combination therapy. In particular, substitutions of arginine (R) by glutamine (Q) at position 70, and/or leucine (L) by methionine (M) at position 91, were common in NVR. The patients with position-70 substitutions often showed little or no decrease in HCV RNA levels during the early phase of IFN-α treatment [21]. Regarding this point, it is interesting that position 70 in the AH1 strain is R (wild type) and that in the O strain is Q (mutant type), whereas position 91 is L (wild type) in both strains. Therefore, wild-type R in position 70 of the AH1 strain may contribute to the high sensitivity to IFN-α in the AH1R assay. Regarding positions 70 and 91 of the HCV Core, it is noteworthy that, among all of the HCV strains used thus far to develop HCV replicon systems, only the AH1 strain possesses double wild-type aa (data not shown). Therefore, the AH1R assay system may be useful for further study of sensitivity to IFN/ribavirin treatment.

The anti-HCV activity of rolipram, which is currently used as an anti-inflammatory drug, is interesting, although its anti-HCV mechanism is unclear. As a selective PDE4 inhibitor [16], rolipram may attenuate fibroblast activities that can lead to fibrosis and may be particularly effective in the presence of transforming growth factor (TGF)-β1-induced fibroblast stimulation [22]. On the other hand, HCV enhances hepatic fibrosis progression through the generation of reactive oxygen species and the induction of TGF-β1 [23]. Taken together, the previous and present results suggest that rolipram may inhibit both HCV RNA replication and HCV-enhanced hepatic fibrosis. However, it is unclear that rolipram shows anti-HCV activity against the majority of HCV strains, because rolipram has been effective for AH1 strain, but not for O strain. Although rolipram's anti-HCV activity would be HCV-strain-specific, it is not clear which HCV strain is the major type regarding the sensitivity to rolipram. Since developed assay systems using genome-length HCV RNA-replicating cells are limited to several HCV strains including O and AH1 strains to date, further analysis using the assay systems of other HCV strains will be needed to clarify this point.

In this study, we demonstrated that the AH1R assay system, which was for the first time developed using an HCV strain derived from a patient with acute hepatitis C, showed different sensitivities against anti-HCV reagents in comparison with assay systems in current use, such as OR6 assay. Therefore, AH1R assay system would be useful for various HCV studies including the evaluation of anti-HCV reagents and the identification of antiviral targets.

**Acknowledgment** This study was supported by grants-in-aid for research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan. K. M. was supported by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science.

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frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 14 February 2012
doi: 10.3389/fmicb.2012.00054

# Exploitation of lipid components by viral and host proteins for hepatitis C virus infection

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Hepatitis C virus (HCV), which is a major causative agent of blood-borne hepatitis, has chronically infected about 170 million individuals worldwide and leads to chronic infection, resulting in development of steatosis, cirrhosis, and eventually hepatocellular carcinoma. Hepatocellular carcinoma associated with HCV infection is not only caused by chronic inflammation, but also by the biological activity of HCV proteins. HCV core protein is known as a main component of the viral nucleocapsid. It cooperates with host factors and possesses biological activity causing lipid alteration, oxidative stress, and progression of cell growth, while other viral proteins also interact with host proteins including molecular chaperones, membrane-anchoring proteins, and enzymes associated with lipid metabolism to maintain the efficiency of viral replication and production. HCV core protein is localized on the surface of lipid droplets in infected cells. However, the role of lipid droplets in HCV infection has not yet been elucidated. Several groups recently reported that other viral proteins also support viral infection by regulation of lipid droplets and core localization in infected cells. Furthermore, lipid components are required for modification of host factors and the intracellular membrane to maintain or up-regulate viral replication. In this review, we summarize the current status of knowledge regarding the exploitation of lipid components by viral and host proteins in HCV infection.

**Keywords: HCV, hepatitis, lipid droplets, host factor**

## INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease including steatosis, cirrhosis, and hepatocellular carcinoma. Epidemiological studies indicate that HCV is also associated with extrahepatic manifestations including type 2 diabetes mellitus, B-cell non-Hodgkin lymphoma, mixed cryoglobulinemia, and Sjögren's syndrome (Jacobson et al., 2010). It has been estimated that there are 170 million patients worldwide, of whom most are infected with HCV. Combination therapy with pegylated interferon (PEG-IFN) and ribavirin has been the standard treatment but it fails to cure ~50% of treated patients (Soriano et al., 2009).

Hepatitis C virus belongs to the genus *Hepacivirus* of the family Flaviviridae. The viral genome of HCV is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb and it encodes a single polypeptide (**Figure 1**). This polyprotein is cleaved by host and viral proteases into structural and non-structural proteins (Harada et al., 1991; Hijikata et al., 1991; Grakoui et al., 1993a,b). Structural proteins, including the core protein and two envelope proteins, and the viroporin p7 are located within one-third of the N-terminal, while the remaining viral proteins are classified as non-structural proteins which form a replication complex with host factors (Grakoui et al., 1993c). HCV core protein is cleaved by signal peptide cleavage and then released from E1 (Santolini et al., 1994). After cleavage by signal peptidase (SP), the C-terminal transmembrane region of the core protein is further cleaved by signal peptide peptidase (SPP; Hussy et al., 1996; McLauchlan et al., 2002). The nucleocapsid, composed of matured core proteins and the viral genome, is surrounded by an envelope composed of host lipids and viral envelope proteins (Wakita et al., 2005). The life cycle of HCV is shown in **Figure 2**. The viral envelope proteins play a role in the binding to host receptors and membrane fusion for uncoating. Recently, several groups reported that the viral particle binds to a very low-density lipoprotein (VLDL), including apolipoprotein E (apoE), which is required for the binding step (Andre et al., 2002; Nielsen et al., 2006; Chang et al., 2007; Benga et al., 2010) as described below. The virus infects hepatocytes via entry factors known as receptors and co-receptors. The viral particle complex composed of the enveloped nucleocapsid and VLDL including apoE (Merz et al., 2011), is reported to bind to heparin sulfate (HS; Barth et al., 2003) and the low-density lipoprotein (LDL) receptor (LDLR; Agnello et al., 1999), although Albecka et al. (2012) recently reported that LDLR is required for optimal replication of the HCV genome rather than entry of the infectious viral particle. Other host factors may be involved in apoE-mediated entry. The HCV viral particle is transferred to the scavenger receptor class B type I (SR-BI; Scarselli et al., 2002; Bartosch et al., 2003) and CD81 (Pileri et al., 1998) through E2 binding and then enters cells with claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Ploss et al., 2009) by endocytosis. The Niemann–Pick C1-like 1 cholesterol absorption receptor has recently been reported to be an HCV cell entry factor that is involved in the entry step between post-binding and

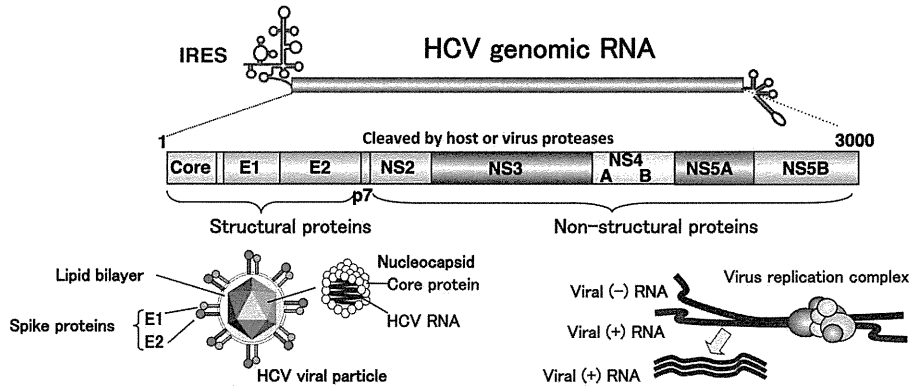


**FIGURE 1 | Structure of HCV.** HCV RNA encodes a polyprotein composed of about 3,000 amino acids. The core protein, and two envelope proteins are classified as structural protein, while NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural proteins.

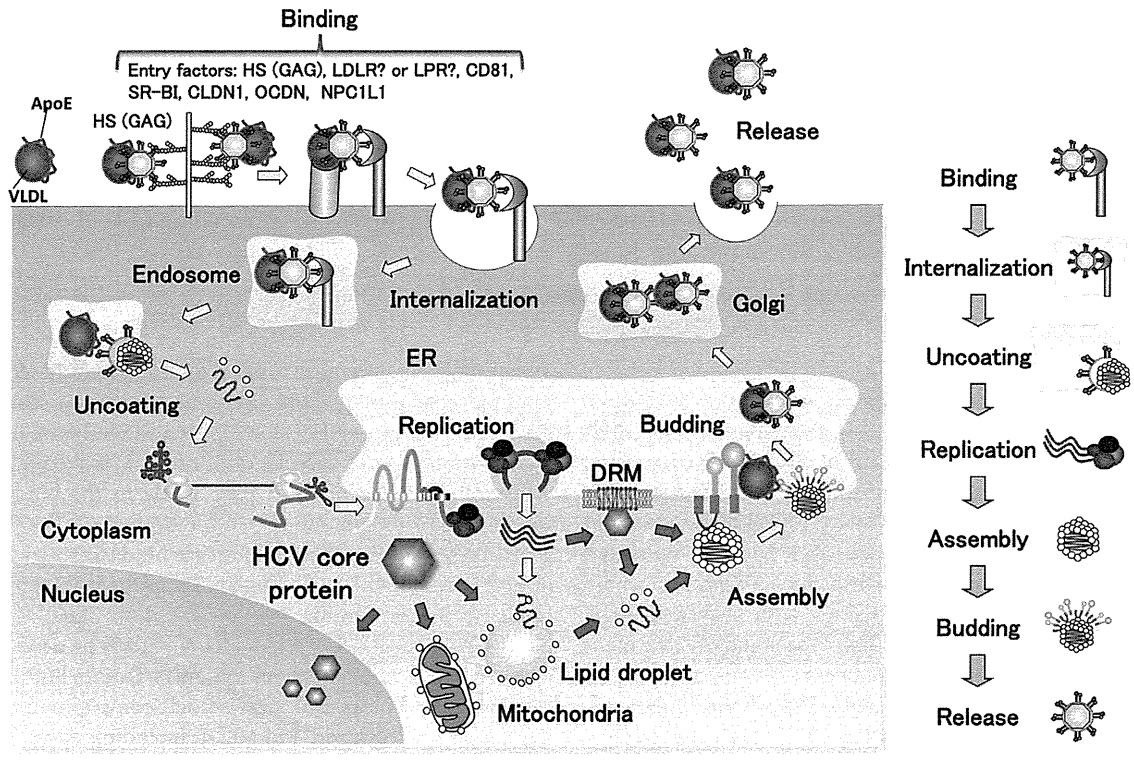


**FIGURE 2 | The infection cycle of HCV.** The HCV complex with VLDL binds to entry factors on the surface of hepatocytes and then enters cells by endocytosis. After uncoating, viral replication is carried out in the convoluted membrane structure called the membranous web. The viral nucleocapsid egresses into the lumen side of the ER and binds to VLDL. The HCV complex with VLDL is released from the infected cells.

pre-fusion (Sainz et al., 2012). The viral envelope fuses with the host plasma membrane in an endosome under a low pH condition (Takikawa et al., 2000; Hsu et al., 2003; Blanchard et al., 2006; Codran et al., 2006; Meertens et al., 2006; Tscherne et al., 2006). The capsid protein and viral genome are expected to be released into the cytoplasm of infected cells. The viral replication, assembly, and budding are summarized in **Figure 3** on the basis of current information. The viral genome is translated dependent on own internal ribosome entry site (Tsukiyama-Kohara et al., 1992) and transcribed by the translated and processed NS3 to NS5B (Lohmann et al., 1999). The viral protein NS4B induces a convoluted membrane structure (termed a membranous web) with host lipid components and proteins, in which the viral replication is carried out (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). The newly synthesized viral positive stranded RNA genome is released from the membranous web and passes to the

core protein via NS5A (Masaki et al., 2008). The core protein is translocated on the surface of the lipid droplet or endoplasmic reticulum (ER) membrane for efficient formation of viral particles, and then encloses the synthesized viral genome to form a capsid near the membranous web (Miyanari et al., 2007; Boson et al., 2011). The capsids are enclosed by an endoplasmic membrane containing the viral envelope proteins E1 and E2 and are then released into ER lumen side, since intracellular envelope proteins are categorized as high-mannose type glycoproteins and the viral particle composed of core proteins and envelope proteins egresses into the lumen side of the intracellular compartment associated with lipid droplets (Miyanari et al., 2007; Vieyres et al., 2010). The viral particle is secreted through a host secretion pathway, although the mechanism by which HCV particles are secreted in infected cells remains poorly understood.

Although no effective vaccine for HCV has been developed, antiviral drugs targeting to the viral and host factors have been reported recently. The HCV replicon system was reported for a screening system based on cultured cells (Lohmann et al., 1999) and has been improved by modification of cell lines and marker genes and introduction of adaptive mutations in the region of the viral RNA genome for high efficiency of viral replication (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001; Ikeda et al., 2002; Pietschmann et al., 2002). The complete infectious cycle of HCV in cultured cells was established in a highly permissive cell line by using the genotype 2a strain JFH1 or its chimeric recombinant virus (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). A system based on the cultured cell line has been an exclusive drug-screening system for finding antiviral compounds that interfere with the process of viral RNA replication under intracellular conditions. NS3 forms a complex with cofactor NS4A (Failla et al., 1994, 1995; Koch et al., 1996). This interaction stabilizes NS3 and retains it on the ER where it acquires the ability of a protease against viral polyprotein (Wolk et al., 2000) and host protein IPS-1/MAVS (Foy et al., 2005; Loo et al., 2006), which is a molecule downstream of the RIG-I sensor molecule (Sumpter et al., 2005; Loo et al., 2008). NS3 serine protease activity is a target of the direct acting HCV antiviral drugs known as telaprevir and boceprevir, which are available clinically by combination therapy with PEG-INF and ribavirin (Hofmann and Zeuzem, 2011). The RNA helicase activity of NS3 and NS5B RNA-dependent RNA polymerase are also used for drug-screening in particular (Hicham Alaoui-Ismaili et al., 2000; Dhanak et al., 2002; Borowski et al., 2003; De Francesco et al., 2003; Boguszewska-Chachulska et al., 2004; Maga et al., 2005; Najda-Bernatowicz et al., 2010). Combination therapy using several compounds targeting host and viral factors may be able to completely eradicate the virus and suppress the pathogenicity induced by HCV infection.

Liver steatosis, which is characterized by accumulation of lipid droplets in hepatocytes, is significantly associated with the incidence of hepatocellular carcinoma in HCV-infected patients (Ohata et al., 2003). Severe liver steatosis has been frequently found in patients infected with the genotype 3a virus (Rubbia-Brandt et al., 2000; Adinolfi et al., 2001). Successful clearance of HCV reduces steatosis in genotype 3a patients, suggesting an association between genotype 3a and severe steatosis. Furthermore, HCV core protein derived from genotype 1 also induced liver steatosis in mouse and cultured cells (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). Lipid droplets containing triglycerides and cholesteryl ester are increased in cells expressing core protein and are surrounded by the core protein (Hope and McLauchlan, 2000). Non-structural proteins associate with the lipid droplets surrounded by HCV core proteins to supply the synthesized viral genome for viral assembly (Miyanari et al., 2007). Other lipid components are reported to be involved in formation of viral particles and the viral RNA replication as described below. This review mainly summarizes the viral and host factors that are associated with lipid metabolism with regard to HCV replication and pathogenicity.

## THE ROLE OF VLDL IN HCV INFECTION

Hepatitis C virus replicates in a convoluted membrane structure as a membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010) and assembles in the area of the ER membrane-associated with lipid droplets surrounded by the core protein (Miyanari et al., 2007). The LDLR has also been proposed to function as one of entry factors described above for HCV entry, in which interaction between LDLR and HCV particles is facilitated though interaction of the virus with host lipoprotein components (Monazahian et al., 1999; Chang et al., 2007; Huang et al., 2007; Miyanari et al., 2007; Gastaminza et al., 2008). HCV RNA containing particles derived from infected human serum were fractionated in densities with a value of 1.03–1.25 g/ml (Thomssen et al., 1992, 1993). The HCV RNA particles of the fraction with a density of lower than 1.06 g/ml possessed infectivity against chimpanzees, while HCV RNA derived from fractions with a higher density showed poor infectious ability (Bradley et al., 1991; Hijikata et al., 1993). The infectious HCV particles form a LDL–virus complex in the sera of human patients (Andre et al., 2002). An LDL–virus complex was found in the fractions with very low to low buoyant densities (1.03–1.25 g/ml), which varied with the stage of infection (Pumeechockchai et al., 2002; Carabaich et al., 2005). HCV particles prepared from infected human serum forms a complex with lipoproteins designated as lipo-viro-particles (LVP; **Figure 3**; Andre et al., 2002; Nielsen et al., 2006). LVP includes triglycerides, HCV RNA, core protein, and apolipoproteins B and E (Andre et al., 2002), which are components of VLDLs and LDL (Brodsky et al., 2004).

Very low-density lipoprotein is formed with a hydrophobic particle composed of triglycerides and cholesteryl ester that is surrounded by a surface coat containing phospholipid, free cholesterol, and two dominant lipoproteins, apoB and apoE (review to see Havel, 2000). Both apoB and apoE were found in a low-density fraction of HCV RNA particles (Andre et al., 2002; Chang et al., 2007). HCV virions could also be precipitated with antibodies against apoB or apoE (Andre et al., 2002; Chang et al., 2007). ApoB and microsomal triglyceride transfer protein (MTP) are required for HCV assembly and production, since knockdown of apoB or a specific antibody to MTP could inhibit HCV production (Huang et al., 2007; Gastaminza et al., 2008). However, another report suggests that knockdown of apoB or antibodies to apoB exhibited no significant effect on HCV infectivity and production (Jiang and Luo, 2009). The monoclonal antibodies against apoE neutralized HCV infection in cultured cells (Chang et al., 2007; Jiang and Luo,

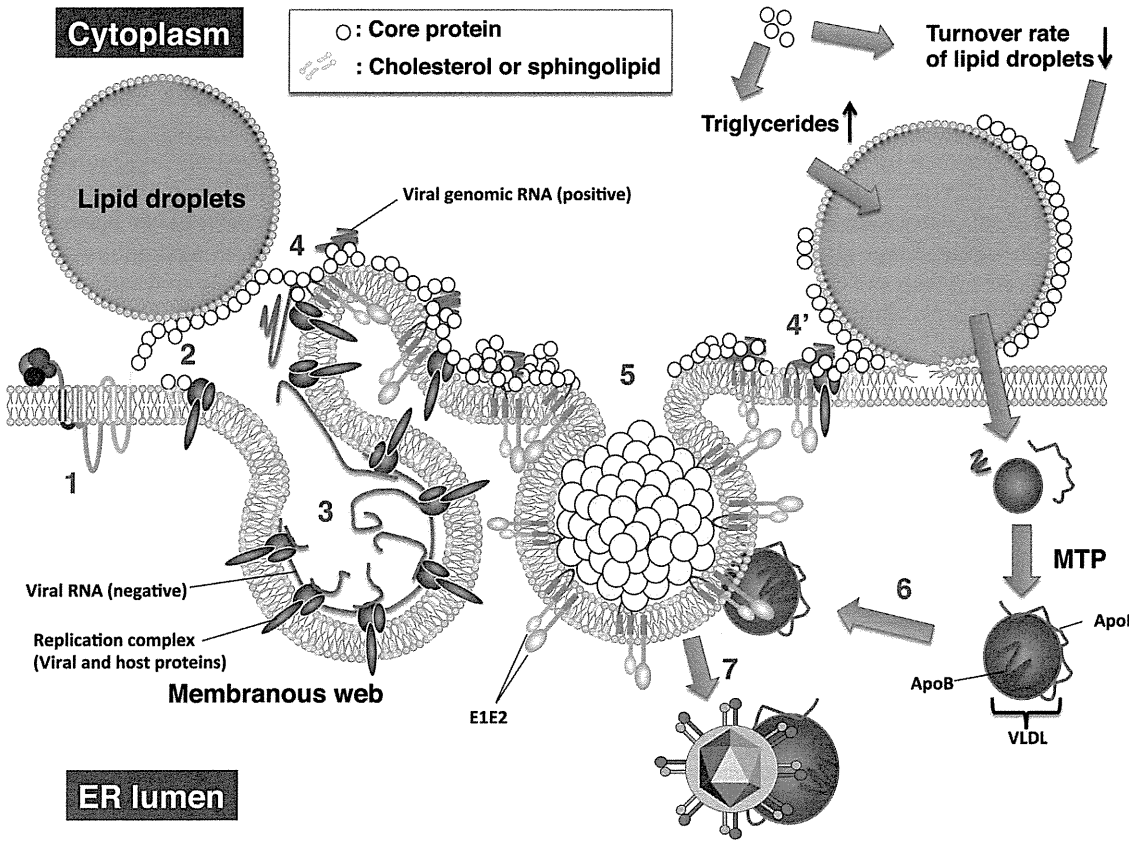


**FIGURE 3 | Hepatitis C virus RNA replication and particle production on lipid droplets and the ER membrane.** HCV RNA replication and viral particle production are thought to be carried out on the lipid droplets and ER membrane. The viral proteins take advantage of host lipid metabolism and intracellular compartments to produce viral components as described in the text. Each step is described below. (1) A newly synthesized or uncoated vial genomic RNA is translated to a viral polyprotein followed by a cleaving polyprotein. (2) Matured non-structural proteins form replication a complex with host factors. The core protein is translocated to lipid droplets by DGAT1. In cells infected with the highly infectious strain (e.g., Jc1), the core protein may be retained on ER membrane by cooperating with non-structural proteins. (3) NS4B is involved in the convoluted membrane structure dubbed the membranous web. Negative and positive strands of viral RNA are synthesized by the replication complex based on each other. (4) The positive strand of genomic RNA is enclosed with the core proteins, cooperating non-structural proteins. Envelope proteins, NS2, and p7 may determine recruitment of the core protein from lipid droplets to the ER membrane along microtubules. Lipid droplets hemi-fused with ER membrane (Wolins et al., 2006) may be involved in recruitment of core protein on the ER membrane (4'). (5) The core proteins enclose the viral RNA genome, and are then surrounded by envelopes. Non-structural proteins and other factors may support formation of nucleocapsid and budding. (6) VLDL binds to viral particles to form LVP before or after budding. (7) The viral particles egress into the ER lumen with VLDL including ApoE. The core protein upregulates synthesis or storage of triglycerides and cholesteryl ester, cooperating with host factors including DGAT1, and so on.

2009), while knockdown of apoE markedly reduced HCV infectivity and infectious viral production without affecting viral entry and replication (Chang et al., 2007; Berger et al., 2009; Jiang and Luo, 2009). Hishiki et al. (2010) suggested that the isoforms 3 and 4, but not 2, of apoE are critical for HCV infectivity dependent of affinity to LDLR. Furthermore, NS5A could interact with apoE in infected cells and colocalization of both proteins supports the notion of intracellular interaction in infected cells (Benga et al., 2010). The C-terminal alpha-helix region spanning from residue 205 to 280 was critical for NS5A–apoE interaction and viral production (Cun et al., 2010). ApoE included in LVP may directly bind to LDLR or LDLR-related proteins in hepatocytes (**Figure 2**), since apoE is a ligand for all members of the LDLR gene family (see review described by Herz et al., 2009). These results suggest that apoE is an essential host factor for HCV entry.

## LOCALIZATION OF THE CORE PROTEIN ON BOTH ER AND LIPID DROPLETS IN INFECTED CELLS

Hepatitis C virus core protein is located at the N-terminus of the HCV polyprotein (**Figure 1**). The HCV core protein is cleaved from a precursor polyprotein by a SP, releasing it from an envelope E1 protein. Then, the C-terminal transmembrane region of the core protein is further processed by a SPP (McLauchlan et al., 2002). The intramembrane processing of the HCV core protein by SPP is critical for the production of infectious viral particles (Okamoto et al., 2008). The C-terminal end of the mature HCV core protein expressed in insect and human cell lines was determined to be $Phe^{177}$ (Ogino et al., 2004; Okamoto et al., 2008). Randall et al. (2007) reported that the introduction of an siRNA targeted to SPP (called HM13) reduced the production of infectious HCV particles, suggesting that SPP is required for HCV

particle production. Our previous report (Okamoto et al., 2008) showed that the production of HCV in cells persistently infected with the JFH1 strain was impaired by treatment with an SPP inhibitor and that JFH1 viruses bearing SPP-resistant mutations in the core protein failed to propagate in a permissive cell line. These data suggest that intramembrane processing of HCV core protein by SPP is required for viral propagation. Matured core protein was found in a detergent-resistant membrane fraction, which was distinct from the classical lipid rafts (Matto et al., 2004). Our data also suggest that cleavage of HCV core protein by SPP is required for localization of HCV core protein in detergent-resistant membrane fractions including cholesterol and sphingolipid (**Figure 3**, step 4 and 5). Detergent-resistant membrane fractions may be derived from the membranous web where the viral replication complex synthesizes the viral RNA genome, since the replication complex is fractionated in lipid raft fractions including Vesicle-associated membrane protein-associated protein (VAP)-A, cholesterol, and sphingolipid (**Figure 3**, step 3; Shi et al., 2003; Aizaki et al., 2004; Gao et al., 2004; Sakamoto et al., 2005). Furthermore, an HCV core protein mutation resistant to SPP results in delayed localization of HCV core protein on lipid droplets and reduction of virus production (Targett-Adams et al., 2008). These reports suggest that cleavage of HCV core protein by SPP is required for its suitable intracellular localization for the viral assembly. Sequence analysis of the core protein suggests that high hydrophobicity is found in the region from amino acid residues 119 to 174, which is called domain 2 (Hope and McLauchlan, 2000). Domain 2 is critical for localization of the core protein on lipid droplets and shares common features with the core protein of GBV-B, but not of other viruses belonging to the Flaviviridae family (Hope et al., 2002). When three hydrophobic amino acids, Leu139, Val140, and Leu144, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, the triple mutations in the core protein led to resistance to SPP cleavage, dislocation of the detergent-resistant membrane, and a reduction in virus production (Okamoto et al., 2004, 2008). Furthermore, comparative analysis between JFH1 and Jc1 suggest that binding strength of domain 2 of core for lipid droplets determines efficiency of virus assembly (Shavinskaya et al., 2007). These results suggest that hydrophobicity of domain 2 in the core protein is required for lipid droplet localization, SPP cleavage, and virus production.

Host lipid biogenesis is responsible for replication and assembly. HCV core protein contributes to the accumulation and production of host lipid components and is detected on the surface of lipid droplets (Hope and McLauchlan, 2000). The core protein is translocated into the lipid droplets near the replication complex and encloses newly synthesized viral RNA to form the nucleocapsid (**Figure 3**, step 2–4 or 4′), egresses into the lumen side of the ER, then is surrounded with host lipid components and viral envelope proteins (**Figure 3**, step 5; Miyanari et al., 2007). HCV core protein interacts with diacylglycerol acyl transferase 1 (DGAT1), which is required for the trafficking of core protein to lipid droplets (**Figure 3**, step 2; Herker et al., 2010). However, the translocation of the core protein to lipid droplets may not be required for efficient production of viral particles. The recombinant virus Jc1 exhibits a higher virus titer than the JFH1 strain (Lindenbach et al., 2006; Pietschmann et al., 2006). The core protein of the Jc1 strain is hardly detected on lipid droplets in infected cells and is mainly localized on ER membranes, together with envelope protein E2 (Miyanari et al., 2007; Shavinskaya et al., 2007; Boson et al., 2011). Expression of p7 increases the ER localization of core protein in the absence of envelope proteins (Boson et al., 2011). However, Miyanari et al. (2007) reported that the core protein of the Jc1 strain was mainly localized with envelope proteins on ER in cells transfected with a complete viral genome, but on lipid droplets in cells that were transfected with the viral genome lacking envelope protein genes. Expression of envelope proteins and p7 may determine intracellular localization of the core protein with regard to viral assembly (**Figure 3**, step 2 and 4 or 4′).

NS2 has been reported to be involved in the assembly process of HCV particles (Jones et al., 2007; Jirasko et al., 2008; Dentzer et al., 2009). NS2, which is composed of three transmembrane regions and a cytoplasmic domain in order after p7 (Lorenz et al., 2006), is known as the autoprotease of which C-terminal cytoplasmic domain is involved in *cis* cleavage at the NS2–NS3 junction (Santolini et al., 1995; Yamaga and Ou, 2002; Lorenz et al., 2006). Genetic interaction was implied between the N-terminal region of NS2 and the upstream structural proteins, since the first transmembrane of NS2 was identified as a genetic determinant for infectivity by construction of chimeric HCV with various genotypes (Pietschmann et al., 2006). Analyses by co-immunoprecipitation and imaging microscopy for interaction between NS2 and other viral proteins in cultured cells suggest that NS2 interacts with p7 and E2 on the ER-derived dotted structure closed to lipid droplets that are surrounded by HCV core protein (Popescu et al., 2011). NS2 also interacts with NS3/4A to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Counihan et al., 2011). HCV p7 is a short hydrophobic protein composed of 63 amino acids and is encoded between the structural and non-structural proteins (Carrere-Kremer et al., 2002). The cytoplasmic loop of p7 is located between the N-terminal and C-terminal transmembrane regions (Carrere-Kremer et al., 2002). HCV p7 is known as a viroprotein that forms homooligomerize to be a ion channel, which is then involved in assembly and release of virus particle in infected cells by modulating pH equilibration in intracellular vesicles (Carrere-Kremer et al., 2002; Jones et al., 2007; Steinmann et al., 2007; Wozniak et al., 2010). Mutations of conserved amino acids required for ion channel activity impaired the production of infectious virus (Jones et al., 2007). However, recruitment of HCV core protein from lipid droplets to the ER assembly site was independent of the ion channel activity of HCV p7 (Boson et al., 2011). HCV p7 enhanced ER localization of the core protein without other viral proteins regardless of viral genotype, although compatibilities between two transmembrane regions of p7 and the first transmembrane domain of NS2 are responsible for ER localization of core protein and infection (Boson et al., 2011). The second transmembrane region of p7, rather than the first, is critical for compatibilities with NS2 regarding recruitment of core protein to the ER assembly site, although both transmembrane regions of p7 are important to sustain infectivity (Boson et al., 2011). These reports speculate that localization of the core protein on lipid droplets may contribute to suppression of virus production and maintenance of persistent HCV infection, while localization of the

core protein on ER may positively support virus production under the fulminant condition.

## REGULATION OF HOST LIPID METABOLISM BY THE CORE PROTEIN

The mechanisms by which the core protein can induce liver diseases and extrahepatic manifestations are unknown. Liver steatosis, which is one of the characteristics associated with persistent HCV infection, develops by accumulation of triglyceride-rich lipids in hepatocytes. However, the precise functions of HCV proteins in the development of fatty liver remain unknown due to the lack of an adequate system to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and hepatocellular carcinoma in transgenic mice (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). The lipid composition of the core-transgenic mouse is similar to that of a hepatitis C patient (Koike et al., 2010; Miyoshi et al., 2011). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and hepatocellular carcinoma. Biosynthesis of triglycerides is mainly regulated by the sterol regulatory element-binding protein (SREBP)-1c. It has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees (Bigger et al., 2004). Our study has demonstrated that the core protein enhances the binding activity of the LXRα–RXRα complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes (Moriishi et al., 2007). The activation may be mediated by the direct interaction between the core protein and RXRα (Tsutsumi et al., 2002). Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and core-transgenic mice (Perlemuter et al., 2002). The MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein E, cholesterol, and triglycerides. In core-transgenic mice, MTP-specific activity is significantly decreased (Perlemuter et al., 2002). In addition, DGAT1, which plays an important role in trafficking core protein from lipid droplets to the ER membrane (Herker et al., 2010), was reported to delay the turnover of lipid droplets that are coated by the core protein (Harris et al., 2011; **Figure 3**). Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication (Kapadia and Chisari, 2005). The core protein can enhance the production of reactive oxygen species (ROS) by induction of induced nitric oxide synthetase (iNOS) or by damage to the mitochondrial electron transport system, contributing to the emergence of hepatocellular carcinoma (Moriya et al., 2001; Okuda et al., 2002; Nunez et al., 2004), suggesting that accumulation of lipids hastens the occurrence of hepatocellular carcinoma by enhancing ROS production. The core protein is reported to be degraded by PA28gamma-dependent, but ubiquitin-independent, proteasome activity, and directly binds to PA28gamma (Moriishi et al., 2003; Suzuki et al., 2009). PA28gamma knockdown diminished liver steatosis, hepatocellular carcinoma, and insulin resistance induced by HCV core protein in the mouse liver (Moriishi et al., 2007). After our reports, several groups found that PA28gamma plays an important role in cell cycling by degradation of SRC-3, p16, p19, and p53 (Li et al., 2006; Chen et al., 2007; Zhang and Zhang, 2008). Furthermore, HCV propagation in a cell culture system is potently suppressed by PA28gamma knockdown, regardless of cell growth (Moriishi et al., 2010). One possibility is that E6AP-dependent ubiquitination of the core protein in cytoplasm is competitively suppressed by peptide fragments deduced from nuclear core protein. However, there is still the possibility of an indirect effect of PA28gamma, since potent reduction of PA28gamma, but not intermediate reduction, can induce nuclear accumulation of HCV core protein in cultured cells and the mouse liver, but both potent and intermediate reductions could suppress viral production (Moriishi et al., 2007, 2010; Cerutti et al., 2011). Further study will be required to clarify the mechanism by which PA28gamma regulates core-induced liver diseases and the HCV life cycle.

## NS3/4A AND LIPID DROPLETS

The NS3 also cleaves the host adaptor proteins IPS-1/MAVS and TRIF to modulate TLR and RIG-I signaling, resulting in inhibition of type I interferon production (Ferreon et al., 2005; Li et al., 2005a,b; Cheng et al., 2006; Loo et al., 2006). It is speculated that NS3 suppresses the activation of host innate immunity induced by HCV RNA and then contributes to persistent infection with HCV. NS3/4A may be responsible for not only the replication, but also the virus assembly and production by interaction with viral and host proteins on a region close to lipid droplets/ER assembly site. NS3/4A interacts with NS2 cooperating with p7 and E2 to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Boson et al., 2011; Counihan et al., 2011; Popescu et al., 2011). HCV NS3/4A also interacts with host protein Y-box-binding protein-1 (YB-1) and influences the equilibrium between viral replication and infectious particle production (Chatel-Chaix et al., 2011). Knockdown of YB-1 impaired HCV RNA replication, regardless of the viral genotype, but did not affect NS3/4A autoprocessing and MAVS cleavage (Chatel-Chaix et al., 2011). JFH1 infection allowed YB-1 to translocate to lipid droplets containing core protein and NS3 (Chatel-Chaix et al., 2011), although knockdown of YB-1 enhanced the production of viral infectious particles (Chatel-Chaix et al., 2011). YB-1 may cooperate with NS3/4A to negatively regulate the steps after replication and to positively regulate viral replication.

## NS5A AND CYCLOPHILINS

The peptide bond *cis/trans* isomerase converts between *cis* and *trans* peptide bonds leading to correct folding of the protein substrate. Peptidyl prolyl *cis/trans* isomerase (PPIase) includes the families of cyclophilin (Fischer et al., 1989), FK506-binding proteins (FKBP; Siekierka et al., 1989a,b) and parvulins (Rahfeld et al., 1994), and the secondary amide peptide bond *cis/trans* isomerase (Schiene-Fischer et al., 2002). Cyclophilin and FKBP are categorized as immunophilins, which are targeted by the immunosuppressants cyclosporin and FK506, respectively (Liu et al., 1991). Some cyclophilins and FKBP8 were shown to interact with NS5B and/or NS5A and to regulate HCV replication (Watashi et al., 2005; Okamoto et al., 2006), suggesting that immunophilins could lead to promising therapies for chronic hepatitis C, as discussed below.

Cyclosporin A and its derivatives, which target cyclophilins, were shown to impair HCV RNA replication and to exhibit efficacy in hepatitis C patients (Watashi et al., 2003; Ishii et al., 2006). Inoue et al. (2003) reported cyclosporin A treatment of HCV in a clinical trial. Cyclosporin derivatives lacking the ability to interact with cyclophilin lost their inhibitory effect on HCV replication (Watashi et al., 2005). Cyclophilin B is reported to be a 20-kDa secreted neurotropic factor for spinal cord cells in chick embryos (Spik et al., 1991), and is secreted into human milk and blood (Spik et al., 1991; Allain et al., 1994). Cyclophilin B specifically interacts with NS5B, the HCV RNA-dependent RNA polymerase around the ER of the HCV replicon cells, and promotes NS5B's association with viral RNA (Watashi et al., 2005). Cyclosporin A (CsA) was shown to disrupt the interaction between NS5B and cyclophilin B (Watashi et al., 2005). Treatment with cyclosporin A and knockdown of cyclophilin B suppressed the replication of HCV. However, several groups reported that interaction between NS5A and cyclophilin A is more important for HCV replication than interaction between NS5B and cyclophilin B. There is a growing consensus that cyclophilin A in particular is a crucial factor during HCV replication. A number of point mutations in both NS5A and NS5B have been reported to be associated with *in vitro* resistance to cyclophilin A (Yang et al., 2008; Chatterji et al., 2009; Kaul et al., 2009). Direct interaction between cyclophilin A and NS5B or NS5A has been observed (Yang et al., 2008). Several CsA-analogs, i.e., NIM811 (Ma et al., 2006), DEB025, and SCY-635 (Hopkins et al., 2010), are currently in preclinical and clinical development. DEB025 disrupts the interaction between NS5A and cyclophilin A and suppresses cyclophilin A isomerase activity. Although experimental differences in cell lines and replicons may affect employment of cyclophilins in HCV replication, the main molecule targeted by the cyclosporin analogs used clinically so far seems to be cyclophilin A.

The treatment with CsA has been associated with increased susceptibility to atherosclerosis and the development of hyperlipidemia (reviewed by Kockx et al., 2010). Treatment with CsA upregulated activity of cholesteryl ester transfer protein and suppressed lipoprotein lipase activity (Tory et al., 2008). Upregulation of cholesteryl ester by cholesteryl ester transfer protein could lead to accumulation of lipoprotein with cholesteryl ester. The report by Anderson et al. (2011) suggests that cyclophilin A and cyclophilin 40 are important for not only viral replication, but also the release of infectious viral particles. NIM811 treatment suppresses virus production and viral RNA replication (Goto et al., 2006). NIM811 treatment led to enlargement of lipid droplets and apoB crescent formation in replicon cells, but not naïve Huh7 cell line, while decreasing apoB secretion and the number of lipid droplets, rendering NS5A dislocation with apoB (Anderson et al., 2011). Knockdown of cyclophilins A and 40 in replicon cells showed the similar changes in lipid droplets size and apoB localization, comparing with NIM811 treatment (Anderson et al., 2011). Cyclophilins A and 40 may regulate lipid trafficking in the presence of HCV proteins to support secretion of viral particles.

## NS5A/B AND MEMBRANE-ASSOCIATED PROTEINS

Host lipids are well known to be essential components in the viral life cycle, including the assembly, budding, and replication of various viruses (Chen et al., 2005; Giese et al., 2006; Mannova et al., 2006; Oomens et al., 2006). In the case of HCV, several types of lipids are required for the HCV life cycle. Saturated and monounsaturated fatty acids, but not polyunsaturated fatty acids, enhance HCV RNA replication (Kapadia and Chisari, 2005), suggesting that lipid biogenesis is involved in HCV replication. HCV particles bind to lipoprotein receptors for entry (Agnello et al., 1999; Scarselli et al., 2002) and are believed to exist with lipoproteins in the serum of infected patients (Thomssen et al., 1992). There is also evidence that HCV uses the VLDL assembly and secretion pathway for maturation and secretion of viral particles (Huang et al., 2007; Gastaminza et al., 2008). Cholesterol and sphingolipids are employed for virion maturation and infectivity, since depletion of cholesterol or down-regulation of sphingomyelin reduces infectivity (Aizaki et al., 2008). Accumulation of lipid components in the liver leads to liver steatosis, and is associated with progression to liver fibrosis and hepatocellular carcinoma, as described above.

Hepatitis C virus replication is suppressed by an inhibitor of geranylgeranyl transferase I, but not by that of farnesyl transferase (Ye et al., 2003). Geranylgeranylate is known as an intermediate found in the mevalonate pathway and is covalently bound to various cellular proteins that are associated with plasma or the intracellular membrane (Horton et al., 2002). Immunoprecipitation analysis revealed that NS5A interacts with FBL2 (Wang et al., 2005a). The F-box motif is located in the N-terminus of FBL2, followed by 11 leucine-rich repeats (Ilyin et al., 1999) and the CAAX motif, which is thought to be modified by geranylgeranylation (Wang et al., 2005a). The F-box motif is generally essential for the formation of the ubiquitin ligase complex (Ilyin et al., 1999), suggesting that FBL2 regulates the ubiquitination of host or viral proteins through the interaction with NS5A. Another possibility is that FBL2 retains the viral replication complex by interacting with NS5A (**Figure 3**, step 3).

Screening of a genome-wide siRNA library revealed phosphatidylinositol 4-kinase III alpha (PI4KA) and COPI vesicle coat complex as a human gene associated with HCV replication (Bigger et al., 2004; Borawski et al., 2009; Li et al., 2009; Tai et al., 2009; Trotard et al., 2009; Vaillancourt et al., 2009; Reiss et al., 2011). Phosphatidylinositol 4-phosphate, which is associated with oxysterol binding protein (OSBP) and CERT (Peretti et al., 2008; Banerji et al., 2010) as described below, is increased by HCV infection (Bigger et al., 2004; Hsu et al., 2010; Reiss et al., 2011; Tai and Salloum, 2011). PI4KA is co-localized with NS5A and double stranded RNA in the replication platform composed of detergent-resistant lipid components, known as a membranous web, and is critical for HCV replication at posttranslational stages in the membranous web (Berger et al., 2009). NS5A can interact with PI4KA (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011) and recruit PI4KA to the membranous web (Berger et al., 2009; Tai et al., 2009; Reiss et al., 2011; Tai and Salloum, 2011). Furthermore, PI4KA, but not phosphatidylinositol 4-kinase III beta, induces the membranous web structure under the non-replicative condition (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011). Biosynthesis of phosphatidylinositol 4-phosphate by PI4KA that is recruited by NS5A in the membranous web may be required for HCV replication and can be an endogenous biomarker of the membranous web (**Figure 3**, step 3).

Vesicle-associated membrane protein-associated proteins were originally identified as proteins that bind to vesicle-associated membrane protein (VAMP) in the nematode *Aplysia* and were designated as VAMP-associated protein 33 kDa (later renamed VAP-A; Skehel et al., 1995). Furthermore, one homolog and its splicing variant were reported as VAP-B and -C, respectively (Nishimura et al., 1999). VAP is classified as a type II membrane protein, and is composed of three functional domains: major sperm protein (MSP), which occupies the N-terminal half region, the coiled-coil domain, and the transmembrane domain. VAP-A shares 60% identity with VAP-B, while VAP-C is the splicing variant of VAP-B that lacks the coiled-coil and transmembrane domains (Nishimura et al., 1999). GST pull-down and immunoprecipitation analyses revealed that NS5A and NS5B interact with human VAP-A/B and that the N-terminal MSP domain and the coiled-coil domain of VAP-A/B are responsible for the binding to NS5B and NS5A, respectively (Tu et al., 1999; Hamamoto et al., 2005). In addition, systematic RNAi screening revealed that 62 target host genes are involved in HCV RNA or proteins including VAP-A/B (Randall et al., 2007). Several reports suggest that HCV replication takes place on the membranous web (Shi et al., 2003; Gao et al., 2004; Sakamoto et al., 2005). NS4B is predominantly associated with a lipid-raft-like detergent-resistant fraction equivalent to the membranous web, and both NS5A and NS5B were co-localized in a similar fraction in the presence of NS4B (Sakamoto et al., 2005). VAP-A was also localized in a detergent-resistant fraction, suggesting that it plays an important role in HCV replication because the dominant negative mutant of VAP-A suppressed the replication of HCV RNA (Gao et al., 2004). VAP-B forms a homodimer and heterodimer with VAP-A, and knockdown of VAP-A or VAP-B led to substantial suppression of HCV replication (Hamamoto et al., 2005). These findings suggest that VAP-A and -B positively regulate HCV replication by binding to NS5A/B.

The physiological function of VAPs was reported to be trafficking of ceramide and cholesterol between ER and the Golgi apparatus. Several VAP-interacting proteins share the FFAT motif (two phenylalanines in an acidic tract), which has the consensus amino acid sequence EFFDAxE, as determined by a comparison among oxysterol binding proteins, OSBP-related proteins (ORPs; Loewen et al., 2003), and the ceramide transport protein CERT (Hanada et al., 2003; Kawano et al., 2006), contributing to the regulation of lipid metabolism. OSBP binds and transports cholesterol or hydroxycholesterol from ER to the Golgi (Ridgway et al., 1992; Wang et al., 2005b), while CERT binds and transports ceramide from ER to the Golgi, where the ceramide is converted to sphingolipids (Kumagai et al., 2005). Altering the sphingomyelin/ceramide ratio of the plasma membrane can effect HCV entry via the cell surface expression of CD81 (Voisset et al., 2008). OSBP mediates HCV secretion while binding to NS5A and VAP-A (Amako et al., 2009). Inhibition of CERT function effectively suppressed HCV release regardless of RNA replication (Aizaki et al., 2008). Phosphorylation of CERT and OSBP by protein kinase D negatively regulates VAPs binding to CERT and OSBP resulting in an effect on HCV infection (Amako et al., 2011). HCV NS5A may allow VAP-A/B to provide ceramide and cholesterol to replication complexes for upregulation of virus propagation (**Figure 3**, step 3).

The VAP-B-splicing variant VAP-C interacts with NS5B via the short form of the MSP domain and then suppresses the HCV replication by disrupting binding of other VAPs to NS5B (Kukihara et al., 2009). Expression of VAP-C is observed in various tissues except for the liver, suggesting that tissue distribution of VAP-C determines the tropism of HCV infection (Kukihara et al., 2009). These findings suggest that VAP-C negatively regulates HCV replication by inhibiting the interaction between VAP-A/B and NS5B. Furthermore, expression of VAP-C was negligible in B cells prepared from chronic hepatitis C patients, in whom B cells included HCV particles (Ito et al., 2010), and expression of the full HCV genome in B cells induced B-cell lymphoma in a conditional transgenic mouse (Kasama et al., 2010), suggesting that HCV infection increases the chance of developing B-cell lymphomas via dysregulation of lipid metabolism.

## CONCLUSION

This review summarizes several recently reported viral and host factors that exploit lipid components to support HCV infection. The mechanism by which HCV proteins cooperate with host factors to exploit lipid components and to regulate lipid metabolism in the infection has not been elucidated completely. The aim of identifying host factors is effective and stable therapy; targeting the host factors might be done to prevent the emergence of resistant viruses. Cyclosporin analogs will be used clinically in the near future. Wide screening and proteomics analyses have revealed novel host factors that are required for HCV replications over the past decade. The mechanism by which HCV infection induces formation of membranous web in infected cells has been unknown yet, although NS4B is involved in formation of membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). We also found several host proteins to be NS4B-associating host factors by proteomics analysis based on the TargetMine program (Tripathi et al., 2010). Further study will be required to identify the prominent factors essential for lipid metabolism that are associated with each step in the HCV life cycle and to develop effective and stable therapies for hepatitis C.

## ACKNOWLEDGMENTS

We thank R. Tanaka for her secretarial work. Our original work cited here were supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare (Research on Hepatitis); the Ministry of Education, Culture, Sports, Science, and Technology.

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