15. Valsecchi M, Mauri L, Casellato R, Prioni S, Loberto N, et al. (2007) Ceramide and sphingomyelin species of fibroblasts and neurons in culture. J Lipid Res 48: 417–424.
16. Watanabe T, Sudoh M, Miyagishi M, Akashi H, Arai M, et al. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther 13: 883–892.
17. Fujita T, Inoue K, Yamamoto S, Ikumoto T, Sasaki S, et al. (1994) Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. J Antibiot (Tokyo) 47: 208–215.
18. Miyake Y, Kozutsumi Y, Nakamura S, Fujita T, Kawasaki T (1995) Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. Biochem Biophys Res Commun 211: 396–403.
19. Park TS, Panek RL, Mueller SB, Hanselman JC, Rosebury WS, et al. (2004) Inhibition of sphingomyelin synthesis reduces atherogenesis in apolipoprotein E-knockout mice. Circulation 110: 3465–3471.
20. Miyanari Y, Hijikata M, Yamaji M, Hosaka M, Takahashi H, et al. (2003) Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. J Biol Chem 278: 50301–50308.
21. Diamond DL, Jacobs JM, Paeper B, Proll SC, Gritsenko MA, et al. (2007) Proteomic profiling of human liver biopsies: hepatitis C virus-induced fibrosis and mitochondrial dysfunction. Hepatology 46: 649–657.
22. Tardif KD, Mori K, Siddiqui A (2002) Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. J Virol 76: 7453–7459.
23. Pettus BJ, Chalfant CE, Hannun YA (2002) Ceramide in apoptosis: an overview and current perspectives. Biochim Biophys Acta 1585: 114–125.
24. Tepper AD, Ruurs P, Wiedmer T, Sims PJ, Borst J, et al. (2000) Sphingomyelin hydrolysis to ceramide during the execution phase of apoptosis results from phospholipid scrambling and alters cell-surface morphology. J Cell Biol 150: 155–164.
25. Liu YY, Han TY, Giuliano AE, Hansen N, Cabot MC (2000) Uncoupling ceramide glycosylation by transfection of glucosylceramide synthase antisense reverses adriamycin resistance. J Biol Chem 275: 7138–7143.
26. Taguchi Y, Kondo T, Watanabe M, Miyaji M, Umehara H, et al. (2004) Interleukin-2-induced survival of natural killer (NK) cells involving phosphatidylinositol-3 kinase-dependent reduction of ceramide through acid sphingomyelinase, sphingomyelin synthase, and glucosylceramide synthase. Blood 104: 3285–3293.
27. Diamond DL, Syder AJ, Jacobs JM, Sorensen CM, Walters KA, et al. (2010) Temporal proteome and lipidome profiles reveal hepatitis C virus-associated reprogramming of hepatocellular metabolism and bioenergetics. PLoS Pathog 6: e1000719.
28. Huitema K, van den Dikkenberg J, Brouwers JF, Holthuis JC (2004) Identification of a family of animal sphingomyelin synthases. EMBO J 23: 33–44.
29. Merrill AH, Jr., Schmelz EM, Dillehay DL, Spiegel S, Shayman JA, et al. (1997) Sphingolipids–the enigmatic lipid class: biochemistry, physiology, and pathophysiology. Toxicol Appl Pharmacol 142: 208–225.
30. Huwiler A, Kolter T, Pfeilschifter J, Sandhoff K (2000) Physiology and pathophysiology of sphingolipid metabolism and signaling. Biochim Biophys Acta 1485: 63–99.
31. Hannun YA, Luberto C, Argraves KM (2001) Enzymes of sphingolipid metabolism: from modular to integrative signaling. Biochemistry 40: 4893–4903.
32. van Genderen IL, Brandimarti R, Torrisi MR, Campadelli G, van Meer G (1994) The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. Virology 200: 831–836.
33. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, et al. (2006) The HIV lipidome: a raft with an unusual composition. Proc Natl Acad Sci U S A 103: 2641–2646.
34. Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y, et al. (2008) Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 82: 5715–5724.
35. Syed GH, Amako Y, Siddiqui A (2010) Hepatitis C virus hijacks host lipid metabolism. Trends Endocrinol Metab 21: 33–40.
36. Berger KL, Cooper JD, Heaton NS, Yoon R, Oakland TE, et al. (2009) Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication. Proc Natl Acad Sci U S A 106: 7577–7582.
37. Borawski J, Troke P, Puyang X, Gibaja V, Zhao S, et al. (2009) Class III phosphatidylinositol 4-kinase alpha and beta are novel host factor regulators of hepatitis C virus replication. J Virol 83: 10058–10074.
38. Tai AW, Benita Y, Peng LF, Kim SS, Sakamoto N, et al. (2009) A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. Cell Host Microbe 5: 298–307.
39. Vaillancourt FH, Pilote L, Cartier M, Lippens J, Liuzzi M, et al. (2009) Identification of a lipid kinase as a host factor involved in hepatitis C virus RNA replication. Virology 387: 5–10.
40. Amemiya F, Maekawa S, Itakura Y, Kanayama A, Matsui A, et al. (2008) Targeting lipid metabolism in the treatment of hepatitis C virus infection. J Infect Dis 197: 361–370.
41. Ishitsuka R, Sato SB, Kobayashi T (2005) Imaging lipid rafts. J Biochem 137: 249–254.
42. Ramstedt B, Slotte JP (2002) Membrane properties of sphingomyelins. FEBS Lett 531: 33–37.
43. He Q, Suzuki H, Sharma N, Sharma RP (2006) Ceramide synthase inhibition by fumonisin B1 treatment activates sphingolipid-metabolizing systems in mouse liver. Toxicol Sci 94: 388–397.
44. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.
45. Inoue K, Umehara T, Ruegg UT, Yasui F, Watanabe T, et al. (2007) Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology 45: 921–928.
46. Kashiwakuma T, Hasegawa A, Kajita T, Takata A, Mori H, et al. (1996) Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J Immunol Methods 190: 79–89.
47. Ikeda K, Shimizu T, Taguchi R (2008) Targeted analysis of ganglioside and sulfatide molecular species by LC/ESI-MS/MS with theoretically expanded multiple reaction monitoring. J Lipid Res 49: 2678–2689.
48. Taguchi R, Nishijima M, Shimizu T (2007) Basic analytical systems for lipidomics by mass spectrometry in Japan. Methods Enzymol 432: 185–211.
49. Ikeda K, Oike Y, Shimizu T, Taguchi R (2009) Global analysis of triacylglycerols including oxidized molecular species by reverse-phase high resolution LC/ESI-QTOF MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 877: 2639–2647.
50. Ikeda K, Mutoh M, Teraoka N, Nakanishi H, Wakabayashi K, et al. (2011) Increase of oxidant-related triglycerides and phosphatidylcholines in serum and small intestinal mucosa during development of intestinal polyp formation in Min mice. Cancer Sci 102: 79–87.

Biochimica et Biophysica Acta 1820 (2012) 1886–1892

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Different mechanisms of hepatitis C virus RNA polymerase activation by cyclophilin A and B in vitro

Leiyun Weng [a], Xiao Tian [a], Yayi Gao [a], Koichi Watashi [b], Kunitada Shimotohno [c], Takaji Wakita [b], Michinori Kohara [d], Tetsuya Toyoda [a,d,e,*]

[a] Unit of Viral Genome Regulation, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 411 Hefei Road, 200025 Shanghai, People's Republic of China
[b] Department of Virology II, National Institute of Health, 1-23-1 Toyama, Shinjuku, Tokyo 132-8640, Japan
[c] Chiba Institute of Technology, 2-17-1 Tsudamuna, Narashino, Chiba 275-0016, Japan
[d] Department of Microbiology and Cell Biology, The Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-Ku, Tokyo 156-8506, Japan
[e] Choju Medical Institute, Fukushimura Hospital, 19-14 Azanakayama, Noyori-cho, Toyohashi, Aichi 441-8124, Japan

ARTICLE INFO

Article history:
Received 26 April 2012
Received in revised form 25 July 2012
Accepted 21 August 2012
Available online 28 August 2012

Keywords:
HCV
RNA polymerase
Cyclophilin A
Cyclophilin B

ABSTRACT

Background: Cyclophilins (CyPs) are cellular proteins that are essential to hepatitis C virus (HCV) replication. Since cyclosporine A was discovered to inhibit HCV infection, the CyP pathway contributing to HCV replication is a potential attractive stratagem for controlling HCV infection. Among them, CyPA is accepted to interact with HCV nonstructural protein (NS) 5A, although interaction of CyPB and NS5B, an RNA-dependent RNA polymerase (RdRp), was proposed first.
Methods: CyPA, CyPB, and HCV RdRp were expressed in bacteria and purified using combination column chromatography. HCV RdRp activity was analyzed in vitro with purified CyPA and CyPB.
Results: CyPA at a high concentration (50× higher than that of RdRp) but not at low concentration activated HCV RdRp. CyPB had an allosteric effect on genotype 1b RdRp activation. CyPB showed genotype specificity and activated genotype 1b and J6CF (2a) RdRps but not genotype 1a or JFH1 (2a) RdRps. CyPA activated RdRps of genotypes 1a, 1b, and 2a. CyPB may also support HCV genotype 1b replication within the infected cells, although its knockdown effect on HCV 1b replicon activity was controversial in earlier reports.
Conclusions: CyPA activated HCV RdRp at the early stages of transcription, including template RNA binding. CyPB also activated genotype 1b RdRp. However, their activation mechanisms are different.
General significance: These data suggest that both CyPA and CyPB are excellent targets for the treatment of HCV 1b, which shows the greatest resistance to interferon and ribavirin combination therapy.

1. Introduction

Hepatitis C virus (HCV[1]), which belongs to the *Flaviviridae* family, has a positive-strand RNA genome, and its replication is regulated by viral and cellular proteins [1]. The genome encodes a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2]. NS5B is an RNA-dependent RNA polymerase (RdRp) [3–5].

HCV frequently establishes a persistent infection that leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [6,7]. More than 170 million individuals worldwide are infected with HCV [8], and the challenge of developing HCV treatment continues. First, combination therapy with pegylated interferon α (Peg-IFNα) and ribavirin led to a sustained virological response (SVR) in approximately 55% of patients infected with any HCV genotype and 42–46% of patients with genotype 1 [9,10]. However, many patients could not tolerate the serious adverse effects. Triple therapy consisting of an NS3/NS4A protease inhibitor (boceprevir or telaprevir), Peg-IFN (α-2a or α-2b), and ribavirin was then introduced, and it has become the standard regimen for genotype 1 infection. SVR improved significantly (from 63% to 75%), and the treatment duration decreased from 12 to 6 months [11,12]. However, triple therapy is more toxic than combination therapy [13].

Nonimmunosuppressant cyclosporine A (CsA) analogues/CyP inhibitors such as DEBIO-025 (Alisporivir) [14], NIM811 [15], and SCY-635 [16] are also the most expected candidates for use as anti-HCV drugs because their resistance selection is rare compared with other direct-acting antiviral agents, and the HCV resistant to

Abbreviations: BSA, bovine serum albumin; CsA, cyclosporine A; CyP, cyclophilin; DTT, dithiothreitol; E, envelope; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase; HCV, hepatitis C virus; NS, nonstructural protein; PPI, peptidyl prolyl *cis*/*trans*-isomerases; Peg-IFN, pegylated interferon-α; PMSF, phenylmethanesulfonylfluoride; RT-PCR, reverse transcription polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis; SVR, sustained virological response; ΔPPI, PPI knockout; wt, wild type

* Corresponding author at: Choju Medical Institute, Fukushimura Hospital, 19-4 Azanakayama, Noyori-cho, Toyohashi, Aichi 441-8124, Japan. Tel.: +81 532 46 7511; fax: +81 532 46 8940.
E-mail address: toyoda_tetsuya@yahoo.co.jp (T. Toyoda).

0304-4165/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2012.08.017

CyP inhibitors acquired mutations that allowed for reduced dependence on CyPs [17,18].

CyP was originally discovered as a cellular factor with high affinity for CsA [19]. CyPs comprise a family of peptidyl prolyl *cis/trans*-isomerases (PPI) that catalyze the *cis–trans* interconversion of peptide bonds amino terminal to proline residues, facilitating protein conformation changes [20]. CyPs are potential antiviral targets because CyPA was found to play a critical role in human immunodeficiency virus-1 infection [21,22]. The role of human CyPs as cellular cofactors in HCV replication was first suggested upon discovery of the anti-HCV effect of CsA [23–26]. Although the completion of a binding assay and the mapping of resistance initially suggested that NS5B was a viral target for CsA [27–29], recent papers have pointed to CyPA and NS5A as the central virus–host interaction involved in HCV replication [30–36]. Despite this unfavorable evidence, we analyzed the effect of CyPA and CyPB on HCV RdRp of various genotypes in vitro and found differences in genotype specificity and the mechanism of HCV RdRp activation.

2. Materials and methods

2.1. Purification of HCV RdRp

HCV RNA RdRps with C-terminal 21 amino acid deletion of 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (JFH1 and J6CF) were expressed in *E. coli* Rosetta/pLysS and purified as described previously [37–40]. The purified HCV RdRps (5 μM, >95% pure) were stocked in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 5% glycerol, and 1 mM phenylmethanesulfonylfluoride (PMSF) at −80 °C. The yield of HCV RdRps is approximately 1.7 mg from a 1-L bacterial culture. The purified HCV RdRps were as shown in Fig. S1 of Weng et al. [38]. The protein purities were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE), using ImageJ 1.46 (http://rsbweb.nih.gov/ij/).

2.2. Construction of CyP-expressing plasmids

Human CyPA and CyPB were cloned from total RNA extracted from 293T cells, using a reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara, Dalian, China) as published previously [29]. After being digested with *Bam*HI and *Eco*RI, they were cloned into the same site of pGEX-6P-3 (GE Healthcare, Bucks, UK), resulting in pGEXCyPA and pGEXCyPB, respectively. CyPBΔPPI, the enzymatic inactive mutant of CyPB, was PCR cloned into pGEX-6P-3 from pCMV-CyPBΔPPIFL [29], resulting in pGEXCyPBΔPPI. CyPAΔPPI was produced by the introduction of the R55A and F60A mutations using a QuickChangeII Site-Directed Mutagenesis Kit (Stratagene, St. Clara, CA, USA) and primers (5′-GTTCCTGCTTTCACGCCATTATTCCAGGGG CCATGTGTCAGGGTG-3′ and 5′-CACCCTGACACATGGCCCCTGGAATAA TGGCGTGAAAGCAGGAAC-3′).

2.3. Purification of CyPs

E. coli Rosetta were transformed using pGEXCyPA, pGEXCyPAΔPPI, pGEXCyPB, and pGEXCyPBΔPPI. GST-tagged CyPA, CyPB, CyPAΔPPI, and CyPBΔPPI were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 18 °C for 4 h. The bacteria were harvested and stocked at −20 °C. After thawing on ice, the bacteria were lysed in 4 packed cell volumes of phosphate-buffered saline, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. After being clarified by centrifugation at 10,000×*g* for 30 min at 4 °C and filtered through a 0.45-μm nitrocellulose filter, the extract was incubated with Glutathione Sepharose 4B (GE Healthcare) for 30 min at 4 °C. After the resin was washed with 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, the GST-CyP was eluted using 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM reduced glutathione, and 1 mM PMSF, followed by gel filtration through a Superdex 200 column (GE Healthcare) in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. The eluted GST-CyP were diluted to 50 mM NaCl and applied to a MonoQ (GE Healthcare) in 20 mM Tris–HCl (pH 9.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. GST-CyPB and GST-CyPBΔPPI were chromatographed using a continuous NaCl gradient of 50–1000 mM. The purified CyPs were stocked at −20 °C.

2.4. In vitro HCV transcription with CyPs

In vitro HCV transcription with CyPs was done as previously described [37–40]. Briefly, the indicated amounts of the CyPs were incubated in 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, 0.5 mM GTP, 200 nM of a 184-nt in vitro transcribed model RNA template (SL12-1S), 100 U/mL of human placental RNase inhibitor, and 100 nM HCV RdRp at 29 °C for 30 min. After preincubation, RdRp was incubated for an additional 90 min with 50 μM ATP, 50 μM CTP, or 5 μM [α-^{32}P]UTP. The RNA products were analyzed using 6% PAGE containing 8 M urea after being purified by phenol/chloroform extraction and ethanol precipitation. The amount of RNA products was analyzed using Typhoon Trio (GE Healthcare).

2.5. RNA filter-binding assay with CyPA and CyPB

An RNA filter-binding assay with CyPA and CyPB was performed as previously described [37,38,40]. Briefly, [^{32}P]-SL12-1S was incubated in 25 μL of 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, and 5 pmol of HCV RdRp with 375 pmol (75×) of CyPA and 25 pmol (5×) of CyPB at 29 °C for 30 min.

2.6. Chemicals and radioisotopes

[α-^{32}P]UTP (800 Ci/mmol, 40 mCi/mL) was purchased from PerkinElmer Life Sciences (Waltham, MA, USA). The nucleotides were purchased from GE Healthcare. The human placental RNase inhibitor T7 RNA polymerase and PrimeSTAR HS DNA polymerase were purchased from Takara. The bacteria were purchased from Novagen (Merck Chemicals, Darmstadt, Germany).

2.7. Statistical analysis

The statistical data were evaluated using Student's *t* test, with $p<0.05$ indicating statistical significance.

3. Results

3.1. Purification of CyPA and B

First, glutathione S-transferase (GST)-tagged CyPA, CyPB, the PPI inactive CyPA (CyPAΔPPI), and CyPB (CyPBΔPPI) were purified using Glutathione Sepharose 4B affinity chromatography. CyPA and CyPAΔPPI were further purified through a Superdex 200 column (Fig. S1). After the Superdex 200 gel filtration, to remove the contaminating nucleic acids, CyPB and CyPBΔPPI were further purified through MonoQ anion exchange chromatography by a continuous NaCl gradient of 50–1000 mM because CyPB has a strong affinity for nucleic acids. Each was eluted with 210–385 mM NaCl (Fig. S2). The purification scheme and purified CyPs are shown in Fig. 1. The yields of CyPA and CyPAΔPPI were approximately 3 mg from a 1-L bacterial culture. CyPA and CyPAΔPPI were >95% pure and stocked at 5 mg/mL in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. CyPB and CyPBΔPPI were stocked at 5 mg/mL in 20 mM Tris–HCl (pH 9.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. The yields of CyPB and CyPBΔPPI were approximately 1 mg from a 1-L bacterial culture. The purities of CyPB and CyPBΔPPI were >95% and >90%, respectively.

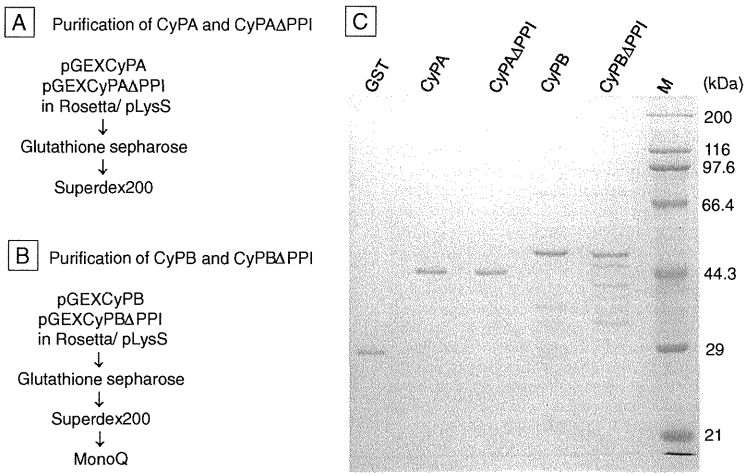

Fig. 1. Cyclophilin purification. The purification schemes of cyclophilin A (CyPA) and the peptidyl prolyl isomerase-inactive mutant protein of CyPA (CyPAΔPPI) (A), cyclophilin B (CyPB) and CyPBΔPPI (B), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (C) with 5 pmol each of purified glutathione S-transferase (GST; 28.3 kDa), GST-CyPA (44.9 kDa), GST-CyPAΔPPI (44.7 kDa), GST-CyPB (52.1 kDa), and GST-CyPBΔPPI (52 kDa) were separated through 10% SDS-PAGE and stained with Coomassie brilliant blue. The sizes of the molecular weight standards (M) are indicated on the right side of the gel. Their final elution profiles are shown in Figs. S1 and S2.

3.2. HCV 1b and JFH1 (2a) transcription in vitro with CyPA and CyPB

The dose–response effects of CyPA and CyPB were examined using an in vitro transcription system of HCR6 (1b) and JFH1 (2a) RdRp wild type (wt). CyPA and CyPB were added to the optimal HCV in vitro transcription condition while the RNA synthesis was in the log phase [4,37]. RdRp (100 nM) was incubated with 0, 50 (ratio to RdRp: 0.5×), 100 (1×), 200 (2×), 500 (5×), and 1000 nM (10×) CyPA and CyPB, GST, or bovine serum albumin (BSA) in GTP (the initiating nucleotide) and an RNA template for 30 min, followed by elongation with ATP, CTP, and UTP for 90 min. CyPA enhancement was further tested using 2 (20×), 5 (50×), 7.5 (75×), and 10 (100×) μM because the enhancement effect of CyPA under 1 μM (10×) was unclear. Fig. S3 shows the autoradiography of HCV HCR6 (1b) and JFH1 (2a) RdRpwt with CyPA and CyPB, the graphs of which were drawn using the data from 3 independent experiments (Fig. 2).

The CyPA activation of both RdRps showed 2 reaction speeds. The first-order ratio of CyPA to HCR6 (1b) RdRpwt<50× is fitted as a linear regression curve, the equation for which is $y=0.07x$ (CyPA-to-RdRp ratio) + 0.7. The linear regression curve fitting of the ratio >50× is $y=0.4x$ (CyPA-to-RdRp ratio) − 17 when calculated from 3 points. That of CyPA to JFH1 (2a) RdRpwt is fitted to a similar linear regression, $y=0.09x$ (CyPA-to-RdRp ratio) + 0.9 (the CyPA-to-RdRp ratio<50×). HCVR6 (1b) and JFH1 (2a) RdRps were activated by 100× CyPA to 25-±0.2- and 19-±1-fold, respectively.

The CyPB activation of HCR6 (1b) RdRpwt occurred in a dose-dependent manner and fitted a sigmoid curve, and the enhancement effect reached a plateau (9.4×) at the ratio of 5×. Neither GST nor BSA enhanced HCR6 (1b) RdRpwt. CyPB, GST, and BSA did not enhance JFH1 (2a) RdRpwt (<1.5×) at the concentrations described earlier.

3.3. Effect of the PPI inactive mutant proteins of CyPA and CyPB

CyP has PPI activity. To test the contribution of PPI activity to HCV HCR6 (1b) and JFH1 (2a) RdRpwt activation, the activation effect of the PPI inactive mutant proteins, CyPAΔPPI at 100× (10 μM) and CyPBΔPPI at 2× (200 nM), were tested together with 100× (10 μM) GST and BSA (Fig. 3). CyPA enhanced JFH1 (2a) RdRpwt 17.6×, whereas CyPAΔPPI enhanced it 16.2×. This difference is statistically significant (Student's *t* test, $p<0.05$). CyPA enhanced HCR6 (1b) RdRpwt activity 27.7×, whereas CyPAΔPPI enhanced it 16.0×. BSA slightly inhibited both RdRps at the same concentration in this experiment. As shown in Fig. 2C and D, it can be concluded that BSA has no effect on HCV transcription. GST enhanced JFH1 (2a) RdRpwt activity 5.0×, but it did not affect HCR6 (1b) RdRpwt activity. CyPB enhanced HCR6 (1b) RdRpwt activity 2.3×, whereas CyPBΔPPI enhanced it 1.7×. This difference is also statistically significant (Student's *t* test, $p<0.05$). JFH1 (2a) RdRpwt was not activated by CyPB or CyPBΔPPI.

3.4. CyP activation steps of HCV transcription

The HCV transcription steps of CyP enhancement were analyzed by the sequential addition of CyPs during in vitro transcription (Fig. 4). CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRpwt, whereas CyPB enhanced HCR6 (1b) RdRpwt when HCV RdRps were incubated with them from the start of transcription (initiation). The CyP effect was then tested after their addition during the elongation period after HCV RdRps was initiated with GTP. CyPA (100×; 10 μM) and CyPB (5×; 500 nM) were added to HCV RdRps after the 30-min incubation with GTP, when 3 GTPs were incorporated at the 5′ end of the products. CyPB did not enhance HCR6 (1b) or JFH1 (2a) RdRp when added during the elongation period, although it enhanced HCV RdRp when added at the start of transcription. CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRp activity only 1.6× (Student's *t* test, $p<0.05$) and 2.1× ($p<0.01$), respectively, when added during the elongation step. These results suggest that CyPA and CyPB activated only the transcription initiation step of HCV RdRps.

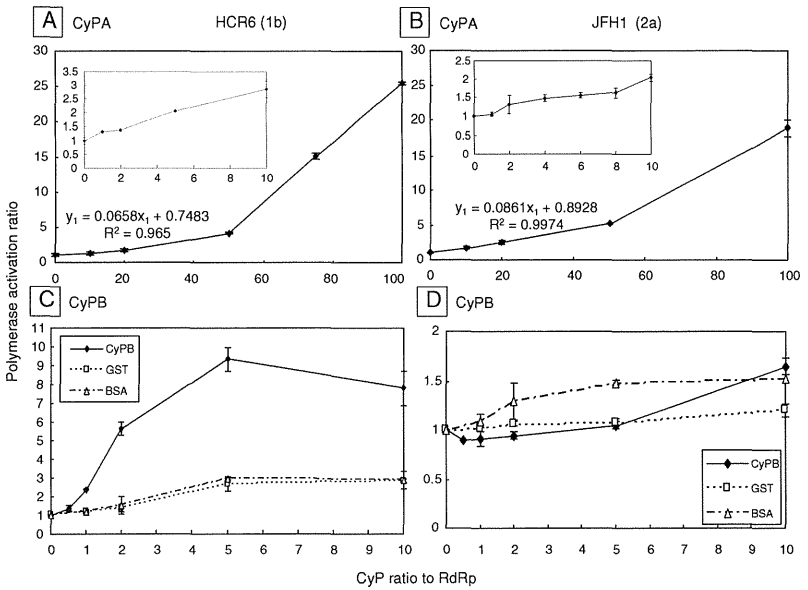

Fig. 2. Dose–response curve of cyclophilin A (CyPA) and cyclophilin B (CyPB) in hepatitis C virus (HCV) transcription in vitro. The dose–response curve of the HCV RdRp activation of CyPA in HCR6 (1b) RdRpwt (A) and JFH1 (2a) RdRpwt (B) CyPB in HCR6 (1b) RdRpwt (C) and JFH1 (2a) RdRpwt was drawn from the image analysis of Fig. S3. Insets A and B indicate that of 0, 0.5×, 1×, 2×, 5×, and 10× of CyPA to RdRp. The first-order ratio of the curves of A and B were fit by linear regression, and the calculated equations are indicated in the graph. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

The effects of 75× CyPA and 5× CyPB on the RNA-binding activity of HCR6 (1b) and JFH1 (2a) RdRp were then tested (Fig. 4E). The effects of HCR6 (1b) and JFH1 (2a) RdRp with CyPA were 10.1-±0.56- and 6.6-±0.68-fold of that without CyPA, respectively. The effect of HCR6 (1b) RdRp with CyPB was 3.1-±0.3-fold of that without CyPB. The RNA-binding activity of HCV RdRps was thus enhanced by the addition of CyPA and CyPB.

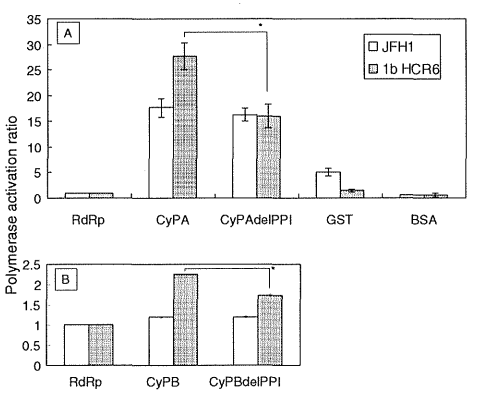

Fig. 3. Effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) with and without peptidyl prolyl isomerases activity on hepatitis C virus (HCV) JFH1 (2a) and HCR6 (1b) RdRp. HCV HCR6 (1b) and JFH1 (2a) RdRpwt (100 nM) were incubated with 100× (10 μM) of CyPA, CyPAΔPPI, glutathione S-transferase (GST), and bovine serum albumin (BSA) (A). HCV RdRps were incubated with 5× (500 nM) of CyPB, CyPBΔPPI, GST, and BSA (B). The mean relative polymerase activity and standard deviation (error bar) were calculated from 3 independent measurements. *$p<0.01$ (Student's t test).

3.5. Effect of CyP activation on RdRp of various HCV genotypes

The CsA sensitivity differed among the HCV genotypes [41]. Therefore, we tested the effects of CyPA and CyPB activation on NN (1b), H77 (1a), RMT (1a), and J6CF (2a) RdRp (Fig. 5). RdRp activity was compared with and without 50× (5 μM) CyPA and 5× (500 nM) CyPB. At their respective concentrations, CyPA activated all of the tested HCV RdRps by 3.9–5.3×, but CyPB activated only 1b RdRps (8–10×). CyPB slightly activated J6CF (2a) RdRp (approximately 4×), but it did not activate the 1a or JFH1 (2a) RdRps (1.4–1.8×).

4. Discussion

Since CsA was discovered to inhibit HCV infection [23–26], the CyP pathway contributing to HCV replication has been proposed as a potential stratagem for controlling HCV infection. Reports about the roles of CyPA in HCV replication via NS5A have been accumulating [33–35,42–44]. However, the effect of CyP inhibitors varied on the RNA-binding activity of NS5B [41,45], and DEBIO-025 decreased CyPB levels in patients [46]. Controversial results of CyPA and CyPB knockout experiments on HCV replicon activity were reported [29,30,47]. Therefore, the effects of CyPA and CyPB on HCV RdRp were carefully analyzed again in vitro.

In this study, we demonstrated that CyPA and CyPB activated HCV 1b RdRp in vitro by completely different kinetics using purified CyPs and HCV RdRps (Fig. 2), which indicated that the mechanism of their HCV RdRp activation differed despite their similar structures [48–50]. Kinetic analysis of CyPA on HCR6 (1b) and JFH1 (2a) RdRp indicated that it had a similar activation mechanism on both HCV RdRps. CyPA did not activate HCV RdRp at low concentrations, but it did activate it at >50× molar excess to it. The unusual dose of CyPA activating HCV RdRp (Fig. 2) postulates that HCV RdRp may be surrounded by CyPA in vitro and factors involving CyPA and HCV RdRp interaction, such as NS5A, in the HCV replication complex of the infected cells [27,28,31,36,51–53] because the interaction of CyPA and HCV RdRp was weak (Fig. S4).

Although some controversial results were obtained from those of Heck et al. [54], the studies agree that CyPB also activated HCV 1b RdRp in vitro. The activation kinetics of CyPB on HCR6 (1b) RdRp showed a sigmoid-like curve (Fig. 2) that suggested an allosteric effect of CyPB on RdRp activity. CyPB may interact with HCV RdRp as a cofactor and directly activate HCR6 (1b) RdRp. The HCV RdRp–CyPB complex was likely to interact more with CyPB, and its activation plateaued at the CyPB/RdRp ratio of 5:1 (Fig. 2C). The CyPB activation curves of Heck et al. [54] also plateaued. These data from the 2 independent groups support the weak interaction between CyPB and HCV 1b RdRp (Fig. S4).

CyPA did not show genotype specificity in the current study (Fig. 5A), a finding that agrees with those of CyPA knockdown, DEBIO-025, and CsA experiments [30,43,55]. CyPB activation showed genotype specificity (Fig. 5B) [54]; CyPB activated 1b and J6CF (2a) RdRp but did not activate 1a or JFH1 (2a) RdRp. Both reports agreed with the finding that JFH1 (2a) subgenomic replicon was independent of CyPB [41]. Although mutations accumulated in the NS5A region of CsA- or DEBIO-025-resistant HCV replicons, some mutations were found in the NS5B region [18,27,28,33,45].

Another controversial result between that of Heck et al. [54] and ours is the Mg^{2+}-dependency of the CyPB activation. The Mg^{2+} concentration in cells is 14–20 mM, and Mg^{2+} ions are distributed almost equally throughout the nuclei, mitochondria, and cytosol/endoplasmic reticulum [56]. The Mn^{2+} concentration in cells varies from report to report [57,58]. The optimal Mn^{2+} and Mg^{2+} concentrations in the HCV in vitro transcription used in this study were

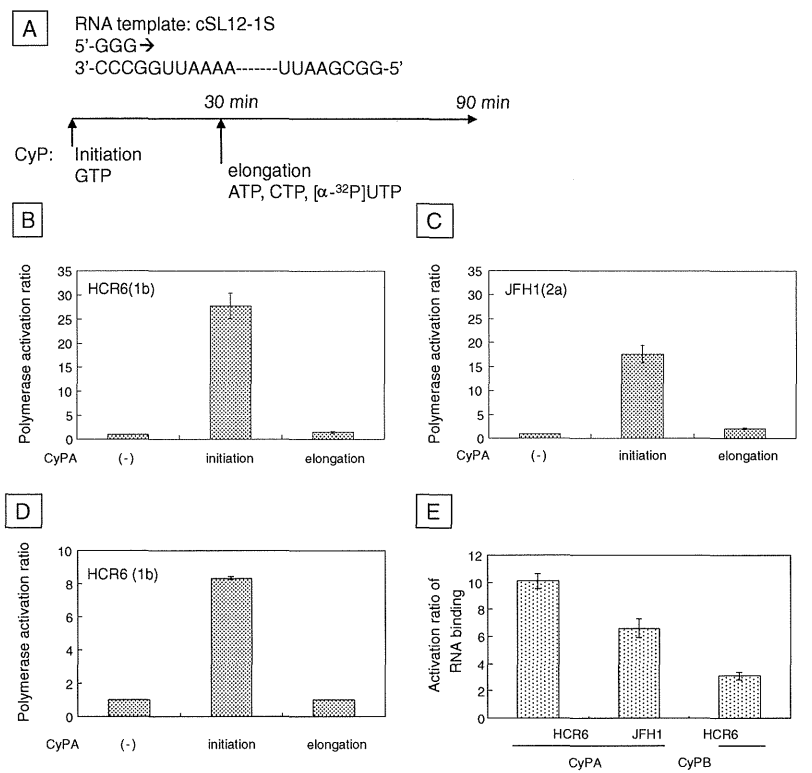

Fig. 4. Hepatitis C virus (HCV) RdRp activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on transcription initiation and elongation. The polymerase activation effect of the timing of the CyPA or CyPB addition was examined. The sequence of the model RNA template (SL12-1S) and experimental design are shown in A. CyPA 100× (10 μM) was incubated with HCR6 (1b) RdRpwt (A) and JFH1 (2a) (B) RdRp during preincubation with 0.5 mM GTP (initiation) or after preincubation (elongation). CyPB 5× (500 nM) was incubated with HCVR6 (1b) RdRpwt during preincubation with 0.5 mM GTP (initiation) or after the preincubation (elongation) (C). The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements. The effect of the 100× CyPA and 5× CyPB on RNA template binding was examined (E).

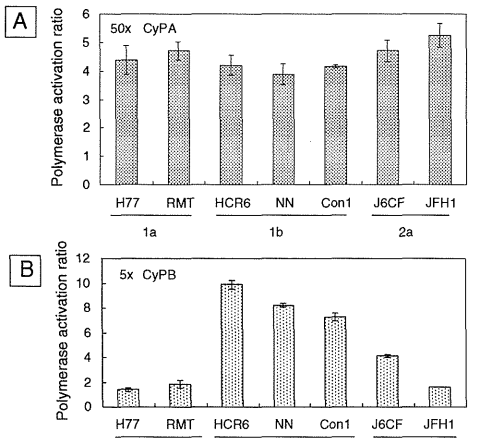

Fig. 5. Activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on hepatitis C virus (HCV) RNA polymerase of genotypes 1a, 1b, and 2a. The polymerase activation effects of CyPA and CyPB on HCV 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (J6CF and JFH1) were examined. HCV RdRp (100 nM) was incubated with 50× CyPA and 5× CyPB. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

different from the physiological concentrations in cells [4,37]. However, under the optimal HCV transcription condition, HCV RdRp activation was observed by CyPA and CyPB (Fig. 1).

The amount of CyPA varies by cell type [59]. In some cells, CyPB may also contribute to HCV 1b replication because it localizes in the endoplasmic reticulum and plasma membranes [60,61], which form a membrane web in which an HCV replication complex exists [1].

PPI activity of CyPs is essential for HCV replicon activation [32,53]. CyP inhibitors (DEBIO-025, NIM811, and SCY-635) inhibit PPI activity. The PPI activity of CyPA contributed to HCV RdRp activation and CyP-NS5A binding [36]. The PPI activity of CyPA partly contributed to the activation of HCR6 (1b) RdRpwt in vitro (Fig. 3A, $p<0.01$). The PPI activity of CyPB may not be essential for RdRp activation because the activation ratio was not large between CyPB and CyPBΔPPI, although the experiment showed a statistically significant difference (Fig. 3B). There may be differences in the RdRp activation mechanisms of CyPA with and without PPI activity. This finding will help with the development of new CyPA inhibitors that target domains other than PPI.

The mechanism of HCV RdRp activation by CyPs is not clear. In the least, CyPA and CyPB enhanced the early stage of HCV transcription, including the template RNA binding of HCV RdRp (Fig. 4) [29,41,45]. The productive template-polymerase binding is the late-limiting step of transcription initiation by HCV RdRp in vitro, and a small fraction of HCV RdRp was active in vitro [62,63]. CyP may enhance this step on many HCV RdRp molecules to show apparent activation of RdRp in vitro.

Considering the controversial reports on CyP and HCV replication [29,33,35,41,43,44], it can be concluded that CyPA is the major factor of HCV genome replication and that the activation of HCV RdRp may require other factors such as NS5A to condense CyPA around the HCV RdRp. Although many HCV treatment approaches have been applied in addition to Peg-IFN, ribavirin, and NS3/NS4a protease inhibitor [64–67], more effort has to be made to ensure an HCV cure. This study and that of Heck et al. [54] demonstrated similar activation kinetics and genotype specificity of CyPB activation (Figs. 2 and 5). CyPB also has the potential to activate HCV 1b genome replication in a limited condition, and it should also be included as the target of inhibitor development because HCV 1b is the genotype that is most resistant to treatment [13].

Acknowledgments

We thank Drs. J. Bukh, C. Rice, and R. Bartenshlager for the HCV J6CF, H77, and Con1 plasmids, respectively. This work was supported by grant-in-aids from the Chinese Academy of Sciences (O514P51131 and O812P1A131) and the Chinese National Key Project (2008ZX10002-014). The GenBank accession numbers of HCV H77, RMT, HCR6, NN, Con1, JFH1, and J6CF are NC_004102, AB520610, AY045702, AB080299, AJ238799, AB047639, and AF177036, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.08.017.

References

[1] S. Lemon, C. Walker, M. Alter, M. Yi, Hepatitis C virus, in: D. Knipe, P. Howley (Eds.), Fields Virology, Lippincott-Raven Publishers, Philadelphia, PA, 2007, pp. 1253–1304.
[2] A. Grakoui, D.W. McCourt, C. Wychowski, S.M. Feinstone, C.M. Rice, A second hepatitis C virus-encoded proteinase, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 10583–10587.
[3] T.L. Tellinghuisen, C.M. Rice, Interaction between hepatitis C virus proteins and host cell factors, Curr. Opin. Microbiol. 5 (2002) 419–427.
[4] T. Kashiwagi, K. Hara, M. Kohara, K. Kohara, J. Iwahashi, N. Hamada, H. Yoshino, T. Toyoda, Kinetic analysis of C-terminally truncated RNA-dependent RNA polymerase of hepatitis C virus, Biochem. Biophys. Res. Commun. 290 (2002) 1188–1194.
[5] S.E. Behrens, L. Tomei, R. De Francesco, Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus, EMBO J. 15 (1996) 12–22.
[6] I. Saito, T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Hepatitis C virus infection is associated with the development of hepatocellular carcinoma, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 6547–6549.
[7] R. Bartenschlager, M. Frese, T. Pietschmann, Novel insights into hepatitis C virus replication and persistence, Adv. Virus Res. 63 (2004) 71–180.
[8] T. Poynard, M.F. Yuen, V. Ratziu, C.L. Lai, Viral hepatitis C, Lancet 362 (2003) 2095–2100.
[9] 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 for initial treatment of chronic hepatitis C: a randomised trial, Lancet 358 (2001) 958–965.
[10] M.W. Fried, M.L. Shiffman, K.R. Reddy, C. Smith, G. Marinos, F.L. Goncales Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, J. Yu, Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection, N. Engl. J. Med. 347 (2002) 975–982.
[11] I.M. Jacobson, J.G. McHutchison, G. Dusheiko, A.M. Di Bisceglie, K.R. Reddy, N.H. Bzowej, P. Marcellin, A.J. Muir, P. Ferenci, R. Flisiak, J. George, M. Rizzetto, D. Shouval, R. Sola, R.A. Terg, E.M. Yoshida, N. Adda, L. Bengtsson, A.J. Sankoh, T.L. Kieffer, S. George, R.S. Kauffman, S. Zeuzem, Telaprevir for previously untreated chronic hepatitis C virus infection, N. Engl. J. Med. 364 (2011) 2405–2416.
[12] F. Poordad, J. McCone Jr., B.R. Bacon, S. Bruno, M.P. Manns, M.S. Sulkowski, I.M. Jacobson, K.R. Reddy, Z.D. Goodman, N. Boparai, M.J. DiNubile, V. Sniukiene, C.A. Brass, J.K. Albrecht, J.P. Bronowicki, Boceprevir for untreated chronic HCV genotype 1 infection, N. Engl. J. Med. 364 (2011) 1195–1206.
[13] M.L. Vachon, D.T. Dieterich, The era of direct-acting antivirals has begun: the beginning of the end for HCV? Semin. Liver Dis. 31 (2011) 399–409.
[14] J. Paeshuyse, A. Kaul, E. De Clercq, B. Rosenwirth, J.M. Dumont, P. Scalfaro, R. Bartenschlager, J. Neyts, The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro, Hepatology (Baltimore, Md) 43 (2006) 761–770.
[15] S. Ma, J.E. Boerner, C. TiongYip, B. Weidmann, N.S. Ryder, M.P. Cooreman, K. Lin, NIM811, a cyclophilin inhibitor, exhibits potent in vitro activity against hepatitis C virus alone or in combination with alpha interferon, Antimicrob. Agents Chemother. 50 (2006) 2976–2982.
[16] S. Hopkins, B. Scorneaux, Z. Huang, M.G. Murray, S. Wring, C. Smitley, R. Harris, F. Erdmann, G. Fischer, Y. Ribeill, SCY-635, a novel nonimmunosuppressive analog of cyclosporine that exhibits potent inhibition of hepatitis C virus RNA replication in vitro, Antimicrob. Agents Chemother. 54 (2010) 660–672.
[17] D.N. Fusco, R.T. Chung, Novel therapies for hepatitis C: insights from the structure of the virus, Annu. Rev. Med. 63 (2011) 373–387.

[18] L. Coelmont, X. Hanoulle, U. Chatterji, C. Berger, J. Snoeck, M. Bobardt, P. Lim, I. Vliegen, J. Paeshuyse, G. Vuagniaux, A.M. Vandamme, R. Bartenschlager, P. Gallay, G. Lippens, J. Neyts, DEB025 (Alisporivir) inhibits hepatitis C virus replication by preventing a cyclophilin A induced cis-trans isomerisation in domain II of NS5A, PLoS One 5 (2010) e13687.
[19] R.E. Handschumacher, M.W. Harding, J. Rice, R.J. Drugge, D.W. Speicher, Cyclophilin: a specific cytosolic binding protein for cyclosporin A, Science (New York, N.Y.) 226 (1984) 544–547.
[20] G. Fischer, T. Tradler, T. Zarnt, The mode of action of peptidyl prolyl cis/trans isomerases in vivo: binding vs. catalysis, FEBS Lett. 426 (1998) 17–20.
[21] G.J. Towers, T. Hatziioannou, S. Cowan, S.P. Goff, J. Luban, P.D. Bieniasz, Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors, Nat. Med. 9 (2003) 1138–1143.
[22] E.K. Franke, H.E. Yuan, J. Luban, Specific incorporation of cyclophilin A into HIV-1 virions, Nature 372 (1994) 359–362.
[23] K. Inoue, K. Sekiyama, M. Yamada, T. Watanabe, H. Yasuda, M. Yoshiba, Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial, J. Gastroenterol. 38 (2003) 567–572.
[24] M. Nakagawa, N. Sakamoto, N. Enomoto, Y. Tanabe, N. Kanazawa, T. Koyama, M. Kurosaki, S. Maekawa, T. Yamashiro, C.H. Chen, Y. Itsui, S. Kakinuma, M. Watanabe, Specific inhibition of hepatitis C virus replication by cyclosporin A, Biochem. Biophys. Res. Commun. 313 (2004) 42–47.
[25] H. Akiyama, H. Yoshinaga, T. Tanaka, K. Hiruma, S. Tanikawa, H. Sakamaki, Y. Onozawa, T. Wakita, M. Kohara, Effects of cyclosporin A on hepatitis C virus infection in bone marrow transplant patients. Bone Marrow Transplantation Team, Bone Marrow Transplant. 20 (1997) 993–995.
[26] K. Watashi, M. Hijikata, M. Hosaka, M. Yamaji, K. Shimotohno, Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes, Hepatology (Baltimore, Md) 38 (2003) 1282–1288.
[27] F. Fernandes, D.S. Poole, S. Hoover, R. Middleton, A.C. Andrei, J. Gerstner, R. Striker, Sensitivity of hepatitis C virus to cyclosporine A depends on nonstructural proteins NS5A and NS5B, Hepatology (Baltimore, Md) 46 (2007) 1026–1033.
[28] J.M. Robida, H.B. Nelson, Z. Liu, H. Tang, Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro, J. Virol. 81 (2007) 5829–5840.
[29] K. Watashi, N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, K. Shimotohno, Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase, Mol. Cell 19 (2005) 111–122.
[30] F. Yang, J.M. Robotham, H.B. Nelson, A. Irsigler, R. Kenworthy, H. Tang, Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro, J. Virol. 82 (2008) 5269–5278.
[31] K. Goto, K. Watashi, D. Inoue, M. Hijikata, K. Shimotohno, Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor, Cancer Sci. 100 (2009) 1943–1950.
[32] U. Chatterji, M. Bobardt, S. Selvarajah, F. Yang, H. Tang, N. Sakamoto, G. Vuagniaux, T. Parkinson, P. Gallay, The isomerase active site of cyclophilin a is critical for hepatitis C virus replication, J. Biol. Chem. 284 (2009) 16998–17005.
[33] F. Fernandes, I.U. Ansari, R. Striker, cyclosporine inhibits a direct interaction between cyclophilins and hepatitis C NS5A, PLoS One 5 (2010) e9815.
[34] A. Kaul, S. Stauffer, C. Berger, T. Pertel, J. Schmitt, S. Kallis, M.Z. Lopez, V. Lohmann, J. Luban, R. Bartenschlager, Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics, PLoS Pathog. 5 (2009) e1000546.
[35] D. Verdegem, A. Badillo, J.M. Wieruszeski, I. Landrieu, A. Leroy, R. Bartenschlager, F. Penin, G. Lippens, X. Hanoulle, Domain 3 of NS5A protein from the hepatitis C virus has intrinsic {alpha}-helical propensity and is a substrate of cyclophilin A, J. Biol. Chem. 286 (2011) 20441–20454.
[36] F. Yang, J.M. Robotham, H. Grise, S. Frausto, V. Madan, M. Zayas, R. Bartenschlager, M. Robinson, A.E. Greenstein, A. Nag, T.M. Logan, E. Bienkiewicz, H. Tang, A major determinant of cyclophilin dependence and cyclosporine susceptibility of hepatitis C virus identified by a genetic approach, PLoS Pathog. 6 (2010).
[37] L. Weng, J. Du, J. Zhou, J. Ding, T. Wakita, M. Kohara, T. Toyoda, Modification of hepatitis C virus 1b RNA polymerase to make a highly active JFH1-type polymerase by mutation of the thumb domain, Arch. Virol. 154 (2009) 765–773.
[38] L. Weng, Y. Hirata, M. Arai, M. Kohara, T. Wakita, K. Watashi, K. Shimotohno, Y. He, J. Zhong, T. Toyoda, Sphingomyelin activates hepatitis C virus RNA polymerase in a genotype-specific manner, J. Virol. 84 (2010) 11761–11770.
[39] A. Murayama, L. Weng, T. Date, D. Akazawa, X. Tian, T. Suzuki, T. Kato, Y. Tanaka, M. Mizokami, T. Wakita, T. Toyoda, RNA polymerase activity and specific RNA structure are required for efficient HCV replication in cultured cells, PLoS Pathog. 6 (2010) e1000885.
[40] L. Weng, M. Kohara, T. Wakita, K. Shimotohno, T. Toyoda, Detergent-induced activation of the hepatitis C virus genotype 1b RNA polymerase, Gene 496 (2012) 79–87.
[41] N. Ishii, K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, K. Shimotohno, Diverse effects of cyclosporine on hepatitis C virus strain replication, J. Virol. 80 (2006) 4510–4520.
[42] H. Grise, S. Frausto, T. Logan, H. Tang, A conserved tandem cyclophilin-binding site in hepatitis C virus nonstructural protein 5A regulates alisporivir susceptibility, J. Virol. 86 (2012) 4811–4822.
[43] U. Chatterji, P. Lim, M.D. Bobardt, S. Wieland, D.G. Cordek, G. Vuagniaux, F. Chisari, C.E. Cameron, P. Targett-Adams, T. Parkinson, P.A. Gallay, HCV resistance to cyclosporin A does not correlate with a resistance of the NS5A-cyclophilin A interaction to cyclophilin inhibitors, J. Hepatol. 53 (2010) 50–56.
[44] T.L. Foster, P. Gallay, N.J. Stonehouse, M. Harris, Cyclophilin A interacts with domain II of hepatitis C virus NS5A and stimulates RNA binding in an isomerase-dependent manner, J. Virol. 85 (2011) 7460–7464.
[45] Z. Liu, J.M. Robida, S. Chinnaswamy, G. Yi, J.M. Robotham, H.B. Nelson, A. Irsigler, C.C. Kao, H. Tang, Mutations in the hepatitis C virus polymerase that increase RNA binding can confer resistance to cyclosporine A, Hepatology (Baltimore, Md) 50 (2009) 25–33.
[46] R. Flisiak, A. Horban, P. Gallay, M. Bobardt, S. Selvarajah, A. Wiercinska-Drapalo, E. Siwak, I. Cielniak, J. Higersberger, J. Kierkus, C. Aeschlimann, P. Grosgurin, V. Nicolas-Metral, J.M. Dumont, H. Porchet, R. Crabbe, P. Scalfaro, The cyclophilin inhibitor Debio-025 shows potent anti-hepatitis C effect in patients coinfected with hepatitis C and human immunodeficiency virus, Hepatology (Baltimore, Md) 47 (2008) 817–826.
[47] M. Nakagawa, N. Sakamoto, Y. Tanabe, T. Koyama, Y. Itsui, Y. Takeda, C.H. Chen, S. Kakinuma, S. Ooka, S. Maekawa, N. Enomoto, M. Watanabe, Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins, Gastroenterology 129 (2005) 1031–1041.
[48] L. Jin, S.C. Harrison, Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13522–13526.
[49] V. Mikol, J. Kallen, G. Pflugl, M.D. Walkinshaw, X-ray structure of a monomeric cyclophilin A-cyclosporin A crystal complex at 2.1 A resolution, J. Mol. Biol. 234 (1993) 1119–1130.
[50] G. Pflugl, J. Kallen, T. Schirmer, J.N. Jansonius, M.G. Zurini, M.D. Walkinshaw, X-ray structure of a decameric cyclophilin–cyclosporin crystal complex, Nature 361 (1993) 91–94.
[51] X. Puyang, D.L. Poulin, J.E. Mathy, L.J. Anderson, S. Ma, Z. Fang, S. Zhu, K. Lin, R. Fujimoto, T. Compton, B. Wiedmann, Mechanism of resistance of hepatitis C virus replicons to structurally distinct cyclophilin inhibitors, Antimicrob. Agents Chemother. 54 (2010) 1981–1987.
[52] U. Chatterji, M.D. Bobardt, P. Lim, P.A. Gallay, Cyclophilin A-independent recruitment of NS5A and NS5B into hepatitis C virus replication complexes, J. Gen. Virol. 91 (2010) 1189–1193.
[53] Z. Liu, F. Yang, J.M. Robotham, H. Tang, Critical role of cyclophilin A and its prolyl-peptidyl isomerase activity in the structure and function of the hepatitis C virus replication complex, J. Virol. 83 (2009) 6554–6565.
[54] J.A. Heck, X. Meng, D.N. Frick, Cyclophilin B stimulates RNA synthesis by the HCV RNA dependent RNA polymerase, Biochem. Pharmacol. 77 (2009) 1173–1180.
[55] R. Crabbe, G. Vuagniaux, J.M. Dumont, V. Nicolas-Metral, J. Marfurt, L. Novaroli, An evaluation of the cyclophilin inhibitor Debio 025 and its potential as a treatment for chronic hepatitis C, Expert Opin. Investig. Drugs 18 (2009) 211–220.
[56] A. Romani, Regulation of magnesium homeostasis and transport in mammalian cells, Arch. Biochem. Biophys. 458 (2007) 90–102.
[57] M. Morello, A. Canini, P. Mattioli, R.P. Sorge, A. Alimonti, B. Bocca, G. Forte, A. Martorana, G. Bernardi, G. Sancesario, Sub-cellular localization of manganese in the basal ganglia of normal and manganese-treated rats. An electron spectroscopy imaging and electron energy-loss spectroscopy study, Neurotoxicology 29 (2008) 60–72.
[58] K. Kalia, W. Jiang, W. Zheng, Manganese accumulates primarily in nuclei of cultured brain cells, Neurotoxicology 29 (2008) 466–470.
[59] H. Gatanaga, D. Das, Y. Suzuki, D.D. Yeh, K.A. Hussain, A.K. Ghosh, H. Mitsuya, Altered HIV-1 Gag protein interactions with cyclophilin A (CypA) on the acquisition of H219Q and H219P substitutions in the CypA binding loop, J. Biol. Chem. 281 (2006) 1241–1250.
[60] E.R. Price, M. Jin, D. Lim, S. Pati, C.T. Walsh, F.D. McKeon, Cyclophilin B trafficking through the secretory pathway is altered by binding of cyclosporin A, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 3931–3935.
[61] E.R. Price, L.D. Zydowsky, M.J. Jin, C.H. Baker, F.D. McKeon, C.T. Walsh, Human cyclophilin B: a second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 1903–1907.
[62] Y. Liu, W.W. Jiang, J. Pratt, T. Rockway, K. Harris, S. Vasavanonda, R. Tripathi, R. Pithawalla, W.M. Kati, Mechanistic study of HCV polymerase inhibitors at individual steps of the polymerization reaction, Biochemistry 45 (2006) 11312–11323.
[63] S.S. Carroll, V. Sardana, Z. Yang, A.R. Jacobs, C. Mizenko, D. Hall, L. Hill, J. Zugay-Murphy, L.C. Kuo, Only a small fraction of purified hepatitis C RNA-dependent RNA polymerase is catalytically competent: implications for viral replication and in vitro assays, Biochemistry 39 (2000) 8243–8249.
[64] D.L. Thomas, C.L. Thio, M.P. Martin, Y. Qi, D. Ge, C. O'Huigin, J. Kidd, K. Kidd, S.I. Khakoo, G. Alexander, J.J. Goedert, G.D. Kirk, S.M. Donfield, H.R. Rosen, L.H. Tobler, M.P. Busch, J.G. McHutchison, D.B. Goldstein, M. Carrington, Genetic variation in IL28B and spontaneous clearance of hepatitis C virus, Nature 461 (2009) 798–801.
[65] D. Ge, J. Fellay, A.J. Thompson, J.S. Simon, K.V. Shianna, T.J. Urban, E.L. Heinzen, P. Qiu, A.H. Bertelsen, A.J. Muir, M. Sulkowski, J.G. McHutchison, D.B. Goldstein, Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance, Nature 461 (2009) 399–401.
[66] Y. Tanaka, N. Nishida, M. Sugiyama, M. Kurosaki, K. Matsuura, N. Sakamoto, M. Nakagawa, M. Korenaga, K. Hino, S. Hige, Y. Ito, E. Mita, E. Tanaka, S. Mochida, Y. Murawaki, M. Honda, A. Sakai, Y. Hiasa, S. Nishiguchi, A. Koike, I. Sakaida, M. Imamura, K. Ito, K. Yano, N. Masaki, F. Sugauchi, N. Izumi, K. Tokunaga, M. Mizokami, Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C, Nat. Genet. 41 (2009) 1105–1109.
[67] M. Gao, R.E. Nettles, M. Belema, L.B. Snyder, V.N. Nguyen, R.A. Fridell, M.H. Serrano-Wu, D.R. Langley, J.H. Sun, D.R. O'Boyle II, J.A. Lemm, C. Wang, J.O. Knipe, C. Chien, R.J. Colonno, D.M. Grasela, N.A. Meanwell, L.G. Hamann, Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect, Nature 465 (2010) 96–100.

Virus Research 163 (2012) 390–395

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

The interaction between human initiation factor eIF3 subunit c and heat-shock protein 90: A necessary factor for translation mediated by the hepatitis C virus internal ribosome entry site

Saneyuki Ujino[a], Hironori Nishitsuji[a], Ryuichi Sugiyama[a], Hitoshi Suzuki[a], Takayuki Hishiki[b], Kazuo Sugiyama[c], Kunitada Shimotohno[b], Hiroshi Takaku[a,*]

[a] Department of Life and Environmental Sciences, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan
[b] Research Institute, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan
[c] Center for Integrated Medical Research, School of Medicine, Keio University, 35 Shinanomachi, Shinjyuku-ku Tokyo 160-8582, Japan

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 4 October 2011
Accepted 6 October 2011
Available online 14 October 2011

Keywords:
eIF3c
Hsp90
HCV IRES
17-AAG
Translation initiation

ABSTRACT

Heat-shock protein 90 (Hsp90) is a molecular chaperone that plays a key role in the conformational maturation of various transcription factors and protein kinases in signal transduction. The hepatitis C virus (HCV) internal ribosome entry site (IRES) RNA drives translation by directly recruiting the 40S ribosomal subunits that bind to eukaryotic initiation factor 3 (eIF3). Our data indicate that Hsp90 binds indirectly to eIF3 subunit c by interacting with it through the HCV IRES RNA, and the functional consequence of this Hsp90–eIF3c–HCV-IRES RNA interaction is the prevention of ubiquitination and the proteasome-dependent degradation of eIF3c. Hsp90 activity interference by Hsp90 inhibitors appears to be the result of the dissociation of eIF3c from Hsp90 in the presence of HCV IRES RNA and the resultant induction of the degradation of the free forms of eIF3c. Moreover, the interaction between Hsp90 and eIF3c is dependent on HCV IRES RNA binding. Furthermore, we demonstrate, by knockdown of eIF3c, that the silencing of eIF3c results in inhibitory effects on translation of HCV-derived RNA but does not affect cap-dependent translation. These results indicate that the interaction between Hsp90 and eIF3c may play an important role in HCV IRES-mediated translation.

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-strand RNA genome (Taylor et al., 1999; Bartenschlager and Lohmann, 2001) encoding a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui et al., 1993; Hijikata et al., 1993). Ishii et al. identified an HCV replicon system in which the full HCV genomic RNA autonomously replicates in the Huh-7 human hepatoma cell line (Fig. 1A) (Ishii et al., 2006). This HCV replicon system allows researchers to study HCV genome replication in cell culture. HCV protein synthesis is initiated by the HCV RNA genome. This genome contains a conserved structure in its 5′-untranslated region (5′-UTR) that acts as an internal ribosome entry site (IRES) (Lukavsky, 2008). Briefly, the small ribosomal subunit (40S) and the eukaryotic initiation factor eIF3 bind specifically to the HCV IRES RNA, allowing for direct recognition of the start codon present in the 5′-UTR of the viral mRNA (Spahn et al., 2001; Collier et al., 2002; Kieft et al., 2002; Fraser and Doudna, 2007; Julien et al., 2009). Consistent with its diverse functions, eIF3 is the largest and most complex initiation factor. The mammalian version, for example, contains 13 nonidentical subunits designated eIF3a to eIF3m. The eIF3 core subunit (eIF3a–c, g, and i) is essential for translation (Kieft et al., 2002; Hinnebusch, 2006; Masutani et al., 2007; Zhou et al., 2008), and eIF3 specifically associates with the apical half of domain III of the HCV IRES (Kieft et al., 2001, 2002; Siridechadilok et al., 2005; Fraser and Doudna, 2007).

Hsp90 is a heat-shock protein that is abundant in the cytosol of eukaryotes and prokaryotes. In contrast to other chaperones, a number of substrates are known to contain Hsp90 (Schulte et al., 1995). Studies of eukaryotes have revealed that these Hsp90 client proteins include a variety of transcription factors (Coumailleau et al., 1995; Garcia-Cardena et al., 1998; Nagata et al., 1999; Sato et al., 2000; Richter and Buchner, 2001; Xu et al., 2001; Waza et al., 2005). Recently, many studies have reported that Hsp90 is involved with not only HCV RNA replication and viral protein but also HCV IRES-mediated translation (Waxman et al., 2001; Kim et al., 2006; Okamoto et al., 2006; Nakagawa et al., 2007; Ujino et al., 2009). In the present study, we demonstrate that eIF3 forms a complex with Hsp90 that is critical for HCV IRES-mediated translation.

* Corresponding author. Tel.: +81 47 478 0407; fax: +81 47 478 0407.
E-mail address: hiroshi.takaku@it-chiba.ac.jp (H. Takaku).

0168-1702/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2011.10.003

Author's personal copy

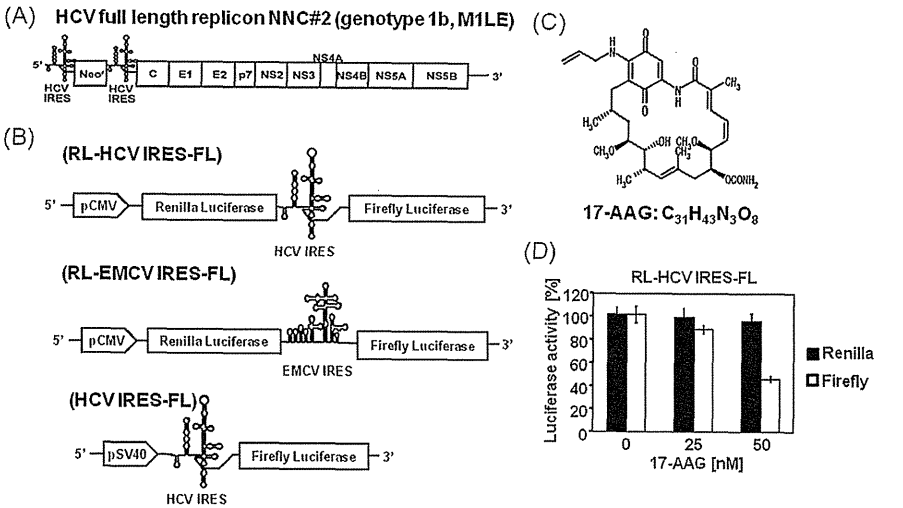

Fig. 1. Inhibition of IRES-mediated translation by an Hsp90 inhibitor. (A) The structure of the HCV replicon RNA molecules comprising the HCV 5′-UTR, including the HCV IRES, the neomycin phosphotransferase gene (Neo^r), and the coding region for the HCV proteins core to NS5B (in the HCV full-length replicon). (B) A schematic representation of the bicistronic HCV IRES or EMCV IRES reporter construct pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL) or pRenilla-EMCV IRES-firefly luciferase (RL-EMCV IRES-FL) driven by the CMV promoter to direct cap-dependent translation of renilla luciferase (RL) and HCV IRES or EMCV IRES-dependent translation of firefly luciferase (FL). The vector construct for HCV IRES-mediated translation of firefly luciferase, pHCV IRES-firefly luciferase (HCV IRES-FL) (Ujino et al., 2010). (C) The structure of the Hsp90 inhibitor 17-AAG (17-allylamino-17-demethoxygeldanamycin, Sigma–Aldrich Chemical Co.). (D) Inhibition of IRES-mediated translation by 17-AAG. Huh-7 cells (1×10^5 cells/well on 12-well plates) treated with 17-AAG (25 and 50 nM) and DMSO as a control for 24 h, and were then transfected with pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL) using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega). The data represent the mean ± standard deviations (SDs) from the experiments performed in triplicate.

To investigate the effects of the *Hsp90* inhibitor 17-AAG on HCV IRES translation, a bicistronic reporter system was used that consisted of an upstream reporter, Renilla luciferase (RL), expressed by cap-dependent translation and a downstream reporter, firefly luciferase (FL), which is under the translational control of the HCV IRES. To construct pcDNA-HCV IRES-firefly Luc, pHCV IRES-firefly Luc (HCV IRES-FL) (Ujino et al., 2010) (Fig. 1B) was digested with BamHI and SalI. The IRES-firefly Luc fragments were inserted into the BamHI-XhoI site of pcDNA3.1 (Invitrogen, Carlsbad, CA). To construct pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL), Renilla luciferase fragments were amplified by PCR from a pFN11A Flexi vector (Promega, Madison, WI), and the PCR products were inserted into the BamHI site of pcDNA-HCV IRES-firefly Luc. The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS). The benzoquinone ansamycin, the antibiotic geldanamycin (GA) and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) (Fig. 1C) (Sigma–Aldrich Chemical Co., St Louis, MO) directly bind to the ATP/ADP binding pocket of Hsp90, thus preventing ATP binding and the completion of client protein refolding (Neckers, 2003). The client proteins of Hsp90 appear to shift the role of the primary chaperone from Hsp90 to Hsp70 in cells treated with Hsp90 inhibitors (Doong et al., 2003). It is also well known that 17-AAG causes a modest increase in Hsp70 levels (Morimoto, 1998; Bagatell et al., 2000; Guo et al., 2005). In our previous report, a significant induction of Hsp70 was detected (Ujino et al., 2009).

For the reporter gene assay, Huh-7 cells were treated with different concentrations of the Hsp90 inhibitor, 17-AAG, or DMSO as a control for 24 h. They were then transfected with the bicistronic reporter construct RL-HCV IRES-FL using Lipofectamine 2000 (Invitrogen), which directs cap-dependent translation of the RL gene and HCV IRES-dependent translation of FL genes (Invitrogen). At 24 h post-transfection, the Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). In cells treated with 10 nM 17-AAG, firefly luciferase activity was reduced by 55% with RL-HCV IRES-FL, whereas Renilla luciferase activity was mostly maintained (Fig. 1D). The inhibition of HCV IRES-mediated translation occurred in a dose-dependent manner. Recently, Kim et al. (2006) demonstrated that Hsp90 regulates ribosomal function by maintaining the stability of 40S ribosomal proteins such as rpS3 and rpS6. The interaction between the 40S ribosomal proteins and Hsp90 has also been associated with ribosomal activities such as protein synthesis. We also found that the Hsp90 inhibitor 17-AAG influences HCV IRES-mediated luciferase activity, suggesting that 17-AAG inhibited HCV RNA replication and HCV IRES-mediated translation.

The HCV IRES is recognized specifically by the small ribosomal subunit and eIF3 before the initiation of viral translation. Although the degradation of rpS3, a component of the small ribosomal subunit, has been shown to occur in the presence of the Hsp90 inhibitor (Kim et al., 2006), the influence of Hsp90 inhibition on eIF3 is not understood. To determine whether 17-AAG affects the expression of the eIF3 subunit, we analyzed eIF3a, eIF3b, eIF3c, eIF3g and eIF3i protein expression by western blot analysis. The HCV replicon cell line NNC#2 (NN/1b/FL), which carries a full genome replicon, was cultured in DMEM with 10% FBS, nonessential amino acids,

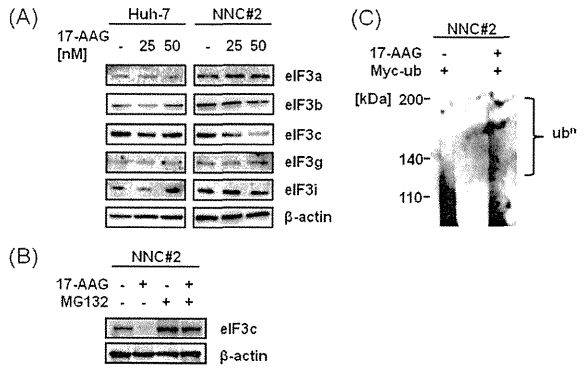

Fig. 2. Effect of 17-AAG treatment on eIF3 expression. (A) Western blot analysis of eIF3 protein expression in Huh-7 or NNC#2 cells treated with 17-AAG (25 nM and 50 nM). The cell lysates were analyzed by western blot 48 h after treatment. The primary antibodies used were monoclonal or polyclonal antibodies against eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (B) The reduction of eIF3c expression was prevented by proteasome inhibitor treatment. NNC#2 cells treated with 17-AAG (50 nM) or DMSO as a control. After 8 h treatment, the cells were treated with the proteasome inhibitor MG-132 (5 μM) or DMSO as a control. The cell lysates were analyzed by western blot 40 h after treatment. The primary antibody used was the eIF3c or β-actin (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (C) eIF3c degradation is mediated by the ubiquitin-dependent protease pathway. NNC#2 cells were transfected with pCMV-Myc-Ubi using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and were then treated with the proteasome inhibitor MG-132 (5 μM) for 16 h. The cell lysates were subjected to an immunoprecipitation assay using an anti-αMyc antibody (Cell Signaling) followed by an immunoblot analysis using anti-eIF3c antibody.

L-glutamine, penicillin/streptomycin, and 1 mg/mL G418 (Invitrogen) at 37 °C in 5% CO_2 (Ishii et al., 2006). For western blot analysis, NNC#2 cells and Huh-7 cells were lysed in 1× chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay buffer (Roche, Basel, Switzerland). The cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked with 5% skimmed milk. The primary antibodies used were monoclonal or polyclonal antibodies against FLAG-M2 (Sigma–Aldrich Chemical Co.), Hsp90 (Cell Signaling Tech., Beverly, MA), eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. When the HCV replicon cell line NNC#2 (NN/1b/FL) and Huh-7 cells were treated with increasing doses of 17-AAG, the expression of the eIF3c subunit was markedly reduced in NNC#2 cells, but the expression in Huh-7 cells was unaffected (Fig. 2A). These results suggest that Hsp90 is involved in eIF3c stability through a physical interaction in the presence of HCV IRES RNA.

Protein degradation in cells is mediated by several protease systems; however, the stability of most proteins is regulated by Hsp90, and they appear to be degraded by proteasomes. To investigate whether the reduction of eIF3c was due to proteasomal degradation, we treated NNC#2 cells with a proteasome inhibitor, MG132, to prevent the 17-AAG-induced degradation of eIF3c. Our results indicated that 17-AAG-induced eIF3c degradation can be blocked by proteasome inhibitors (Fig. 2B). Proteasome inhibitors substantially prevented the degradation of eIF3c in cells treated with 17-AAG. This is most likely because the disruption of Hsp90 by the Hsp90 inhibitor treatment destabilized the eIF3c protein. Therefore, it is clear that proteasome-dependent degradation results in the decreased level of eIF3c protein. This indicates that the stability of eIF3c was supported by Hsp90, and unstable eIF3c was removed by proteasomes. Furthermore, we investigated whether the ubiquitination of eIF3c was affected by the Hsp90 inhibitor, 17-AAG. We transfected pCMV-Myc-Ubi (provided by Dr. A. Ryo) using Lipofectamine 2000 (Invitrogen) into NNC#2 cells, which were then treated with 17-AAG (50 μM). After treatment, the cells were then treated with 5 μM MG132 and subjected to immunoprecipitation with an anti-αMyc antibody (Cell Signaling) followed by an immunoblot analysis using an anti-eIF3c antibody. Notably, polyubiquitinated forms of eiF3c was detected in cells treated with 17-AAG (Fig. 2C). These results suggest that the destabilized eIF3c protein is degraded by proteasome-dependent proteolysis mediated by ubiquitin conjugation, and Hsp90 plays an important role in maintaining the stable form of the eIF3c protein in vivo.

To investigate the influence of eIF3c silencing on HCV IRES-mediated translation, Huh-7 cells were transfected with siRNA targeted to eIF3c at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen), and they were then transfected with RL-HCV IRES-FL. Control small interference RNA (siRNA) and eIF3 p110 (eukaryotic translation initiation factor 3, subunit 8, 110 kDa) siRNA were purchased from Santa Cruz Biotechnology. The protein levels of eIF3c were examined by western blot analysis, and HCV IRES-mediated translation was analyzed with a Dual-Luciferase Assay. As demonstrated in Fig. 3A, when compared to Huh-7 cells treated with control siRNA, the eIF3c protein level was markedly reduced in Huh-7 cells transfected with eIF3 p110 siRNA targeting eIF3c. Furthermore, firefly luciferase activity in RL-HCV IRES-FL was also reduced by approximately 63% in the cells treated with siRNA targeted to eIF3c, whereas Renilla luciferase activity was mostly maintained (Fig. 3B). The inhibition of HCV IRES-mediated translation by siRNA against eIF3c indicates that the suppression of HCV IRES-mediated translation by Hsp90 inhibition leads to a reduction in eIF3c. To further characterize the HCV IRES inhibitory effect of siRNA targeting eIF3c, we used additional bicistronic reporter plasmids for transient transfection assays with Huh-7 cells. Since we were mainly interested in viral IRESs, we chose to investigate the effect of the luciferase activities on translation derived from the IRES of EMCV in place of the HCV IRES (Fig. 1B). To generate pcDNA-EMCV IRES-firefly Luc, EMCV IRES fragments were created by PCR using the following primers: 5′-GAC TGG ATC CCC CCC CCT AAC-3′ and 5′-CAG TGG GCC CTA TTA TCG TGT TTT TCA AAG GAA AAC C-3′. The PCR products were inserted into the BamHI and

Author's personal copy

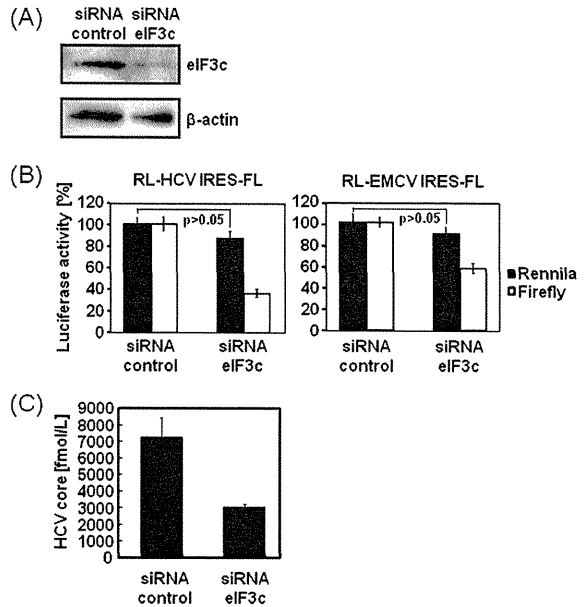

Fig. 3. Knockdown of eIF3c expression inhibits HCV IRES-mediated translation. (A) eIF3c protein expression in Huh-7 cells transfected with control siRNA or eIF3c siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen). Cell lysates were analyzed by western blot 24 h post-treatment. eIF3c or β-actin antibodies were used as the primary antibodies (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (B) Huh-7 cells were transfected with siRNA targeted to eIF3c (50 nM) or nontarget control siRNA (50 nM) using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were then transfected with RL-HCV IRES-FL or RL-EMCV IRES-FL using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES or EMCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega). Results are representative of three independent experiments, and error bars indicate the ± standard deviations (SDs) of the means. $P > 0.05$ (Student's t-test). (C) HCV IRES-mediated translational inhibition with eIF3 p110 siRNA by HCV full-genome RNA (NN/1b/FL). Huh-7 cells were transfected with eIF3 p110 siRNA or control siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were transfected with 2 μg of HCV full-genome RNA (NN/1b/FL) using Lipofectamine 2000 (Invitrogen). After 24 h, intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions (Aoyagi et al., 1999). The relative chemiluminescence units were measured and used to determine the concentration of the HCV core antigen according to a standard curve generated using recombinant HCV core antigen. The concentration was expressed in units of femtomole/L (fmol/L). The data represent the mean ± standard deviations (SDs) from the experiments performed in triplicate.

ApaI sites of pcDNA3.1, and firefly Luc fragments were cloned into the ApaI site of the resulting plasmid. To construct pcDNA-Renilla-EMCV IRES-firefly Luc (RL-EMCV IRES-FL), pcDNA-Renilla-EMCV IRES-firefly Luc was digested with BamHI, and the renilla Luc fragments were inserted into BamHI site of pcDNA-EMCV IRES-firefly Luc (Fig. 1B). Huh-7 cells were transfected with control small interference RNA (siRNA) or eIF3 p110 siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen) and then transfected with RL-EMCV IRES-FL. Following transient transfection in Huh-7 cells, firefly luciferase activity in RL-EMCV IRES-FL was also reduced by approximately 43% in cells treated with siRNA targeting eIF3c, but Renilla luciferase activity was mostly maintained (Fig. 3B). The knockdown of eIF3c expression also resulted in inhibitory effects on translation derived from HCV and EMCV (Fig. 3B) but did not affect cap-dependent translation. These results indicate that eIF3c may play a more important initiation factor in IRES-mediated translation than cap-dependent translation. However, it remains the subject of future investigation to determine whether only eIF3c proteins are subject to the IRES-mediated translation.

We also examined the HCV IRES-mediated translational inhibition with eIF3 p110 siRNA by HCV full-genome RNA ((NN/1b/FL) (Ishii et al., 2006). Huh-7 cells were transfected with eIF3 p110 siRNA or control siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were transfected with HCV full-genome RNA (NN/1b/FL). After 24 h, the intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions (Aoyagi et al., 1999). The core-protein expression in cells treated with eIF3 p110 siRNA was reduced by approximately 61% when compared to cells treated with control siRNA (Fig. 3C). These findings further confirmed that HCV IRES-mediated translational inhibition occurs through a reduction of eIF3c expression caused by the Hsp90 inhibitor-mediated disruption of the interaction between eIF3c and Hsp90 with HCV IRES RNA.

To investigate the role of Hsp90 in HCV IRES-mediated translation further, we confirmed the interaction of eIF3c and Hsp90 by immunoprecipitation. The pFLAG-eIF3c vector was constructed by subcloning a DNA fragment encoding full-length human eiF3c into the EcoRI and XbaI sites of the pFLAG CMV™-2 expression vector (Sigma–Aldrich Chemical Co.) so that the amino-terminal FLAG epitope was fused in-frame with eIF3c. The pFLAG-eIF3c expression vector or the control vector pFLAG-CMV2 was transfected into NNC#2 cells or Huh-7 cells. After 48 h, the immunoprecipitates

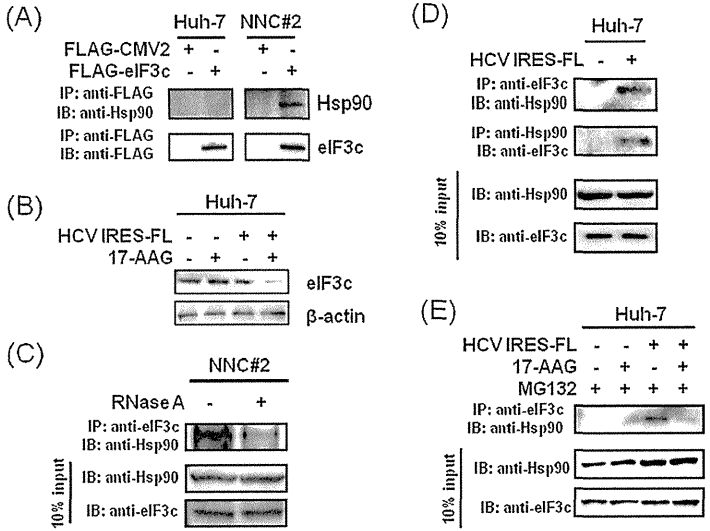

Fig. 4. An interaction between eIF3c and Hsp90 was induced by HCV IRES. (A) The pFLAG-eIF3c vector was constructed by subcloning a DNA fragment encoding full-length human eIF3c into the EcoRI and XbaI sites of the pFLAG CMV™-2 expression vector (Sigma–Aldrich Chemical Co.) such that the amino-terminal FLAG epitope was fused in-frame with eIF3c. Huh-7 and NNC#2 cells were transfected with pFLAG-eIF3c or pFLAG-CMV2 control plasmids using Lipofectamine 2000 (Invitrogen). Cell lysates were immunoprecipitated by the anti-FLAG M2 antibody 48 h after transfection. The precipitates were analyzed by western blot using the anti-Hsp90 antibody. (B) The Huh-7 cells were transfected with or without pHCV IRES-firefly luciferase (HCV IRES-FL) (Fig. 1B) and then treated with or without 17-AAG (50 nM). Cell lysates were analyzed by western blot 48 h post-treatment with an eIF3c primary antibody (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma-Aldrich Chemical Co.) was used as the secondary antibody. β-Actin was used as an internal control. (C) The interruption of Hsp90–eIF3c interaction by RNase A treatment. NNC#2 cell lysates were treated with RNase A (5 U/μL) (Sigma–Aldrich Chemical Co.). After 4 h, the cell lysates were subjected to immunoprecipitation using an anti-eIF3c antibody, followed by immunoblot analysis using an anti-Hsp90 antibody. (D) eIF3c or Hsp90 co-immunoprecipitates with Hsp90 or eIF3c from cells transfected or untransfected with pHCV IRES-FL. Huh-7 cells were transfected with pHCV IRES-FL using Lipofectamine 2000 (Invitrogen) and subject to immunoprecipitation using the indicated antibodies at 24 h post-transfection. The precipitates were analyzed by western blot using the indicated antibodies. (E) The inhibitor 17-AAG dissociates Hsp90 and eIF3c from the HCV IRES complex. Huh-7 cells were transfected with pHCV IRES-FL using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and were then treated with MG132 (5 μM) for 16 h. Cell lysates were subjected to immunoprecipitation using an anti-eIF3c antibody, and the precipitates were analyzed by western blot using the anti-Hsp90 antibody.

(anti-FLAG antibody) were determined by western blot analysis (Fig. 4A). The western blot analysis clearly indicated that eIF3c and Hsp90 coprecipitated in NNC#2 cells, whereas they did not coprecipitate in Huh-7 cells, suggesting that eIF3c was bound to the chaperone complex that formed with Hsp90 in NNC#2 cells (Fig. 4A). This interaction between Hsp90 and eIF3c in NNC#2 cells suggests that HCV translation is due to the interaction between eIF3c and Hsp90. Given the observed binding of eIF3 with the HCV IRES RNA, this Hsp90-eIF3c interaction occurring in HCV replicon cells was likely mediated by HCV IRES RNA. To address this question, Huh-7 cells were transfected with pHCV IRES-firefly luciferase (HCV IRES-FL) (Fig. 1B) using Lipofectamine 2000 (Invitrogen) and then treated with 17-AAG (50 nM) or DMSO as a control. After treatment, the cell lysates were analyzed by western blot (Fig. 4B). The level of eIF3c was reduced by the 17-AAG treatment in cells transfected with pHCV IRES-FL compared to control DMSO, but eIF3c was not reduced in cells transfected with pHCV IRES-FL. The disruption of Hsp90 activity by the Hsp90 inhibitor, 17-AAG, appears to dissociate eIF3c from the Hsp90-eIF3c-HCV IRES complex and induce the degradation of the free forms of eIF3c. To verify this result, NNC#2 cell lysates were further treated with RNase A (5 U/μL) (Sigma–Aldrich Chemical Co.). After treatment, anti-eIF3c antibody immunoprecipitates were determined by western blot analysis (Fig. 4C). The analysis clearly indicated that eIF3c and Hsp90 coprecipitated in NNC#2 cell lysates, whereas they did not coprecipitate in NNC#2 cells lysates treated with RNase A. The interaction between Hsp90 and eIF3c was interrupted by RNase A treatment. These results suggest that the interaction of eIF3c and Hsp90 is dependent on HCV IRES binding. Next, to further demonstrate whether HCV IRES is required for the interaction between eIF3c and Hsp90, we performed a co-immunoprecipitation assay using the extracts of cells transfected with pHCV IRES-FL. eIF3c co-immunoprecipitated with anti-Hsp90 (Fig. 4D). The interaction of eIF3c and Hsp90 was further confirmed by reverse co-immunoprecipitation of Hsp90 and eIF3c (Fig. 4D). These results also indicated that the interaction of eIF3c and Hsp90 was supported by the HCV IRES. Furthermore, we performed an immunoprecipitation assay to confirm that eIF3c and Hsp90 interaction was influenced by the treatment with 17-AAG in cells transfected with pHCV IRES-FL. Huh-7 cells were transfected with pHCV IRES-FL using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and then MG132 (5 μM) for 16 h. An immunoprecipitation assay with the cell lysates was performed using the anti-eIF3c antibody, and the precipitates were analyzed by western blot using the anti-Hsp90 antibody. Although treatment with both 17-AAG and MG132 in cells transfected with pHCV IRES-FL resulted in a reduction in the interaction of Hsp90 and eIF3c, eIF3c expression recovered upon treatment with the proteasome inhibitor MG132 (Fig. 4E), but the interaction between Hsp90 and eIF3c was not restored (Fig. 4E). Furthermore, eIF3c was not detected in cells not treated with MG132 (Fig. 2B), which indicates that eIF3c is a client protein for active Hsp90 (Fig. 2C). In contrast, an interaction between Hsp90 and eIF3c was observed in cells not treated with 17-AAG (Fig. 4E). These results suggest that the interaction between Hsp90 and eIF3c is specific to HCV IRES-expressing cells. However, the interaction of Hsp90 and eIF3c in the presence of 17-AAG, which blocks the association of Hsp90 with eIF3c, may dissociate Hsp90 and eIF3c from the overall HCV IRES complex (Figs. 2B and C, and 4B and E).

In conclusion, our results demonstrate that HCV IRES-mediated translational inhibition occurs through a reduction of eIF3c expression caused by the Hsp90 inhibitor-mediated disruption of the interaction between eIF3c and Hsp90 with HCV IRES RNA. Furthermore, the interaction between Hsp90 and eIF3c requires HCV IRES RNA. Taken together, our results suggest that the interaction between Hsp90 and eIF3c plays an important role in HCV IRES-mediated translation. More experiments are needed to verify the relationship between eukaryotic initiation factor 3 (eIF3) and Hsp90.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Dr. A. Ryo for providing pCMV-Myc-Ubi. We are grateful to S. Yamaguchi, M. Sato, and Y. Katumura for their excellent technical assistance. This work was supported by a Grant-in-Aid for HCV Research from the Ministry of Health, Labor, and Welfare of Japan, by a Grant-in-Aid for High Technology Research (HTR) from the Ministry of Education, Science, Sports, and Culture of Japan, and a Grant from the Strategic Research Foundation Grant-aided Project for Private Universities from the Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT).

References

Aoyagi, K., Ohue, C., Iida, K., Kimura, T., Tanaka, E., Kiyosawa, K., Yagi, S., 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. 37 (6), 1802–1808.
Bagatell, R., Paine-Murrieta, G.D., Taylor, C.W., Pulcini, E.J., Akinaga, S., Benjamin, J., Whitesell, L., 2000. Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of hsp90-binding agents. Clin. Cancer Res. 6 (8), 3312–3318.
Bartenschlager, R., Lohmann, V., 2001. Novel cell culture systems for the hepatitis C virus. Antivir. Res. 52 (1), 1–17.
Collier, A.J., Gallego, J., Klinck, R., Cole, P.T., Harris, S.J., Harrison, G.P., Aboul-Ela, F., Varani, G., Walker, S., 2002. A conserved RNA structure within the HCV IRES eIF3-binding site. Nat. Struct. Biol. 9 (5), 375–380.
Coumailleau, P., Poellinger, L., Gustafsson, J.A., Whitelaw, M.L., 1995. Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild type ligand binding affinity and specificity. J. Biol. Chem. 270 (42), 25291–25300.
Doong, H., Rizzo, K., Fang, S., Kulpa, V., Weissman, A.M., Kohn, E.C., 2003. CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: accumulation of poly-ubiquitinated Hsp90 client proteins. J. Biol. Chem. 278 (31), 28490–28500.
Fraser, C.S., Doudna, J.A., 2007. Structural and mechanistic insights into hepatitis C viral translation initiation. Nat. Rev. Microbiol. 5 (1), 29–38.
Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., Sessa, W.C., 1998. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature 392, 821–824.
Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., Rice, C.M., 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67 (3), 1385–1395.
Guo, F., Rocha, K., Bali, P., Pranpat, M., Fiskus, W., Boyapalle, S., Kumaraswamy, S., Balasis, M., Greedy, B., Armitage, E.S., Lawrence, N., Bhalla, K., 2005. Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res. 65 (22), 10536–10544.
Hijikata, M., Mizushima, H., Shimotohno, K., 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. 67 (8), 4665–4675.
Hinnebusch, A.G., 2006. eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem. Sci. 10, 553–562.
Ishii, N., Watashi, K., Hishiki, T., Goto, K., Inoue, D., Hijikata, M., Wakita, T., Kato, N., Shimotohno, K., 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. 80 (9), 4510–4520.
Julien, P., Rodolfo, R., Jan, M., Isabel, A., Jerome, B., Emmanuel, D., Florence, B., 2009. Human initiation factor eIF3 subunit b interacts with HCV IRES RNA through its N-terminal RNA recognition motif. FEBS Lett. 583 (1), 70–74.
Kieft, J.S., Zhou, K., Jubin, R., Doudna, J.A., 2001. Mechanism of ribosome recruitment by hepatitis C IRES RNA. RNA 7 (2), 194–206.
Kieft, J.S., Zhou, K., Grech, A., Jubin, R., Doudna, J.A., 2002. Crystal structure of an RNA tertiary domain essential to HCV IRES-mediated translation initiation. Nat. Struct. Biol. 9 (5), 370–374.
Kim, T.S., Jang, K., Kim, C.-Y., Lee, H.D., Ahn, J.Y., Kim, B.-Y., 2006. J. Interaction of Hsp90 with ribosomal proteins protects from ubiquitination and proteasome-dependent degradation. Mol. Biol. Cell 17 (2), 824–833.
Lukavsky, P.J., 2008. Structure and function of HCV IRES domains. Virus Res. 139 (2), 166–171.
Masutani, M., Sonenberg, N., Yokoyama, S., Imataka, H., 2007. Reconstitution reveals the functional core of mammalian eIF3. EMBO J. 26 (14), 3373–3383.
Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12 (24), 3788–3796.
Nagata, Y., Anan, T., Yoshida, T., Mizukami, T., Taya, Y., Fujiwara, T., Kato, H., Saya, H., Nakao, M., 1999. The stabilization mechanism of mutant-type p53 by impaired ubiquitination: the loss of wild-type p53 function and the hsp90 association. Oncogene 18 (44), 6037–6049.
Neckers, L., 2003. Development of small molecule Hsp90 inhibitors: utilizing both forward and reverse chemical genomics for drug identification. Curr. Med. Chem. 10 (9), 733–739.
Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., Kohara, M., 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem. Biophys. Res. Commun. 353 (4), 882–888.
Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Moriishi, K., Matsuura, Y., 2006. hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. 25 (20), 5015–5025.
Richter, K., Buchner, J., 2001. Hsp90, chaperoning signal transduction. J. Cell. Physiol. 188 (3), 281–290.
Sato, S., Fujita, N., Tsuruo, T., 2000. Modulation of Akt kinase activity by binding to Hsp90. Proc. Natl. Acad. Sci. U.S.A. 97 (20), 10832–10837.
Schulte, T.W., Blagosklonny, M.V., Ingui, C., Neckers, L., 1995. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem. 270 (41), 24585–24588.
Siridechadilok, B., Fraser, C.S., Hall, R.J., Doudna, J.A., Nogales, E., 2005. Structural roles for human translation factor eIF3 in initiation of protein synthesis. Science 310, 1513–1515.
Spahn, C.M., Kieft, J.S., Grassucci, R.A., Penczek, P.A., Zhou, K., Doudna, J.A., Frank, J., 2001. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. Science 291, 1959–1962.
Taylor, D.R., Shi, S.T., Tomano, P.R., Barber, G.N., Lai, M.M., 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285, 107–110.
Ujino, S., Yamaguchi, S., Shimotohno, K., Takaku, H., 2009. Heat-shock protein 90 is essential for stabilization of the hepatitis C virus nonstructural protein NS3. J. Biol. Chem. 284 (11), 6841–6846.
Ujino, S., Yamaguchi, S., Shimotohno, K., Takaku, H., 2010. Combination therapy for hepatitis C virus with heat-shock protein 90 inhibitor 17-AAG and proteasome inhibitor MG132. Antivir. Chem. Chemother. 20 (4), 161–167.
Waxman, L., Whitney, M., Pollok, B.A., Kuo, L.C., Darke, P.L., 2001. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc. Natl. Acad. Sci. U.S.A. 98 (24), 13931–13935.
Waza, M., Adachi, H., Katsuno, M., Minamiyama, M., Sang, C., Tanaka, F., Inukai, A., Doyu, M., Sobue, G., 2005. 17-AAG, an Hsp90 inhibitor, ameliorates polyglutamine-mediated motor neuron degeneration. Nat. Med. 11 (10), 1088–1095.
Xu, W., Mimnaugh, E., Rosser, M.F., Nicchitta, C., Marcu, M., Yarden, Y., Neckers, L., 2001. Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J. Biol. Chem. 276 (5), 3702–3708.
Zhou, M., Sandercock, A.M., Fraser, C.S., Ridlova, G., Stephens, E., Schenauer, M.R., Yokoi-Fong, T., Barsky, D., Leary, J.A., Hershey, J.W., Doudna, J.A., Robinson, C.V., 2008. Mass spectrometry reveals modularity and a complete subunit interaction map of the eukaryotic translation factor eIF3. Proc. Natl. Acad. Sci. U.S.A. 105 (47), 18139–18144.

frontiers in MICROBIOLOGY

MINI REVIEW ARTICLE
published: 10 January 2012
doi: 10.3389/fmicb.2011.00278

Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

Ikuo Shoji*, Lin Deng and Hak Hotta

Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

Edited by:
Yasuko Yokota, National Institute of Infectious Diseases, Japan

Reviewed by:
Koji Ishii, National Institute of Infectious Diseases, Japan
Kohji Moriishi, University of Yamanashi, Japan

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

Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)-α-based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Pazienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

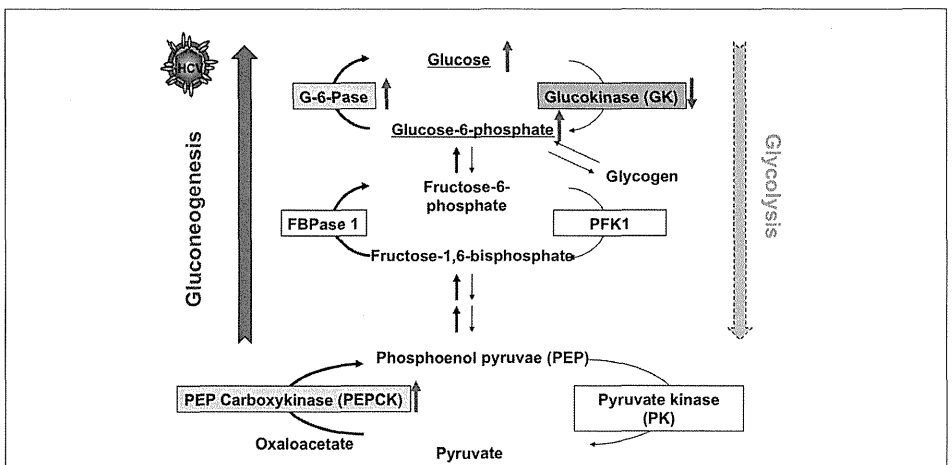

FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells. HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.

2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (**Figure 2**). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

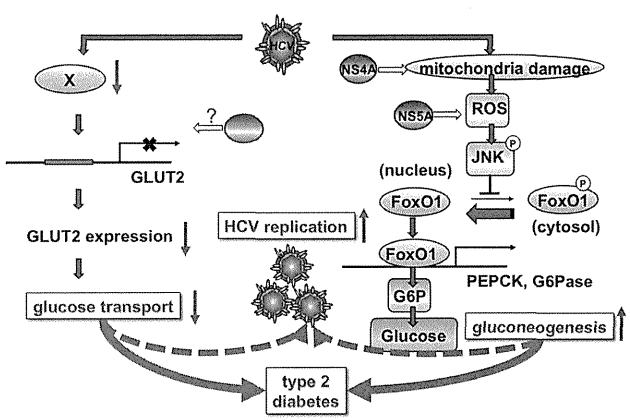

FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2**. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregualtion, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

ACKNOWLEDGMENTS

The authors are grateful to all of their co-workers who contributed to the studies cited here. This work was supported in part by grants-in-aid for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan, and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) program of Ministry of Education, Culture, Sports, Science and Technology, Japan. This study was also carried out as part of the Global *Center of Excellence program of Kobe University Graduate School* of Medicine, and the Science and Technology Research Partnership for Sustainable Development (SATREPS) program of Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA).

REFERENCES

Banerjee, A., Meyer, K., Mazumdar, B., Ray, R. B., and Ray, R. (2010). Hepatitis C virus differentially modulates activation of forkhead transcription factors and insulin-induced metabolic gene expression. *J. Virol.* 84, 5936–5946.

Bungyoku, Y., Shoji, I., Makine, T., Adachi, T., Hayashida, K., Nagano-Fujii, M., Ide, Y. H., Deng, L., and Hotta, H. (2009). Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. *J. Gen. Virol.* 90, 1681–1691.

Burdette, D., Olivarez, M., and Waris, G. (2010). Activation of transcription factor Nrf2 by hepatitis C virus induces the cell-survival pathway. *J. Gen. Virol.* 91, 681–690.

Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby,

R., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991). Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 88, 2451–2455.

Clore, J. N., Stillman, J., and Sugerman, H. (2000). Glucose-6-phosphatase flux in vitro is increased in type 2 diabetes. *Diabetes* 49, 969–974.

Deng, L., Adachi, T., Kitayama, K., Bungyoku, Y., Kitazawa, S., Ishido, S., Shoji, I., and Hotta, H. (2008). Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. *J. Virol.* 82, 10375–10385.

Deng, L., Shoji, I., Ogawa, W., Kaneda, S., Soga, T., Jiang, D. P., Ide, Y. H., and Hotta, H. (2011). Hepatitis C virus infection promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway. *J. Virol.* 85, 8556–8568.

Dionisio, N., Garcia-Mediavilla, M. V., Sanchez-Campos, S., Majano, P. L., Benedicto, I., Rosado, J. A., Salido, G. M., and Gonzalez-Gallego, J. (2009). Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. *J. Hepatol.* 50, 872–882.

Godoy, A., Ulloa, V., Rodriguez, F., Reinicke, K., Yanez, A. J., Garcia Mde, L., Medina, R. A., Carrasco, M., Barberis, S., Castro, T., Martinez, F., Koch, X., Vera, J. C., Poblete, M. T., Figueroa, C. D., Peruzzo, B., Perez, F., and Nualart, F. (2006). Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. *J. Cell. Physiol.* 207, 614–627.

Gross, D. N., van den Heuvel, A. P., and Birnbaum, M. J. (2008). The role of FoxO in the regulation of metabolism. *Oncogene* 27, 2320–2336.

Hirota, K., Sakamaki, J., Ishida, J., Shimamoto, Y., Nishihara, S., Kodama, N., Ohta, K., Yamamoto, M., Tanimoto, K., and Fukamizu, A. (2008). A combination of HNF-4 and Foxo1 is required for reciprocal transcriptional regulation of glucokinase and glucose-6-phosphatase genes in response to fasting and feeding. *J. Biol. Chem.* 283, 32432–32441.

Karpac, J., and Jasper, H. (2009). Insulin and JNK: optimizing metabolic homeostasis and lifespan. *Trends Endocrinol. Metab.* 20, 100–106.

Kasai, D., Adachi, T., Deng, L., Nagano-Fujii, M., Sada, K., Ikeda, M., Kato, N., Ide, Y. H., Shoji, I., and Hotta, H. (2009). HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters. *J. Hepatol.* 50, 883–894.

Kawaguchi, T., Yoshida, T., Harada, M., Hisamoto, T., Nagao, Y., Ide, T., Taniguchi, E., Kumemura, H., Hanada, S., Maeyama, M., Baba, S., Koga, H., Kumashiro, R., Ueno, T., Ogata, H., Yoshimura, A., and Sata, M. (2004). Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am. J. Pathol.* 165, 1499–1508.

Koike, K. (2007). Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways. *J. Gastroenterol. Hepatol.* 22(Suppl. 1), S108–S111.

Lemon, S. M., Walker, C., Alter, M. J., and Yi, M. (2007). "Hepatitis C virus," in *Fields' Virology,* 5th Edn, eds B. N. Fields, D. M. Knipe, and P. M. Howley (Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams and Wilkins), 1291–1304.

Leturque, A., Brot-Laroche, E., and Le Gall, M. (2009). GLUT2 mutations, translocation, and receptor function in diet sugar managing. *Am. J. Physiol. Endocrinol. Metab.* 296, E985–E992.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A., and Rice, C. M. (2005). Complete replication of hepatitis C virus in cell culture. *Science* 309, 623–626.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285, 110–113.

Macheda, M. L., Rogers, S., and Best, J. D. (2005). Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J. Cell. Physiol.* 202, 654–662.

Mason, A. L., Lau, J. Y., Hoang, N., Qian, K., Alexander, G. J., Xu, L., Guo, L., Jacob, S., Regenstein, F. G., Zimmerman, R., Everhart, J. E., Wasserfall, C., Maclaren, N. K., and Perrillo, R. P. (1999). Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 29, 328–333.

Miyamoto, H., Moriishi, K., Moriya, K., Murata, S., Tanaka, K., Suzuki, T., Miyamura, T., Koike, K., and Matsuura, Y. (2007). Involvement of the PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. *J. Virol.* 81, 1727–1735.

Nakashima, K., Takeuchi, K., Chihara, K., Hotta, H., and Sada, K. (2011). Inhibition of hepatitis C virus replication through adenosine monophosphate-activated protein kinase-dependent and -independent pathways. *Microbiol. Immunol.* 55, 774–782.

Negro, F. (2011). Mechanisms of hepatitis C virus-related insulin resistance. *Clin. Res. Hepatol. Gastroenterol.* 35, 358–363.

Negro, F., and Alaei, M. (2009). Hepatitis C virus and type 2 diabetes. *World J. Gastroenterol.* 15, 1537–1547.

Nomura-Takigawa, Y., Nagano-Fujii, M., Deng, L., Kitazawa, S., Ishido, S., Sada, K., and Hotta, H. (2006). Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. *J. Gen. Virol.* 87, 1935–1945.

Pazienza, V., Clement, S., Pugnale, P., Conzelman, S., Foti, M., Mangia, A., and Negro, F. (2007). The hepatitis C virus core protein of genotypes 3a and 1b downregulates insulin receptor substrate 1 through genotype-specific mechanisms. *Hepatology* 45, 1164–1171.

Poynard, T., Yuen, M. F., Ratziu, V., and Lai, C. L. (2003). Viral hepatitis C. *Lancet* 362, 2095–2100.

Rozance, P. J., Limesand, S. W., Barry, J. S., Brown, L. D., Thorn, S. R., LoTurco, D., Regnault, T. R., Friedman, J. E. and Hay, W. W. Jr. (2008). Chronic late-gestation hypoglycemia upregulates hepatic PEPCK associated with increased PGC1alpha mRNA and phosphorylated CREB in fetal sheep. *Am. J. Physiol. Endocrinol. Metab.* 294, E365–E370.

Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Tsukamoto, K., Kimura, S., Moriya, K., and Koike, K. (2004). Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 126, 840–848.

Takashima, M., Ogawa, W., Hayashi, K., Inoue, H., Kinoshita, S., Okamoto, Y., Sakaue, H., Wataoka, Y., Emi, A., Senga, Y., Matsuki, Y., Watanabe, E., Hiramatsu, R., and Kasuga, M. (2010). Role of KLF15 in regulation of hepatic gluconeogenesis and metformin action. *Diabetes* 59, 1608–1615.

Tzivion, G., Dobson, M., and Ramakrishnan, G. (2011). FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochim. Biophys. Acta* 1813, 1938–1945.

van der Horst, A., and Burgering, B. M. (2007). Stressing the role of FoxO proteins in lifespan and disease. *Nat. Rev. Mol. Cell Biol.* 8, 440–450.

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

Wang, A. G., Lee, D. S., Moon, H. B., Kim, J. M., Cho, K. H., Choi, S. H., Ha, H. L., Han, Y. H., Kim, D. G., Hwang, S. B., and Yu, D. Y. (2009). Non-structural 5A protein of hepatitis C virus induces a range of liver pathology in transgenic mice. *J. Pathol.* 219, 253–262.

Wu, X., and Freeze, H. H. (2002). GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80, 553–557.

Zhao, X., Gan, L., Pan, H., Kan, D., Majeski, M., Adam, S. A., and Unterman, T. G. (2004). Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. *Biochem. J.* 378, 839–849.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 December 2011; accepted: 25 December 2011; published online: 10 January 2012.

Citation: Shoji I, Deng L and Hotta H (2012) Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders. Front. Microbio. 2:278. doi: 10.3389/fmicb.2011.00278

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

ELSEVIER

Institut Pasteur

Microbes and Infection 14 (2012) 69–78

www.elsevier.com/locate/micinf

Original article

Generation of a recombinant reporter hepatitis C virus useful for the analyses of virus entry, intra-cellular replication and virion production

Kazuya Kamada [a], Ikuo Shoji [a], Lin Deng [a], Chie Aoki [a,b], Suratno Lulut Ratnoglik [a,b], Takaji Wakita [c], Hak Hotta [a,b,*]

[a] *Division of Microbiology, Kobe University, Graduate School of Medicine, 7-5-1 Kusunoki-cho, Cyuo-ku, Kobe, Hyogo 650-0017, Japan*
[b] *JST/JICA SATREPS, Japan*
[c] *Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan*

Received 21 February 2011; accepted 18 August 2011
Available online 31 August 2011

Abstract

The lack of a culture system that efficiently produces progeny virus has hampered hepatitis C virus (HCV) research. Recently, the discovery of a novel HCV isolate JFH1 and its chimeric derivative J6/JFH1 has led to the development of an efficient virus productive culture system. To construct an easy monitoring system for the viral life cycle of HCV, we generated bicistronic luciferase reporter virus genomes based on the JFH1 and J6/JFH1 isolates, respectively. Transfection of the J6/JFH1-based reporter genome to Huh7.5 cells produced significantly greater levels of progeny virus than transfection of the JFH1 genome. Furthermore, the expression of dominant-negative Vps4, a key molecule of the endosomal sorting complex required for transport machinery, inhibited the virus production of JFH1, but not that of J6/JFH1. These results may account for the different abilities to produce progeny virus between JFH1 and J6/JFH1. Using the J6/JFH1/Luc system, we showed that the two polyanions heparin and polyvinyl sulfate decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner. We also analyzed the function of microRNA on HCV replication and found that miR-34b could affect the replication of HCV. The reporter virus generated in this study will be useful for investigating the nature of the HCV life cycle and for identification of HCV inhibitors.

Keywords: HCV; Reporter virus; Virus production; ESCRT; microRNA

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus and has a positive-stranded RNA genome of about 9.6 kb [1,2]. HCV persistently infects hepatocytes, and the persistent infection can lead to liver cirrhosis and hepatocellular carcinoma. Considering that approximately 170 million people are infected with HCV worldwide [3], HCV is a major public health problem throughout the world. A combination therapy of pegylated interferon-α and ribavirin has been established as the standard of care for treating HCV infection [3,4]. Nonetheless, approximately 50% of individuals with chronic HCV infection are still unable to resolve infection [4,5]. For this reason, more effective therapies are greatly needed against the disease caused by HCV infection [6].

The HCV genome encodes a 3000 amino acid polyprotein which is cleaved by host and viral proteases to yield the mature structural proteins, composed of core and glycoprotein E1 and E2, and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [1–3]. Translation of the HCV open reading frames is mediated via the 5′ untranslated region and a part of the core coding region carrying the internal ribosome entry site (IRES) [1,7].

In 1999, Bartenschlager and his colleagues produced the HCV replicon system, a tissue culture system that recapitulated the RNA replication of HCV in a human hepatoma cell line [8]. In the initial subgenomic replicon system, genes

* Corresponding author. Division of Microbiology, Kobe University, Graduate School of Medicine, 7-5-1 Kusunoki-cho, Cyuo-ku, Kobe, Hyogo 650-0017, Japan. Tel.: +81 78 382 5500; fax: +81 78 382 5519.
E-mail address: hotta@kobe-u.ac.jp (H. Hotta).

1286-4579/$ - see front matter © 2011 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2011.08.009

unessential for RNA replication that contained the core, E1, E2, p7 and NS2 of the HCV genome were replaced with a genetic cassette carrying an antibiotics resistance gene and IRES from encephalomyocarditis virus (EMCV). The development of a subgenomic replicon system became a driving force for the studies on the mechanism of HCV replication, and these studies revealed numerous biological features of HCV replication. However, the resulting systems were unable to produce progeny virus. Therefore, the nature of the HCV, i.e., the virus production and virus entry, remained unclear for a long while.

Wakita and his colleagues isolated a full-length HCV genome from the sera of a patient with fulminant hepatitis [9]. The HCV strain, designated JFH1, belongs to genotype 2a. The transfection of the Huh7 hepatoma cell line with the JFH1 genome yields a progeny virus called HCVcc that is infectious both *in vivo* and *in vitro*. The HCVcc system allowed us to perform virological studies to investigate the nature of HCV [9,10]. However, the analyses using HCVcc have not been suitable for carrying out high-throughput screening due to the labor-intensive quantitative reverse transcription-PCR methods used in screening and the difficulties presented by the low signal-to-noise ratios.

In this study, to develop a robust tool for use in the screening of HCV replication, we have constructed a genome-length luciferase reporter HCV derived from the JFH1 and J6/JFH1 strains, and used it to analyze the intra-cellular RNA replication and extra-cellular progeny virus production. We demonstrated here that our recombinant reporter HCV system was useful for studying viral genome replication, virus entry, and virion production of HCV.

2. Materials and methods

2.1. Plasmids

The plasmid pFGR-JFH1/Luc, which encodes bicistronic constructs of HCV IRES-driven firefly luciferase reporter genes and the EMCV IRES-driven full-genomic JFH1 genome, was constructed by insertion of the JFH1 full genome of pJFH1 [9] into pSGR-JFH1 [11]. The plasmid pFL-J6/JFH1, which contains a chimeric full-genome composed of the 5′NCR to NS2 region derived from J6 and NS3 to the 3′NCR region from JFH1 [10], was kindly supplied by C.M. Rice of the Center for the Study of Hepatitis C, Rockefeller University. To yield the bicistronic luciferase reporter construct composed of full-length J6/JFH1, the JFH1 full genome of pFGR-JFH1/Luc was replaced with the J6/JFH1 full genome of pFL-J6/JFH1 by digestion with BstZ17I, and the resultant plasmid was designated as pFGR-J6/JFH1/Luc. As a negative control for the HCV replication, a non-synonymous mutation at NS5B (GDD to GND), which disrupts NS5B polymerase activity, was introduced into the pFGR-J6/JFH1/Luc NS5B region by site-directed mutagenesis, and the resultant plasmid was designated pFGR-J6/JFH1/Luc (GND).

2.2. Cell culture and indirect immunofluorescence

All experiments described in this study were performed by using Huh7.5 human hepatoma cells, a highly HCV-susceptible subclone of Huh7 cells. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 0.01% streptomycin, and were subcultured twice weekly. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis as previously reported [12]. The primary antibody used was derived from an HCV-infected patient's serum. The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (MBL, Nagoya, Japan).

2.3. In vitro transcription and electroporation

Plasmid DNA was linearized with XbaI, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in RNase-free water. The purified DNA was used for *in vitro* RNA transcription using a T7 Megascript kit (Ambion, Austin, TX) following the manufacturer's protocols. The concentration was determined by measurement of the optical density at 260 nm, and the RNA integrity was checked by agarose gel electrophoresis. The *in vitro*-transcribed RNA (10 μg) was transfected into Huh7.5 cells by means of electroporation (975 μF, 270 V) using a Gene Pulser (Bio-Rad, Hercules, CA). The cells were then cultured in complete medium. The culture fluid of transfected cells was harvested and cleared by passing through 0.45-μm-pore-size filters and stored at −80 °C until use.

2.4. Luciferase assay

The *firefly* luciferase activity was measured by a luciferase assay system (Promega, Madison, WS). The cells were harvested, washed twice with dication-free phosphate buffered saline (PBS), and lysed in a passive lysis buffer supplied by the manufacturer. A 20-μl sample of the lysate was subjected to a luciferase assay. The luminescence was measured at 10 s after an initial 2 s delay according to the manufacturer's instructions, using a Lumat LB9501 luminometer (Berthold, Freiburg, Germany). The assays were performed in duplicate at least three times, and the mean and standard error were computed.

2.5. Vectors of ESCRT family proteins and DNA transfection

The cDNA of the endosomal sorting complex required for transport (ESCRT) family proteins was amplified from Huh7.5 cells by RT-PCR and cloned into pcDNA3.1-FLAG [13], an expression vector containing a CMV promoter and FLAG tag sequence in pcDNA3.1 (Invitrogen, Carlsbad, CA). For the expression of each ESCRT family protein, Huh7.5 cells were transfected with each ESCRT expression vector by using TransIT LT1 transfection reagents (Takara, Kyoto, Japan). The expression levels of the three ESCRT family proteins in transfected Huh7.5 cells were monitored by immunoblotting using the anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO).

2.6. Quantification of HCV core protein

HCV core protein in the cells or cell-culture supernatants was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics). To determine the intra-cellular amounts of core, cell lysates were prepared as described by Schaller et al. [14].

2.7. Blocking of virus attachment and entry with anti-CD81 antibody

Blocking of virus attachment and entry with anti-CD81 antibody was performed essentially as described previously [9]. Huh7.5 cells (6×10^4 cells/well of a 24-well plate) were pre-treated with anti-CD81 antibody (clone JS-81; BD Biosciences) or an isotype-matched control antibody (purified mouse IgG1, isotype control; BD Biosciences) as indicated for 1 h. Cells were then infected with the reporter viruses for 6 h. The viruses were removed, and then the culture medium was replaced with complete DMEM. On day 2 post-infection, the cells were lysed with a passive lysis buffer as mentioned above. The efficiency of infection was monitored by measuring the luciferase activity of the cell lysate.

2.8. Transfection of microRNA inhibitor

Huh7.5 cells were electroporated with luciferase reporter HCV RNA as mentioned above, and then the cells were seeded in a well of a 24-well plate. To analyze the effect of inhibition of microRNA (miR), both a specific miRNA inhibitor (Anti-miR™ miRNA) and a non-targeting negative control (Anti-miR™miRNA Inhibitors—Negative Control) were purchased from Ambion, Inc. 50 pmol of a specific miRNA inhibitor or negative control were transfected into luciferase reporter RNA-electroporated Huh7.5 cells by using a siPORT™ NeoFX™ Transfection Agent (Ambion) according to the manufacturer's instructions. At 48 h post-transfection, the cells were harvested, and viral replication was determined by luciferase assay of the cell lysate.

2.9. Polyions

The polyanions heparin (mol. wt. 3000), dextran sulfate (mol. wt. 50,000) and polyvinyl sulfate (mol. wt. 150,000), and the polycations polybrene (mol. wt. 3000), DEAE-dextran (mol. wt. 100,000), and poly-L-lysine (mol. wt. 500,000) (all purchased from Sigma) were dissolved in PBS.

3. Results

3.1. Construction and characterization of luciferase reporter HCV

To construct a reporter HCV that can permit easy monitoring of both virus production and intra-cellular viral growth kinetics, we constructed the bicistronic HCV constructs by inserting a luciferase reporter gene into the 5′ end of the coding sequence of the JFH1 or J6/JFH1 full-genome plasmids clone as shown in Fig. 1A. In the transcript derived from bicistronic reporter HCV clone, the HCV and EMCV IRESs are responsible for the translation of the luciferase protein and all HCV proteins, respectively. A reporter construct with NS5B GDD to GND mutation, which disrupts viral polymerase function, was also constructed by site-directed mutagenesis, and served as a negative control for viral genome replication. To examine the replication level of reporter HCVs, we prepared the RNAs from each construct by *in vitro* transcription, and then transfected them into Huh7.5 cells by an electroporation technique. The viral replication was quantified up to 10 days post-transfection by using an HCV core-specific ELISA and luciferase reporter assay. As shown in Fig. 1B, the transfection of RNAs of both the JFH1/Luc and J6/JFH1/Luc reporter clones induced intra-cellular HCV core protein expression, which peaked on day 2 post-transfection. Both JFH1/Luc and J6/JFH1/Luc showed similar kinetics, and the high level core protein expression continued until day 10 post-transfection. As expected, the GND mutant exhibited 100-fold lower intra-cellular core protein expression on day 2 post-transfection. The level of core expression by the GND mutant continued to decline thereafter, and fell below the detection limit on day 10 post-transfection. As shown in Fig. 1C, both JFH1/Luc and J6/JFH1/Luc induced similar levels of luciferase activity in Huh7.5 cells at 4 h after electroporation. This result indicated that both RNAs were electroporated with similar efficiency because RNA replication had not started at that time and all the luciferase was translated from the input RNA. At 4 days post-electroporation, the luciferase activities of both JFH1/Luc and J6/JFH1/Luc were 10-fold greater than those measured at 4 h after electroporation. Subsequently, JFH1/Luc and J6/JFH1/Luc showed almost the same kinetics of luciferase activity until 10 days post-transfection. At 3 days post-electroporation, both JFH1/Luc and J6/JFH1/Luc electroporated cells were stained with HCV-positive patient sera, and the rate of intra-cellular replication was then visualized using immunofluorescent microscopy as previously reported [12]. As a result, the HCV-positive rates were 17% and 19% for JFH1/Luc and J6/JFH1/Luc, respectively (Fig. 1D). These results indicated that the luciferase activity of reporter HCV-transfected cells reflected the intra-cellular viral replication, and also suggested that both JFH1 and J6/JFH1 had similar intra-cellular replication ability in Huh7.5 cells.

3.2. Production of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we assessed the potential of the reporter HCV to produce infectious progeny virions. Huh7.5 cells were electroporated with the reporter RNAs, and the culture supernatant was collected at various time points. To analyze the release of progeny virions from the reporter RNA-electroporated cells, the amounts of core protein in culture supernatants were

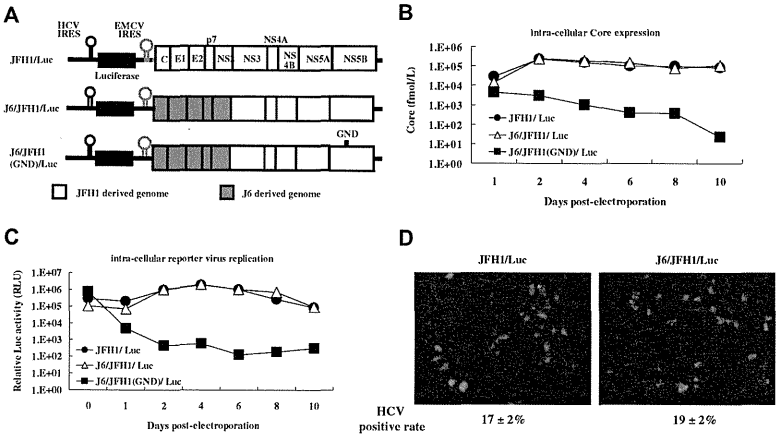

Fig. 1. Schematics of luciferase reporter HCV in this study. (A) Organization of luciferase reporter HCV. The luciferase gene is depicted as a black box. The JFH1-derived open reading frame and J6-derived open reading frame are depicted as a gray box and white box, respectively. As a negative control, a GND mutation was introduced to NS5B RdRp. (B, C) Virus replication kinetics in Huh7.5 cells of luciferase reporter HCV. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and the cells were assayed for core protein ELISA (B) and luciferase activity (C) at intervals as indicated. The assays were repeated at least three times, and the mean values are presented. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis at 3 days post-electroporation (D). Cells were incubated with an HCV-infected patient's serum followed by FITC-labeled goat anti-human IgG (green). In parallel, the cells were stained with Hoechst 33342 to visualize the nuclei (blue). The HCV-positive rate was calculated by counting the number of HCV-positive cells among the total cells, and the data represent the means and SE of three independent experiments.

analyzed by ELISA. As shown in Fig. 2A, electroporation of both reporter viral RNAs with Huh7.5 cells released the HCV core protein into the culture supernatants. The levels of core protein released from both reporter HCV RNAs peaked at 6 days post-electroporation. The amount of core protein of the J6/JFH1/Luc supernatants was 2–4 fold greater than that of JFH1/Luc among all the time points tested. In parallel, to analyze the infectivity of progeny virions produced from reporter RNA-electroporated cells, these supernatants were used as inocula for naïve Huh7.5 cells. The cells inoculated with these supernatants were harvested at 48 h post-inoculation, and the luciferase activity of the cell lysate was analyzed (Fig. 2B). These supernatants infected naïve Huh7.5 cells, and transduced luciferase activity in the cells. The infectious virus of both reporter HCVs was initially detected on day 2 and peaked on day 4 post-electroporation. However, the infectivity was decreased after day 6 post-electroporation. Furthermore, the infectivity of J6/JFH1/Luc supernatants was significantly higher than that of JFH1/Luc (approximately 10-fold). To compare the luciferase activity and the virus titer, we

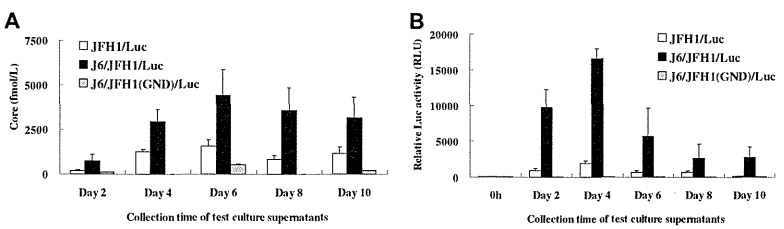

Fig. 2. Progeny virus production from luciferase reporter RNA-transfected Huh7.5 cells. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and culture supernatants of the cells were collected at the indicated time points. The amount of progeny virus in the supernatant was measured by the HCV core protein ELISA. (A) In parallel, the supernatants were added to naïve Huh7.5 cells. At 48 h post-addition, the cells were lysed, and assayed for luciferase activity to assess the infectivity of progeny virus from reporter HCV RNA. (B) The assays were repeated at least three times, and the mean values are presented.

performed standard virion titration by immunofluorescent antibody staining. The result showed that the virus titer of J6/JFH/Luc supernatant, collected at day 4 post-RNA transfection, was 5×10^3 fluorescent-focus forming units (ffu) per ml. In contrast, the titer of JFH/Luc supernatant was below the detection limit ($<1 \times 10^2$ ffu/ml). Interestingly, the peaks of the core release and the infectivity were slightly different, i.e., the peak of the core release of J6/JFH1 was on day 6, and that of the infectivity was on day 4 post-electroporation. Collectively, these data revealed that J6/JFH1 had a greater ability to release progeny virions than JFH1, though the levels of intracellular replication were comparable between J6/JFH1/and JFH1.

3.3. Characterization of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we examined whether the J6/JFH1/Luc-derived supernatants had the features of a virus and thus could be used as a surrogate for HCV. The supernatants collected from each culture of reporter RNA-electroporated cells were irradiated with ultra-violet (UV) for 5 min, and the supernatants were then inoculated into naïve Huh7.5 cells. As shown in Fig. 3A, the infectivity of the reporter virus was completely abrogated by UV-irradiation. The results indicated that the luciferase activity transduced by the supernatants was derived from the genome of the reporter virus, not from incorporation of the luciferase protein into the virion. The entry of the HCV virion was mediated by binding between the cellular surface protein CD81 and the HCV envelope protein E2 [15]. Therefore, the naïve Huh7.5 cells were pre-treated with a recombinant monoclonal antibody against CD81. After 1 h pre-treatment, the J6/JFH1/Luc supernatant was inoculated into the cells, and the luciferase activity of cells was analyzed at 48 h post-inoculation (Fig. 3B). Normal mouse IgG showed no effect on the infectivity of the J6/JFH/Luc supernatant. In contrast, the infectivity of the J6/JFH/Luc supernatant was decreased by pre-treatment with anti-CD81 antibody in a dose-dependent manner. The results suggested that the supernatant from luciferase reporter J6/JFH1/Luc-transfected cells contained a virus with characteristics similar to HCV, and that this reporter virus could be utilized to investigate all the steps of virus replication, including the intra-cellular viral replication, the virus production and the virus entry as a surrogate model of HCV.

3.4. Analysis of a potential role for ESCRT family proteins in HCV virus production

Prior to the recent establishment of the JFH1-based cell-culture system, there was no system for producing the HCV virus, and thus many aspects of the virus production of HCV still remain poorly understood. Generally, the production of the enveloped virus requires a multi-step process that includes the proper transport of viral proteins and organization of viral proteins on the cellular membrane, and these steps are coordinated by a variety of cellular factors [16,17]. From numerous intensive studies, it has been revealed that the process of budding of many enveloped viruses utilizes the ESCRT machinery, which is responsible for the formation of luminal vesicles of endosomal multivesicular bodies (MVB) [16,18–20]. The ESCRT machinery consists of a number of cellular proteins that make up three functional sub-complexes – ESCRT-I, ESCRT-II and ESCRT-III – and other related factors; i.e., Vps4 and AIP/Alix are also participated in the function of ESCRT machinery [20]. A series of analyses about ESCRT networks has revealed the consensus amino acid motifs of viral proteins; the P(T/S)AP motif was observed to interact with Tsg101, and the YPxL motif was seen in the case of AIP/Ailx [19]. We searched for these motifs in the J6 and JFH1 genomes, and found one AIP/Alix interacting the YPxL motif in the NS5B region (aa. 2604 to 2607; YPDL). Therefore, the relation between ESCRT and HCV was examined by analyzing the virus production using a luciferase reporter HCV system. First, we constructed the expression plasmids of the ESCRT-I protein Tsg101, and the ESCRT-associated proteins Nedd4L and AIP/Alix. The ESCRT expression plasmids were transfected into the J6/JFH1/Luc or JFH1/Luc RNA-transfected Huh7.5 cells. After 48 h of transfection, the culture supernatants were collected and inoculated into the culture of the naïve Huh7.5 cells. The effects of over-expression of ESCRT proteins on intra-cellular virus

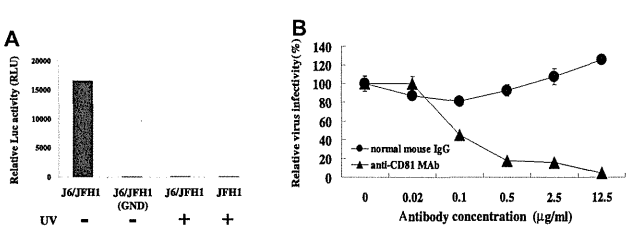

Fig. 3. Anti-CD81 antibody blocks luciferase reporter HCV infection. The reporter viruses containing supernatants were prepared as described in Materials and methods. (A) The JFH1/Luc and J6/JFH1/Luc supernatants were irradiated with UV at 5 min, and then added to naïve Huh7.5 cells. The infectivity was analyzed by luciferase assay. (B) Huh7.5 cells were pre-treated with anti-CD81 monoclonal antibody or control mouse IgG at 1 h before infection. Cells were then infected with J6/JFH1/Luc reporter viruses for 6 h. At 48 h post-infection, the cells were lysed and assayed for luciferase activity. Activities are expressed as the relative activity compared to that of the null antibody-treated sample. The assays were repeated at least three times, and the mean values are presented.

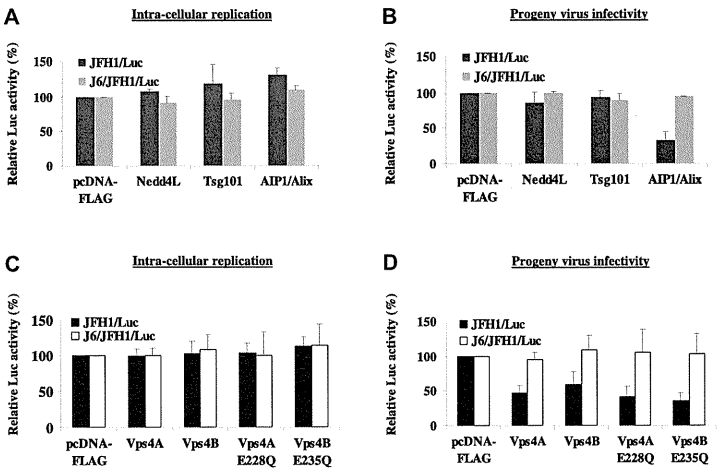

Fig. 4. Effect of ESCRT family protein expression on intra-cellular replication and progeny virus production in Huh7.5 cells. Huh7.5 cells were electroporated with JFH1/Luc and J6/JFH1/Luc RNA, respectively. The RNA-electroporated cells were then transfected with ESCRT protein expression plasmids. At 96 h after transfection, the culture supernatants were collected, and the cells were harvested. (A, C) Cell lysates were assayed for luciferase activity to assess intra-cellular virus replication. (B, D) Collected supernatants were added to naïve Huh7.5 cells and incubated for 48 h, and then the luciferase activity of the cells was analyzed to assess progeny virus infectivity. The data relative to that of luciferase activity in the absence of ESCRT protein (pcDNA-FLAG) is indicated. The assays were repeated at least three times, and the mean and standard error are presented.

replication and virus production were analyzed by monitoring the luciferase activity of reporter RNA-transfected cells (Fig. 4A), and the luciferase activity expressed by supernatant virus (Fig. 4B). As shown in Fig. 4A, the overexpression of Nedd4L, Tsg101, and AIP/Alix had no effect on the intra-cellular replication of either reporter HCV. As shown in Fig. 4B, the virus production from J6/JFH1/Luc also was not affected by these ESCRT protein expressions. In contrast, the expression of AIP/Alix decreased the virus production from JFH1/Luc by 50%. This result implied that the ESCRT machinery might have played some role in the difference in the efficacy of virus production observed between JFH1 and J6/JFH1. AAA-ATPase Vps4, which is present in humans in two isoforms (Vps4A and Vps4B), is a key modulator protein for the final step of ESCRT machinery. To analyze the role of ESCRT in HCV virus production, we constructed expression vectors for Vps4A and 4B, as well as expression vectors for a dominant-negative Vps4A(E228Q) and Vps4B(E235Q) [19]. As shown in Fig. 4C, the intra-cellular replications of JFH1 and J6/JFH1 were not influenced by the wild-type or dominant-negative Vps4 expression. In contrast, the levels of virus production of JFH1/Luc were reduced up to 50% by the expression of both dominant-negative Vps4 mutants (Fig. 4D). Interestingly, neither dominant-negative Vps4 influenced the virus production of J6/JFH1/Luc. These results implied that JFH1 might utilize the ESCRT machinery for release of infectious virus particles.

3.5. Effect of polyions on the infectivity of the J6/JFH1/Luc reporter virus

Next, we tested the usefulness of the J6/JFH1/luc reporter system for virus entry analysis. The binding of the viral and cellular receptors is coordinated with the ionic conditions, indicating that compounds that affect the ionic charge of the receptor surface might be potent inhibitors of virus infection [21,22]. Polyions with a positive or negative charge are frequently used for virus entry analyses, and exhibit inhibitory activity on virus infection [21,22]. Therefore, we investigated the effect of different polyions on the infectivity of the J6/JFH1/Luc virus in order to clarify the influence of electrostatic interactions in virus binding to cell membranes. As candidate compounds, we used both polymers having a positive charge (polybrene (size of 3000 Da), DEAE-dextran (100,000 Da), and poly-L-lysine (500,000 Da)) and those having a negative charge (heparin (15,000 Da), dextran sulfate (50,000 Da), and polyvinyl sulfate (150,000 Da)). These polymers were added to the Huh7.5 cells at 1 h before inoculation of the J6/JFH1/Luc virus into the cells. After 48 h of inoculation, the cells were harvested and the luciferase activity was analyzed (Fig. 5A and B). As shown in Fig. 5A, two polyanions, heparin and polyvinyl sulfate, decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner, whereas one polyanion, dextran sulfate, enhanced the infectivity up to 2-fold. In the case of polycations, the addition of polybrene enhanced virus

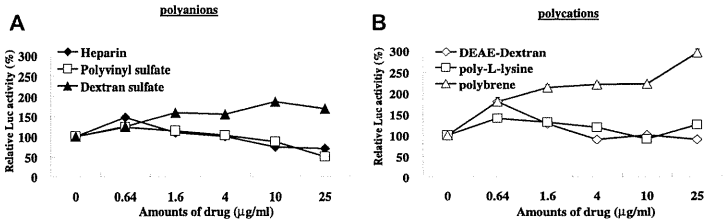

Fig. 5. Effect of multiple polyions on J6/JFH1/Luc virus infection of Huh7.5 cells. Huh7.5 cells were infected with J6/JFH1/Luc virus in the presence of each of the polyions for 6 h, and a luciferase assay was performed 48 h later. The data was expressed as the relative activity compared to the luciferase activity in the absence of polyions. The assays were repeated at least three times, and the mean values are presented.

infection up to 3-fold in a dose-dependent manner, although poly-L-lysine and DEAE-dextran showed no effect on the infectivity of the J6/JFH1/Luc virus (Fig. 5B). The effect shown by compounds belonging to positive and negative polyions suggested that the electric charge is not sufficient by itself to explain the inhibitory or enhancing activity of these drugs on the HCV virus entry. These results indicated that the J6/JFH1/Luc virus was useful to easily monitor HCV virus entry.

3.6. Screening of microRNA inhibition on intra-cellular HCV replication

To confirm the usefulness of the J6/JFH1/Luc reporter system in the analysis targeting intra-cellular replication of HCV, we analyzed the possible involvement of micro RNAs (miRNAs) in HCV infection. miRNAs are evolutionarily conserved, small, non-coding RNA molecules that regulate gene expression at the level of translation [23,24]. Recently, it has been reported that some miRNAs influence the replication of HCV in the cells [25–27]. For example, the expression of miR-122 in the cells might be essential for HCV replication [25]. In addition, the number of miRNAs has been increasing due to numerous strenuous analyses in recent years. Therefore, we compared the full sequences of the viral genome among 4 different HCV strains (H77C, Con1, J6, JFH1) with the sequences of 630 human miRNAs using the miRNA database program (RegRNA: http://regrna.mbc.nctu.edu.tw/index.php), and then identified 54 miRNAs that matched with at least one HCV strain. 10 of the 54 miRNAs matched with all four HCV strains. Hence, we focused on analysis of the function of the 10 miRNAs on HCV replication and prepared commercially available miRNA inhibitors (Anti-miR™ miRNA inhibitor, Ambion) that were chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous target miRNA molecules. The J6/JFH1/Luc RNA-electroporated cells were transfected with each of the 10 specific miRNA inhibitors and the luciferase activities were analyzed at 48 h post-transfection of the inhibitors. None of the miRNA inhibitors significantly affected the cell viability (data not shown). As shown in Fig. 6A, the inhibition of miR-122 reduced the level of intra-cellular virus replication by up to 50% as previously reported [25]. A similar reduction of viral replication was also observed by treatment with the miR-34b inhibitor. The treatment with an anti-miR negative control that is a random sequence anti-miR molecules that has been extensively tested in human cell lines and validated to not produce identifiable effects on known miRNA functions showed no significant effect on HCV replication. None of the other inhibitors showed any significantly greater effect on the

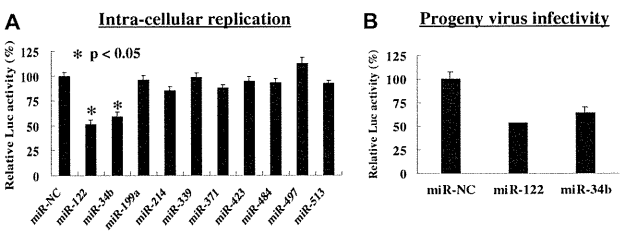

Fig. 6. Effect of miRNA inhibitor on intra-cellular replication of J6/JFH1/Luc RNA in Huh7.5 cells. Target cells were electroporated with J6/JFH1/Luc RNA, and then transfected with a miRNA-specific or a non-target negative control miRNA inhibitor. At 48 h post-transfection, cells were harvested and analyzed for luciferase activity (A). In parallel, the culture supernatants were collected at 48 h post-transfection to assess the effect of miRNA inhibitors on the virus production. The supernatants were added to naïve Huh7.5 cells, and the progeny virus infectivities were then analyzed by luciferase assay. (B) The data relative to the luciferase activity obtained from a non-target negative control miRNA inhibitor are indicated. The assays were repeated at least three times, and the mean and standard error are presented. Statistical significance relative to the negative control miRNA samples as calculated by *t*-test is shown (*$p < 0.05$).

virus replication than the anti-miR negative control. The miR-34b and miR-122 inhibitors decreased the virus production to the levels 64% and 53% of the control, respectively (Fig. 6B). Since the extent of the reduction in virus production was comparable with that of intra-cellular HCV RNA levels (Fig. 6A), it was likely that these miRNA inhibitors affected the intra-cellular viral replication rather than interfering with the particle formation and the release of the virion. These results suggest that the function of miR-34b could affect the replication of HCV, and also suggested that the J6/JFH1/Luc system was useful to analyze the intra-cellular replication of HCV.

4. Discussion

In this report, we generated two bicistronic luciferase reporter HCV clones from JFH1 and J6/JFH1, and established a unifying system that can monitor intra-cellular viral replication, virion production, and virus entry. Using two constructs, we initially compared the potential of intra-cellular viral replication and virus production. After transfection of reporter RNAs, the level of the intra-cellular core protein and the luciferase activity in RNA-transfected cells showed similar kinetics for JFH1/Luc and J6/JFH1/Luc (Fig. 1B and C). In contrast, both the efficacy of core protein production into the culture supernatant and the infectivity of supernatant virus from J6/JFH1/Luc were significantly higher than that of JFH1/Luc (Fig. 2A and B). These results indicated two possibilities that JFH1 and J6/JFH1 utilize different machinery for progeny virus packaging and budding, or that they utilize the same machinery for the virus production but to a different degree. To evaluate the difference in the virus production between JFH1 and J6/JFH1, we analyzed the role of ESCRT machinery in virus production (Fig. 4A–D). Dominant-negative Vps4 expression inhibited JFH1/Luc virus production, but did not influence J6/JFH1/Luc virus production. In the course of preparing this manuscript, Corless et al. reported that HCV requires late components of the ESCRT pathway for release of infectious virus particles [28]. They showed that a dominant-negative Vps4 expression inhibited the production of virus-like particles derived from JFH1 in a dose-dependent manner. The findings reported by Corless et al. and the findings of our present study emphasize that the ESCRT machinery plays an essential role in JFH1 virus production.

To examine the virus entry, we analyzed the effect of anti-CD81 antibody and polyions on reporter virus infectivity (Figs. 3B and 5A and B). The pre-treatment with anti-CD81 antibody decreased the infectivity of the reporter J6/JFH virus in a dose-dependent manner. The result suggested that the reporter J6/JFH1 virus, similar to HCVcc, utilized the CD81 as a major entry receptor, and that our reporter virus could be used as a surrogate model of HCV entry analysis. As a result of polyions analysis, one of the polycations (dextran sulfate) and one of the polyanions (polybrene) increased the reporter virus infectivity, and the remainder of the polyions inhibited the virus infectivity. These results indicate the possibility that not only the electrostatic condition of polyions but also their molecular weight may be a determinant of the receptor binding of HCV. Considering that several membrane molecules have been identified as candidate cellular receptors for HCV entry [15,29,30], the polyions could interact with a different molecule(s) to influence virus production. As for heparin, it was reported that cell surface heparan sulfate proteoglycans play an important role in mediating HCV envelope–target cell interaction [31]. Basu et al. [32] also reported that heparin treatment completely blocked HIV/HCV E1-E2 pseudotype infection. In their analysis, however, the inhibitory effect of heparin against cell culture-grown HCV H77 was somewhat lower than that of HIV/HCV E1-E2 pseudotypes. In our present study, the level of inhibitory effect of heparin on J6/JFH1 reporter virus infection was not so prominent. Collectively, these data suggest a possibility that cell surface heparan sulfate proteoglycans contribute to the infection of both HIV/HCV E1–E2 pseudotype and cell culture-grown HCV with a different degree. Therefore, to develop a polyion-based anti-HCV drug, a more detailed assessment of the interaction between each candidate receptor and polyion is necessary.

Using microRNA inhibitors, the decrease of miR-34b expression suppressed intra-cellular HCV replication (Fig. 6A). miR-34b belongs to the evolutionary conserved microRNA family of miR-34s [33], known for their role in the p53 tumor suppressor network [34]. miR-34s have been shown to be controlled in a tissue-specific manner by p53. Both wild-type and mutant-type p53 protein expressions in serum and cytoplasm of liver tissue were more pronounced in patients with hepatocellular carcinoma associated with HCV infection [35]. Wild-type p53 binds to a transcriptional regulatory element of miR-34s, thereby up-regulating miR-34 expression [34]. However, it is not understood whether the mutant-type p53 increases miR-34b expression. Furthermore, HCV replication in chronic hepatitis is higher than that of hepatocellular carcinoma [36]. Therefore, more detailed research is needed to reveal the significance of miR-34b expression in HCV replication and hepatocellular carcinoma.

As mentioned above, we have generated a recombinant luciferase reporter HCV, and have shown that the reporter HCV could be used for the quantitative analyses of intra-cellular replication, virus entry, and virion production. In general, the intra-cellular HCV replication has been analyzed by the quantitative real-time RT-PCR method that could detect a small amount of viral RNA because of the greatly high sensitivity. However, the real-time RT-PCR method involves multi-step procedures of the RNA extraction, the reverse transcription and the PCR reaction, which require skillfulness to perform. The high sensitivity and the multiple-steps of the real-time RT-PCR system sometimes cause an experimental error(s) when conducted by less-experienced individuals. On the other hand, our HCV luciferase reporter system is simpler and easier to perform compared to the real-time PCR system. The significant advantage of the reporter HCV is that it can analyze a large number of samples at a time in a time- and cost-saving manner. Also, it can be used to evaluate all the events of viral life cycle. By using it, we have started the screening of anti-HCV substances from the natural resource chemical libraries and found a number of potential candidates for the analysis. Thus, this system can be applicable for robust screening analyses of chemical compounds to discover a potential therapeutic target of HCV.

Acknowledgments

The authors are grateful to Dr C.M. Rice (Center for the Study of Hepatitis C, the Rockefeller University, New York, NY, USA) for providing pFL-J6/JFH1 and Huh7.5 cells. We also thank the members of our department for their helpful discussion. This study was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan, and a SATREPS Grant from Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA). This study was also carried out as part of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), Ministry of Education, Culture, Sports, Science and Technology, and the Global Center of Excellence (G-COE) Program at Kobe University Graduate School of Medicine.

References

[1] T.L. Tellinghuisen, M.J. Evans, T. von Hahn, S. You, C.M. Rice, Studying hepatitis C virus: making the best of a bad virus, J. Virol. 81 (2007) 8853–8867.
[2] N. Kato, M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, K. Shimotohno, Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 9524–9528.
[3] S. Chevaliez, J.M. Pawlotsky, Hepatitis C virus: virology, diagnosis and management of antiviral therapy, World J. Gastroenterol. 13 (2007) 2461–2466.
[4] M. Laguno, C. Cifuentes, J. Murillas, S. Veloso, M. Larrousse, A. Payeras, L. Bonet, F. Vidal, A. Milinkovic, A. Bassa, C. Villalonga, I. Perez, C. Tural, M. Martinez-Rebollar, M. Calvo, J.L. Blanco, E. Martinez, J.M. Sanchez-Tapias, J.M. Gatell, J. Mallolas, Randomized trial comparing pegylated interferon alpha-2b versus pegylated interferon alpha-2a, both plus ribavirin, to treat chronic hepatitis C in human immunodeficiency virus patients, Hepatology 49 (2009) 22–31.
[5] A. El-Shamy, M. Nagano-Fujii, N. Sasase, S. Imoto, S.R. Kim, H. Hotta, Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy, Hepatology 48 (2008) 38–47.
[6] D.P. Webster, P. Klenerman, J. Collier, K.J. Jeffery, Development of novel treatments for hepatitis C, Lancet Infect. Dis. 9 (2009) 108–117.
[7] P.J. Lukavsky, Structure and function of HCV IRES domains, Virus. Res. 139 (2009) 166–171.
[8] V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.
[9] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.
[10] B.D. Lindenbach, M.J. Evans, A.J. Syder, B. Wolk, T.L. Tellinghuisen, C.C. Liu, T. Maruyama, R.O. Hynes, D.R. Burton, J.A. McKeating, C. M. Rice, Complete replication of hepatitis C virus in cell culture, Science 309 (2005) 623–626.
[11] T. Kato, T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, T. Wakita, Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system, J. Clin. Microbiol. 43 (2005) 5679–5684.
[12] L. Deng, T. Adachi, K. Kitayama, Y. Bungyoku, S. Kitazawa, S. Ishido, I. Shoji, H. Hotta, Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway, J. Virol. 82 (2008) 10375–10385.
[13] K. Kamada, T. Igarashi, M.A. Martin, B. Khamsri, K. Hatcho, T. Yamashita, M. Fujita, T. Uchiyama, A. Adachi, Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 16959–16964.
[14] T. Schaller, N. Appel, G. Koutsoudakis, S. Kallis, V. Lohmann, T. Pietschmann, R. Bartenschlager, Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes, J. Virol. 81 (2007) 4591–4603.
[15] 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.
[16] A. Calistri, C. Salata, C. Parolin, G. Palu, Role of multivesicular bodies and their components in the egress of enveloped RNA viruses, Rev. Med. Virol. 19 (2009) 31–45.
[17] S. Welsch, B. Muller, H.G. Krausslich, More than one door – budding of enveloped viruses through cellular membranes, FEBS Lett. 581 (2007) 2089–2097.
[18] B. Strack, A. Calistri, S. Craig, E. Popova, H.G. Gottlinger, AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding, Cell 114 (2003) 689–699.
[19] P.D. Bieniasz, Late budding domains and host proteins in enveloped virus release, Virology 344 (2006) 55–63.
[20] J.H. Hurley, P.I. Hanson, Membrane budding and scission by the ESCRT machinery: it's all in the neck, Nat. Rev. Mol. Cell Biol. 11 (2010) 556–566.
[21] O. Bagasra, P. Whittle, B. Heins, R.J. Pomerantz, Anti-human immunodeficiency virus type 1 activity of sulfated monosaccharides: comparison with sulfated polysaccharides and other polyions, J. Infect. Dis. 164 (1991) 1082–1090.
[22] J. Haldar, D. An, L. Alvarez de Cienfuegos, J. Chen, A.M. Klibanov, Polymeric coatings that inactivate both influenza virus and pathogenic bacteria, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 17667–17671.
[23] A. Ventura, T. Jacks, microRNAs and cancer: short RNAs go a long way, Cell 136 (2009) 586–591.
[24] D.P. Bartel, microRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233.
[25] C.L. Jopling, M. Yi, A.M. Lancaster, S.M. Lemon, P. Sarnow, Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA, Science 309 (2005) 1577–1581.
[26] G. Randall, M. Panis, J.D. Cooper, T.L. Tellinghuisen, K.E. Sukhodolets, S. Pfeffer, M. Landthaler, P. Landgraf, S. Kan, B.D. Lindenbach, M. Chien, D.B. Weir, J.J. Russo, J. Ju, M.J. Brownstein, R. Sheridan, C. Sander, M. Zavolan, T. Tuschl, C.M. Rice, Cellular cofactors affecting hepatitis C virus infection and replication, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 12884–12889.
[27] Y. Murakami, H.H. Aly, A. Tajima, I. Inoue, K. Shimotohno, Regulation of the hepatitis C virus genome replication by miR-199a, J. Hepatol. 50 (2009) 453–460.
[28] L. Corless, C.M. Crump, S.D. Griffin, M. Harris, Vps4 and the ESCRT-III complex are required for the release of infectious hepatitis C virus particles, J. Gen. Virol. 91 (2010) 362–372.
[29] 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.
[30] 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.

[31] H. Barth, C. Schafer, M.I. Adah, F. Zhang, R.J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T.H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H.E. Blum, T.F. Baumert, Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate, J. Biol. Chem. 278 (2003) 41003–41012.
[32] A. Basu, T. Kanda, A. Beyene, K. Saito, K. Meyer, R. Ray, Sulfated homologues of heparin inhibit hepatitis C virus entry into mammalian cells, J. Virol. 81 (2007) 3933–3941.
[33] M. Fabbri, C.M. Croce, G.A. Calin, microRNAs, Cancer J. 14 (2008) 1–6.
[34] L. He, X. He, S.W. Lowe, G.J. Hannon, microRNAs join the p53 network – another piece in the tumour-suppression puzzle, Nat. Rev. Cancer 7 (2007) 819–822.
[35] A.M. Attallah, G.E. Shiha, H. Ismail, S.E. Mansy, R. El-Sherbiny, I. El-Dosoky, Expression of p53 protein in liver and sera of patients with liver fibrosis, liver cirrhosis or hepatocellular carcinoma associated with chronic HCV infection, Clin. Biochem. 42 (2009) 455–461.
[36] M. Colombo, Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma, J. Hepatol. 31 (Suppl. 1) (1999) 25–30.

Journal of Medical Virology 84:229–234 (2012)

A Point Mutation at Asn-534 That Disrupts a Conserved *N*-Glycosylation Motif of the E2 Glycoprotein of Hepatitis C Virus Markedly Enhances the Sensitivity to Antibody Neutralization

Mikiko Sasayama,[1] Ikuo Shoji,[1] Myrna Adianti,[1,2] Da-Peng Jiang,[1] Lin Deng,[1] Takafumi Saito,[3] Hisayoshi Watanabe,[3] Sumio Kawata,[3] Chie Aoki,[1,4] and Hak Hotta[1,4]*

[1]*Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan*
[2]*Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia*
[3]*Faculty of Medicine, Department of Gastroenterology, Yamagata University, Yamagata, Japan*
[4]*Japan Science and Technology Agency (JST) / Japan InternationalCooperation Agency (JICA), SATREPS, Tokyo, Japan*

The molecular basis of antibody neutralization against hepatitis C virus (HCV) is poorly understood. The E2 glycoprotein of HCV is critically involved in viral infectivity through specific binding to the principal virus receptor component CD81, and is targeted by anti-HCV neutralizing antibodies. A previous study showed that a mutation at position 534 (N534H) within the sixth *N*-glycosylation motif of E2 of the J6/JFH1 strain of HCV genotype 2a (HCV-2a) was responsible for more efficient access of E2 to CD81 so that the mutant virus could infect the target cells more efficiently. The purpose of this study was to analyze the sensitivity of the parental J6/JFH1, its cell culture-adapted variant P-47 possessing 10 amino acid mutations and recombinant viruses with the adaptive mutations to neutralization by anti-HCV antibodies in sera of HCV-infected patients. The J6/JFH1 virus was neutralized by antibodies in sera of patients infected with HCV-2a and -1b, with mean 50% neutralization titers being 1:670 and 1:200, respectively ($P < 0.00001$). On the other hand, the P-47 variant showed 50- to 200-times higher sensitivity to antibody neutralization than the parental J6/JFH1 without genotype specificity. The N534H mutation, and another one at position 416 (T416A) near the first *N*-glycosylation motif to a lesser extent, were shown to be responsible for the enhanced sensitivity to antibody neutralization. The present results suggest that the residues 534, and 416 to a lesser extent, of the E2 glycoprotein are critically involved in the HCV infectivity and antibody neutralization. ***J. Med. Virol. 84:229–234, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: humoral immune mechanism; evasion; glycan

INTRODUCTION

Hepatitis C virus (HCV), a member of the family *Flaviviridae*, the genus *Hepacivirus*, is an enveloped, positive-stranded RNA virus that infects an estimated 170 million people worldwide. The virus evades the

Grant sponsor: Science and Technology Research Partnership for Sustainable Development (SATREPS) Program of Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA); Grant sponsor: Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) Program of Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant sponsor: Research on Hepatitis, Health and Labour Sciences Research Grants of Ministry of Health, Labour and Welfare, Japan; Grant sponsor: Global Center of Excellence (G-COE) Program of Kobe University Graduate School of Medicine.

Mikiko Sasayama present address is Mahidol-Osaka Center for Infectious Diseases, Bangkok, Thailand.

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

Accepted 7 September 2011
DOI 10.1002/jmv.22257
Published online in Wiley Online Library (wileyonlinelibrary.com).

host immune system to establish chronic infection, which often leads to serious liver diseases, such as cirrhosis and hepatocellular carcinoma. Even with a current standard treatment with pegylated interferon *plus* ribavirin, sustained viral clearance is obtained for only approximately 50% of patients infected with HCV genotype 1b (HCV-1b). Neither antibody-based prophylaxis nor an effective vaccine is currently available.

A better understanding of the interplay between viral and host factors that determine HCV clearance or persistence is needed for the design of effective passive immunotherapy and effective vaccines. A growing body of evidence from studies in humans and chimpanzees suggests that HCV-specific T-cell immunity plays an important role in the viral clearance [Bowen and Walker, 2005]. Also, several studies have indicated a role for humoral immunity in HCV infection [Bartosch et al., 2003; Logvinoff et al., 2004; Lavillette et al., 2005; Netski et al., 2005; Pestka et al., 2007; Dowd et al., 2009]. However, this aspect remains poorly characterized.

The E2 glycoprotein of HCV plays an important role in viral attachment and, therefore, becomes a major target of anti-HCV neutralizing antibodies. Identification of protective epitopes in E2 conserved among different HCV strains is a major challenge in vaccine design [Tarr et al., 2006; Helle et al., 2007; Gal-Tanamy et al., 2008; Keck et al., 2008]. The development of infectious retroviral pseudoparticles (HCVpp) bearing HCV envelope glycoproteins helps us study interactions between E2 epitopes and the virus receptor CD81 or neutralizing antibodies [Bartosch et al., 2003; Logvinoff et al., 2004; Lavillette et al., 2005; Pestka et al., 2007; Dowd et al., 2009]. More significantly, authentic HCV particles produced by the HCV cell culture system (HCVcc) are currently available for this purpose [Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005; Fournier et al., 2007].

Recently, it was demonstrated using HCVcc that a mutation at position 534 from Asn to His (N534H) in the E2 glycoprotein of the HCV J6/JFH1 strain confers an advantage to the mutant viruses at the entry level probably through more efficient access to CD81 [Bungyoku et al., 2009]. The Asn-534 is located in the sixth of 11 *N*-linked glycosylation sites and the N534H mutation is predicted to remove this glycosylation. The present study has shown that the N534H mutation in the E2 glycoprotein of HCV J6/JFH1 markedly enhances the sensitivity of the virus to neutralization by specific neutralizing antibodies in sera of patients infected with HCV.

MATERIALS AND METHODS

Cells and Viruses

Huh-7.5 cells [Blight et al., 2002] and pFL-J6/JFH1 [Lindenbach et al., 2005] were kindly provided by Dr. C. M. Rice (Rockefeller University, New York, NY, USA). Huh-7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Invitrogen) at 37°C in a CO_2 incubator. Propagation of HCV J6/JFH1, its cell culture-adapted mutant P-47 and recombinant viruses possessing each of the adaptive mutations was described previously [Deng et al., 2008; Bungyoku et al., 2009].

Human Sera and Anti-HCV Neutralization Test

Sera were collected from 89 patients infected chronically with HCV-1b or HCV-2a, who were treated with pegylated interferon α-2b and ribavirin, as described previously [El-Shamy et al., 2007, 2008]. Sera were also collected from 11 patients with acute HCV-1b infection, either severe acute hepatitis or mild self-resolving hepatitis. The study protocol was approved by the Ethic Committees in Kobe University and Yamagata University and informed written consent provided by patients and volunteers. Sera collected from healthy volunteers who were negative for anti-HCV antibodies served as a control. The sera were inactivated at 56°C for 30 min before being used for the virus neutralization test.

An HCV neutralization test was performed as described previously [Sasayama et al., 2010]. In brief, serially diluted serum samples were mixed with the same amount of HCV solution containing 1×10^4 cell-infecting units. After incubation at 37°C for 1 hr, the mixtures were inoculated to Huh-7.5 cells (2×10^5 cells per well in 24-well plates) and incubated in a 5% CO_2 incubator. After 3 hr, the inocula were removed and fresh complete DMEM were added to the cells. At 24 hr postinfection, cells were fixed with ice-cold methanol, blocked with 5% goat serum in phosphate-buffered saline and subjected to immunofluorescence analysis using mouse monoclonal antibody against HCV core antigen (2H9) [Wakita et al., 2005] and Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR). The immunostained cells were counterstained with Hoechst 33342 (Molecular Probes) at room temperature for 5 min and observed under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The number of HCV-infected cells in each well was counted by using a software BZ-H1C (Keyence). The serum dilutions that neutralized 50% of the virus infectivity was calculated by curvilinear regression analysis [Abe et al., 2003]. Titers were expressed as 50% neutralization titers (NT_{50}).

Statistical Analysis

Student's *t*-test was used to compare the data between different groups. A *P*-value of <0.05 was considered to be significant.

RESULTS

Anti-HCV Neutralizing Antibodies in Sera of Patients Infected With HCV

Sera were obtained from patients chronically infected with HCV-1b or -2a, and tested for anti-HCV neutralizing activities. Representative results of neutralization curves using the parental J6/JFH1 and the P-47 mutant as challenge viruses are shown in Figure 1. When measured against J6/JFH1, NT_{50} titers of sera of patients infected with HCV-1b ranged from 1:10 to 1:700, with the mean NT_{50} titer being 1:197, whereas those of patients infected with HCV-2a ranged from 1:100 to 1:1,500, with the mean value being 1:670 (Table I). The difference in NT_{50} between patients infected with HCV-1b and -2a was statistically significant ($P < 0.00001$). When measured against P-47, on the other hand, unexpectedly high NT_{50} titers were obtained ranging from 1:4,000 to 1:182,000, with the mean values being 1:40,500 and 1:32,900 for patients infected with HCV-1b and -2a, respectively. These results suggest the possibility that an adaptive mutation(s) of P-47, most probably present in the envelope glycoproteins, confers higher sensitivity to neutralization by anti-HCV antibodies.

Unlike the case with J6/JFH1, when P-47 was used as a challenge virus, no significant difference in NT_{50} titers was observed between patients infected with HCV-1b and -2a (Table I). This result suggests the possible presence of a genotype-dominant neutralization epitope(s) on the envelope glycoproteins of J6/JFH1 although anti-HCV neutralizing antibodies in patients' sera are reactive to both HCV-1b and -2a. The broad reactivity of the neutralizing antibodies in patients' sera across different HCV genotypes is consistent with previous observations by other researchers [Logvinoff et al., 2004; Meunier et al., 2005; Fournier et al., 2007; Pestka et al., 2007; Scheel et al., 2008].

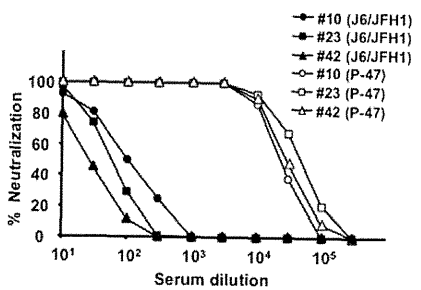

Fig. 1. Neutralization curves (NT_{50} assay) of sera obtained from HCV-infected patients against HCV J6/JFH1 and its adaptive mutant P-47. J6/JFH1 or P-47 was incubated with serial dilutions of HCV-infected patients (nos. 10, 23, and 42; all infected with HCV-1b) and tested for neutralization activities. The neutralization rates at each dilution were plotted. Filled and open symbols indicate data obtained with J6/JFH1 and P-47, respectively.

Sera obtained from patients with acute hepatitis C contained much lower titers of anti-HCV neutralizing antibodies compared to those in sera from chronic hepatitis patients, with the average NT_{50} titers against J6/JFH1 and the adaptive mutant P-47 being 1:15 and 1:126, respectively (Table I). Two patients with severe acute hepatitis C with elevated serum alanine aminotransferase levels of >1,000 IU/ml [Saito et al., 2004; unpublished], possessed relatively high NT_{50} titers against P-47 (1:150 and 1:1,100) compared to the remaining nine patients who experienced mild self-resolving hepatitis (<1:10 to 1:50).

A Single-Point Mutation (N534H or T416A) of the HCV E2 Glycoprotein Increases Sensitivity to Neutralization by Anti-HCV Antibodies

Neutralization of virus infectivity by antibodies usually involves their interaction with viral envelope glycoproteins. It has been reported that the cell culture-adapted mutant P-47 possesses 10 amino acid mutations, including four mutations in E2, compared to the parental J6/JFH1 [Bungyoku et al., 2009]. To examine which mutation(s) in E2 is responsible for the increased sensitivity of P-47 to neutralization by antibodies in patients' sera, recombinant viruses possessing each one of the four mutations in E2 were used (Fig. 2A). The result obtained revealed that a recombinant virus possessing a single-point mutation at position 534 from Asn to His (N534H) and another one possessing four mutations (E2) were as sensitive as P-47 to neutralization by sera of chronic hepatitis patients (Fig. 2B) and the two patients with acute hepatitis C (data not shown). The T416A and T396A mutants were also significantly more sensitive than J6/JFH1, but less sensitive than P-47, N534H, and E2 mutants, to neutralization by antibodies in patients' sera. In this connection, it was recently reported that a JFH1 virus-based T416A mutant showed increased sensitivity to antibody neutralization [Dhillon et al., 2010].

DISCUSSION

The present results revealed that sera of patients infected with HCV-1b possessed cross-genotypic neutralizing antibodies against the J6/JFH1 strain of HCV-2a, albeit with significantly lower titers (ca. one-third) compared to the homotypic neutralization titers observed for patients infected with HCV-2a (Table I). When measured against the adaptive mutant P-47 derived from J6/JFH1, neutralizing antibody titers of the patients sera increased markedly to the level 50- to 200-times higher than that measured against J6/JFH1. Also, the partial genotype-specificity observed with J6/JFH1 was no longer evident when measured against P-47. The marked increase in the sensitivity of P-47 to antibody neutralization was assigned to a mutation at position 534 (N534H), and another one at position 416 (T416A) to a lesser extent, of the E2 glycoprotein (Fig. 2).

TABLE I. NT_{50} Titers in Sera of HCV-Infected Patients With Chronic or Acute Hepatitis C

CH/AH	Genotype	NT_{50} titer[a] measured against J6/JFH1	P-47
CH	HCV-1b (n = 69)	197 ± 164 (1)	40,500 ± 31,800 (206)
CH	HCV-2a (n = 20)	670 ± 652[b] (3.4)	32,900 ± 26,500[c] (167)
AH	HCV-1b (n = 11)	15 ± 28 (0.08) (<10–100)	126 ± 326 (0.6) (<10–1,100)

CH, chronic hepatitis; AH, acute hepatitis.
[a]Mean ± SD. The number in the parenthesis means the ratio when compared to the mean titer that was obtained with sera of HCV-1b-infected CH patients against J6/JFH1.
[b]$P < 0.00001$, compared to the mean titer obtained with sera of HCV-1b-infected patients against J6/JFH1 (Student's *t*-test).
[c]$P = 0.33$, compared to the mean titer obtained with sera of HCV-1b-infected patients against P-47 (Student's *t*-test).

The N534H and T416A mutations are located at the sixth, and in close proximity to the first, respectively, of the conserved 11 *N*-linked glycosylation sites of the HCV E2 glycoprotein [Helle et al., 2007; Bungyoku et al., 2009]. It was recently reported that the positions 416 and 534 are conformationally located in the former and the latter halves of the central domain 1 (DIa and DIb), respectively, of E2 and that the two parts of DI domain interact to form the CD81-binding region [Helle et al., 2010; Krey et al., 2010; Albecka et al., 2011]. This region is, therefore, considered as the possible target for neutralizing antibodies that inhibit E2-CD81 interactions [Helle and Dubuisson, 2008; Law et al., 2008; Owsianka et al., 2008; Perotti et al., 2008].

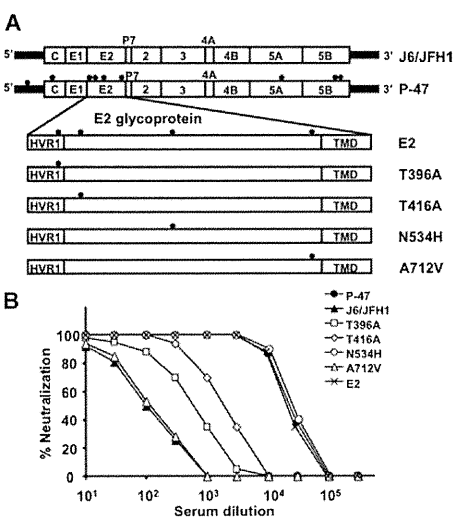

Fig. 2. Effects of amino acid mutations at positions 396, 416, 534, and 712 of the HCV E2 glycoprotein on neutralization by anti-HCV antibodies in patients' sera. **A**: A schematic diagram of the mutations seen in the adaptive mutant P-47 and recombinant viruses carrying each (T396A, T416A, N534H, and A712V) and all (E2) of the four mutations in E2. Filled circles indicate the positions of the mutations. **B**: A representative result of virus neutralization by anti-HCV antibodies in an HCV-infected patient (no. 10; HCV-1b).

The N534H mutation removes glycans at this position as it disrupts the consensus sequence for *N*-linked glycosylation. The removal of glycans at positions 417, 532, and 645 (the first, sixth, and eleventh glycosylation site, respectively) of the H77 isolate (HCV-1a) was shown to increase the sensitivity of HCVpp to neutralizing antibodies and to enhance the access of CD81 to its binding site on E2 [Falkowska et al., 2007; Helle et al., 2007]. It should be noted, however, that the HCVpp system relies on retroviral pseudoparticles bearing HCV envelope glycoproteins that assemble at the plasma membrane or in multivesicular bodies whereas HCV virions assemble on the endoplasmic reticulum membranes that are closely associated with lipid droplet [Miyanari et al., 2007; Helle and Dubuisson, 2008]. Therefore, the virus neutralization data obtained with HCVpp should be verified using the HCVcc system in which virion assembly and maturation take place through the authentic process.

By using the HCVcc system, it was shown that a variant virus possessing the N534K mutation spread faster than the parental JFH1 virus [Delgrange et al., 2007], with the result suggesting the possibility that removal of glycans on residue 534 resulted in more efficient access of E2 to CD81. It is also possible that removal of glycans on this residue might allow more efficient access of neutralizing antibodies to the CD81-binding region of E2, resulting in increased sensitivity to antibody neutralization. In fact, Helle et al. [2010] recently reported that removal of glycans at five (the first, second, fourth, sixth, and eleventh) *N*-linked glycosylation sites in E2 markedly increased the sensitivity of JFH1 virus to antibody neutralization, suggesting that the glycans interfere with the access of neutralizing antibodies to a determinant crucial for virus infectivity. It was also reported that mutations at positions 415 (N415D) and 416 (T416A) near the first glycosylation site of JFH1 virus increased the sensitivity to neutralizing antibodies in patients' sera [Dhillon et al., 2010]. Also, a mutation at position 451 (G451R), which is located in the domain 2 (DII) but still in close proximity to DI [Helle et al., 2010; Krey et al., 2010; Albecka et al., 2011], increased the sensitivity of JFH1 virus to antibody neutralization [Grove et al., 2008].

In conclusion, the present study using J6/JFH1 virus, another HCVcc strain, has demonstrated that the N534H mutation within the sixth *N*-glycosylation site of the E2 glycoprotein, and the T416A mutation near the first *N*-glycosylation site to a lesser extent, markedly enhances sensitivity to neutralization by antibodies in sera of HCV-infected patients. These results suggest that glycans on Asn-534 of the HCV E2 glycoprotein plays an important role in protecting the virus from humoral immune mechanisms of the host.

ACKNOWLEDGMENTS

We are grateful to Dr. C.M. Rice (Center for the Study of Hepatitis C, the Rockefeller University, New York, NY, USA) for providing pFL-J6/JFH1 and Huh-7.5 cells.

REFERENCES

Abe M, Kuzuhara S, Kino Y. 2003. Establishment of an analyzing method for a Japanese encephalitis virus neutralization test in vero cells. Vaccine 21:1989–1994.

Albecka A, Monserret R, Krey T, Tarr AW, Diesis E, Ball JK, Descamps V, Duverlie G, Rey F, Penin F, Dubuisson J. 2011. Identification of new functional regions in hepatitis C virus envelope glycoprotein E2. J Virol JVI accepts, 85:1777–1792.

Bartosch B, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. 2003. In vitro assay for neutralizing antibody to hepatitis C virus: Evidence for broadly conserved neutralization epitopes. Proc Natl Acad Sci USA 100:14199–14204.

Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 76:13001–13014.

Bowen DG, Walker CM. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 436:946–952.

Bungyoku Y, Shoji I, Makine T, Adachi T, Hayashida K, Nagano-Fujii M, Ide YH, Deng L, Hotta H. 2009. Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J Gen Virol 90:1681–1691.

Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouillé Y, Dubuisson J, Wakita T, Duverlie G, Wychowski C. 2007. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J Gen Virol 88:2495–2503.

Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, Shoji I, Hotta H. 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 82:10375–10385.

Dhillon S, Witteveldt J, Gatherer D, Owsianka AM, Zeisel MB, Zahid MN, Rychłowska M, Foung SK, Baumert TF, Angus AG, Patel AH. 2010. Mutations within a conserved region of the hepatitis C virus glycprotein that influence virus-receptor interaction and sensitivity to neutralizing antibodies. J Virol 84: 5494–5507.

Dowd KA, Netski DM, Wang XH, Cox AL, Ray SC. 2009. Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. Gastroenterology 136:2377–2386.

El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol 51:471–482.

El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48:38–47.

Falkowska E, Kajumo F, Garcia E, Reinus J, Dragic T. 2007. Hepatitis C virus envelope glycoprotein E2 glycans modulate entry, CD81 binding, and neutralization. J Virol 81:8072–8079.

Fournier C, Duverlie G, François C, Schnuriger A, Dedeurwaerder S, Brochot E, Capron D, Wychowski C, Thibault V, Castelain S. 2007. A focus reduction neutralization assay for hepatitis C virus neutralizing antibodies. Virol J 4:35.

Gal-Tanamy M, Keck ZY, Yi M, McKeating JA, Patel AH, Foung SK, Lemon SM. 2008. In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. Proc Natl Acad Sci USA 105:19450–19455.

Grove J, Nielsen S, Zhong J, Bassendine MF, Drummer HE, Balfe P, McKeating JA. 2008. Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and sensitivity to neutralizing antibodies. J Virol 82:12020–12029.

Helle F, Dubuisson J. 2008. Hepatitis C virus entry into host cells. Cell Mol Life Sci 65:100–112.

Helle F, Goffard A, Morel V, Duverlie G, McKeating J, Keck ZY, Foung S, Penin F, Dubuisson J, Voisset C. 2007. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. J Virol 81:8101–8111.

Helle F, Vieyres G, Elkrief L, Popescu CI, Wychowski C, Descamps V, Castelain S, Roingeard P, Duverlie G, Dubuisson J. 2010. Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. J Virol 84:11905–11915.

Keck ZY, Olson O, Gal-Tanamy M, Xia J, Patel AH, Dreux M, Cosset FL, Lemon SM, Foung SK. 2008. A point mutation leading to hepatitis C virus escape from neutralization by a monoclonal antibody to a conserved conformational epitope. J Virol 82:6067–6072.

Krey T, d'Alayer J, Kikuti CM, Saulnier A, Damier-Piolle L, Petitpas I, Johansson DX, Tawar RG, Baron B, Robert B, England P, Persson MA, Martin A, Rey FA. 2010. The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary organization of the molecule. PLoS Pathog 6:e1000762.

Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. 2005. 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 79:6023–6034.

Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR. 2008. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. Nat Med 14:25–27.

Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623–626.

Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc Natl Acad Sci USA 101:10149–10154.

Meunier JC, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, Emerson SU, Cosset FL, Purcell RH, Bukh J. 2005. Evidence for cross-genotype neutralization of hepatitis C virus pseudoparticles and enhancement of infectivity by apolipoprotein C1. Proc Natl Acad Sci USA 102:4560–4565.

Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 9:1089–1097.

Netski DM, Mosbruger T, Depla E, Maertens G, Ray SC, Hamilton RG, Roundtree S, Thomas DL, McKeating J, Cox A. 2005. Humoral immune response in acute hepatitis C virus infection. Clin Infect Dis 41:667–675.

Owsianka AM, Tarr AW, Keck ZY, Li TK, Witteveldt J, Adair R, Foung SK, Ball JK, Patel AH. 2008. Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. J Gen Virol 89:653–659.

Perotti M, Mancini N, Diotti RA, Tarr AW, Ball JK, Owsianka A, Adair R, Patel AH, Clementi M, Burioni R. 2008. Identification of a broadly cross-reacting and neutralizing human monoclonal

antibody directed against the hepatitis C virus E2 protein. J Virol 82:1047–1052.

Pestka JM, Zeisel MB, Bläser E, Schürmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc Natl Acad Sci USA 104:6025–6030.

Saito T, Watanabe H, Shao L, Okumoto K, Hattori E, Sanjo M, Misawa K, Suzuki A, Takeda T, Sugahara K, Ito JI, Saito K, Togashi H, Kawata S. 2004. Transmission of hepatitis C virus quasispecies between human adults. Hepatol Res 30:57–62.

Sasayama M, Deng L, Kim SR, Ide Y, Shoji I, Hotta H. 2010. Analysis of neutralizing antibodies against hepatitis C virus in patients who were treated with pegylated-interferon plus ribavirin. Kobe J Med Sci 56:E60–E66.

Scheel TK, Gottwein JM, Jensen TB, Prentoe JC, Hoegh AM, Alter HJ, Eugen-Olsen J, Bukh J. 2008. Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. Proc Natl Acad Sci USA 105:997–1002.

Tarr AW, Owsianka AM, Timms JM, McClure CP, Brown RJ, Hickling TP, Pietschmann T, Bartenschlager R, Patel AH, Ball JK. 2006. Characterization of the hepatitis C virus E2 epitope defined by the broadly neutralizing monoclonal antibody AP33. Hepatology 43:592–601.

Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796.

Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. 2005. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci USA 102:9294–9299.

Original Paper

Intervirology

Intervirology 2012;55:1–11
DOI: 10.1159/000322219

Received: May 12, 2010
Accepted after revision: October 14, 2010
Published online: February 4, 2011

Polymorphisms of Hepatitis C Virus Non-Structural Protein 5A and Core Protein and Clinical Outcome of Pegylated-Interferon/Ribavirin Combination Therapy

Ahmed El-Shamy[a, c] Soo-Ryang Kim[b] Yoshi-Hiro Ide[a] Noriko Sasase[b]
Susumu Imoto[b] Lin Deng[a] Ikuo Shoji[a] Hak Hotta[a]

[a]Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, and [b]Division of Gastroenterology, Kobe Asahi Hospital, Kobe, Japan; [c]Department of Virology, Suez Canal University Faculty of Veterinary Medicine, Ismalia, Egypt

Key Words
Hepatitis C virus · Non-structural protein 5A · Interferon/ribavirin resistance-determining region · Interferon sensitivity-determining region · Core protein · Sustained virological response · Prediction

Abstract
Objective: Hepatitis C virus (HCV genome) polymorphisms are thought to influence the outcome of pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy. This study aimed to examine non-structural protein 5A (NS5A) polymorphisms, e.g. IFN/RBV resistance-determining region (IRRDR) and IFN sensitivity-determining region (ISDR), and core protein polymorphism as predictive therapeutic markers. ***Methods:*** Pretreatment sequences of NS5A and core regions were analyzed in 68 HCV-1b-infected patients treated with PEG-IFN/RBV. ***Results:*** Of 24 patients infected with HCV having an IRRDR with 6 or more mutations (IRRDR≥6), 18 (75%) patients achieved sustained virological response (SVR), whereas only 11 (25%) of 44 patients infected with HCV having IRRDR≤5 did. IRRDR≥6 was significantly associated with SVR ($p < 0.0001$). On the other hand, ISDR≥2 was significantly associated with relapse (either before [breakthrough] or after end-of-treatment response [ETR-relapse]) ($p < 0.05$) and a point mutation of the core protein from Arg to Gln at position 70 (Gln^{70}) was significantly associated with null-response ($p < 0.05$). Multivariate analysis identified IRRDR≥6 as the only viral genetic factor that independently predicted SVR. ***Conclusion:*** NS5A (IRRDR and ISDR) and core protein polymorphisms are associated with the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR≥6 is a useful marker for prediction of SVR.

Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver diseases worldwide [1]. As a consequence of the long-term persistence of chronic hepatitis C, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years [2]. To reduce the impact of this worldwide health problem, efficient treatment is required. Currently, a combination therapy of pegylated-interferon-α and ribavirin (PEG-IFN/RBV) is a

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/12/0551–0001$38.00/0

Accessible online at:
www.karger.com/int

Hak Hotta, MD, PhD
Division of Microbiology, Center for Infectious Diseases
Kobe University Graduate School of Medicine
7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017 (Japan)
Tel. +81 78 382 5500, Fax +81 78 382 5519, E-Mail hotta@kobe-u.ac.jp

standard treatment for chronic hepatitis C [3]. However, this therapy is sometimes difficult to tolerate and results in a sustained virological response (SVR) in only ~50% of patients, especially those infected with the most resistant genotypes, HCV-1a and HCV-1b [3]. Given the considerable side effects, the possibility of discontinuation and the high cost of this treatment, prediction of treatment outcome is needed. An expanded range of predictors may assist clinicians and patients in more accurately assessing the likelihood of an SVR and thus in making more informed treatment decisions [4].

Since the HCV genotype is one of the major factors affecting the IFN-based therapy response, IFN resistance is, at least partly, genetically encoded by HCV itself [5]. In this context, non-structural protein 5A (NS5A) has been widely discussed for its correlation with IFN responsiveness. Enomoto et al. [6] proposed that sequence variations within a region in NS5A spanning from amino acids (aa) 2,209 to 2,248, called the IFN sensitivity-determining region (ISDR), is correlated with IFN responsiveness. Recently, we identified a new region near the C-terminus of NS5A spanning from aa 2,334 to 2,379, which we referred to as the IFN/RBV resistance-determining region (IRRDR) [7]. The degree of sequence variation within IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV combination therapy. On the other hand, prediction of SVR by aa substitutions within the core protein in Japanese patients infected with HCV-1b has also been proposed [8, 9]. In multivariate analysis, the criterion of double-wild core, presence of Arg at position 70 and Leu at position 91 (Arg^{70}/Leu^{91}), was identified as an independent SVR predictor.

This study aimed to examine NS5A polymorphisms, including those in IRRDR and ISDR, and core polymorphism as predictive markers for HCV treatment outcome. The core protein with Arg^{70}/Leu^{91} was defined as wild-core while the other patterns as non-wild-core. The possible correlation of either Arg^{70} alone or Leu^{91} alone with the clinical outcome of PEG-IFN/RBV therapy was also examined.

Patients and Methods

Patients

A total of 68 patients seen at Kobe Asahi Hospital in Kobe, Japan, who were chronically infected with HCV-1b, with diagnoses based on anti-HCV antibody detection and HCV-RNA detection, were enrolled in the study. HCV subtype was determined as according to the method of Okamoto et al. [10]. Patients were treated with PEG-IFNα-2b (Pegintron®; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/kg b.w., once weekly, s.c.) and RBV (Rebetol®; Schering-Plough) (600–800 mg daily, per os), according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. All patients received >80% of scheduled dosage of PEG-IFN and RBV. Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV RNA and core antigen titers as reported previously [11].

The study protocol was approved beforehand by the Ethic Committee in Kobe Asahi Hospital, and written informed consent was obtained from each patient prior to the treatment.

Sequence Analysis of HCV NS5A and Core

HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). Amplification of full-length NS5A and core regions of the HCV genome were performed as described elsewhere [7, 11, 12]. The sequences of the amplified fragments of NS5A and core regions were determined by direct sequencing. The aa sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical Analysis

Statistical differences in the patients' baseline parameters according to the degree of IRRDR polymorphism were determined by Student's t test for numerical variables and Fisher's exact probability test for categorical variables. Likewise, statistical differences in treatment responses according to NS5A and core polymorphisms were determined by Fisher's exact probability test. Kaplan-Meier HCV survival curve analysis was performed based on serum HCV-RNA positivity data during the treatment period (48 weeks) according to NS5A and core polymorphisms. The data obtained were evaluated by the log-rank test. Uni- and multivariate logistic analyses were performed to identify variables that independently predicted the treatment outcome. Variables with a p value of <0.1 in univariate analysis were included in a multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All statistical analyses were performed using SPSS version 16 software (SPSS Inc., Chicago, Ill., USA). Unless otherwise stated, a p value <0.05 was considered as statistically significant.

Nucleotide Sequence Accession Numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB285035 through AB285081, AB354116 through AB354118, and AB518774 through AB518861.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy

Among 68 patients enrolled in this study, HCV-RNA negativity was achieved by 8 (12%) patients at week 4 (rapid virological response [RVR]), 36 (53%) patients at week 12 (early virological response [EVR]), 47 (69%) patients at week 48 (end-of-treatment response [ETR]) and 29 (43%) patients at week 72 (SVR) (table 1). A total of 39 patients (57%) failed to achieve SVR and they were referred to as non-SVR. Non-SVR can be further divided into three categories: (i) null-response, which is defined by continued presence of serum HCV RNA during the entire period of the treatment and follow-up; (ii) breakthrough, defined as transient disappearance of HCV RNA followed by its reappearance before the end of the 48-week treatment, and (iii) ETR-relapse, defined by reappearance of HCV RNA after ETR has been achieved. Seventeen (25%) patients were null-response while 18 (26%) and 4 (6%) patients were ETR-relapse and breakthrough, respectively (table 1).

Table 1. Proportions of various virological responses of patients treated with PEG-IFN/RBV

Virological response	Proportion, patients	
	n/total	%
RVR	8/68	12
EVR	36/68	53
ETR	47/68	69
SVR	29/68	43
Non-SVR	39/68	57
Null-response	17/68	25
ETR-relapse	18/68	26
Breakthrough	4/68	6

PEG-IFN/RBV = Pegylated-interferon/ribavirin; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response; SVR = sustained virological response.

Correlation between NS5A Polymorphism and Treatment Responses

Using a receiver operating characteristic curve analysis, 6 mutations in IRRDR were previously estimated as an optimal cutoff number of mutations for SVR prediction [7]. Initially the correlation between the patients' demographical, hematological, biochemical and virological baseline parameters and the degree of IRRDR polymorphism was examined. This analysis revealed that patient's sex was the only factor that significantly correlated to the degree of IRRDR polymorphism since 49% (17/35) of males were infected with HCV isolates having IRRDRs with 6 mutations or more (IRRDR≥6) compared to 21% (7/33) of females ($p = 0.02$) (table 2). HCV-RNA titers or HCV core antigen titers did not differ significantly between patients infected with HCV isolates of IRRDR≥6 and those of IRRDR≤5.

Next, the possible correlation between IRRDR polymorphism and the ultimate treatment responses was examined. Among 24 patients infected with HCV isolates of IRRDR≥6, 18 (75%), 6 (25%), 3 (12.5%) and 3 (12.5%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3). By contrast, among 44 patients infected with HCV isolates of IRRDR≤5, 11 (25%), 33 (75%), 14 (32%) and 19 (43%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. The proportions of different treatment responses among HCV isolates with IRRDR≥6 and IRRDR≤5 were significantly different. Furthermore, patients infected with HCV isolates with Ala at position 2360 (Ala^{2360}) in IRRDR had a more significant likelihood of SVR than those infected with HCV isolates with non-Ala^{2360}, who tended to be non-SVR, in particular null-response (table 3; fig. 1).

As the IRRDR polymorphism was closely correlated with the ultimate treatment responses, it was also significantly correlated with the on-treatment responses, in particular EVR and ETR (table 4). However, there was no significant correlation between the IRRDR polymorphism and RVR. Also, the presence of Ala^{2360} was correlated significantly with ETR.

Regarding the analysis of ISDR polymorphism and its correlation to the treatment responses, first, the criterion of ISDR with 4 mutations or more (ISDR≥4), the initial criterion of IFN responsiveness proposed by Enomoto et al. [6] was tested. Since the prevalence of ISDR≥4 was only 9% (6/68) of all isolates analyzed, this criterion did not significantly correlate with the treatment responses (data not shown). Next, the correlation between the treatment responses and ISDR mutations at a cutoff point of 2 mutations, a newly proposed ISDR criterion of PEG-IFN/RBV responsiveness [13, 14] was tested. Although there was no significant difference in the proportions of SVR and non-SVR between HCV isolates with ISDR of 2 mutations or more (ISDR≥2) and those of ISDR≤1, a small but significant difference in the proportions of SVR and relapse (ETR-relapse *plus* breakthrough) was observed between ISDR≥2 and ISDR≤1 (table 3). Interestingly, ISDR polymorphism was the only virological factor examined in this study that showed a significant correlation with RVR (table 4). However, this correlation disappeared when further time points of treatment course, such as EVR and ETR, were considered.

Table 2. Correlation between IRRDR polymorphism and patients' demographic characteristics

Factor	IRRDR≥6	IRRDR≤5	p value
Age, mean ± SD	58.71 ± 8.44	59.61 ± 10.30	0.71
Sex, male/female	17/7	18/26	0.02
Body weight, kg	59.87 ± 9.56	58.20 ± 11.92	0.56
Platelets, $\times 10^4/mm^3$	17.22 ± 5.5	14.96 ± 4.71	0.16
Hemoglobin, g/dl	14.25 ± 1.48	13.55 ± 1.77	0.11
γ-GTP, IU/l	49.50 ± 44.29	55.60 ± 65.60	0.69
GPT, IU/l	47.54 ± 33.09	49.33 ± 34.78	0.84
HCV-RNA, KIU/ml	2,070.21 ± 1,720.27	2,038.57 ± 1,963.05	0.95
HCV core antigen, fmol/l	6,750.87 ± 6,859.82	9,320.52 ± 10,636.48	0.30

IRRDR = Interferon/ribavirin resistance-determining region; γ-GTP = γ-guanosine triphosphate; GPT = glutamic pyruvate transaminase.

Table 3. Correlation between NS5A and core protein polymorphisms and ultimate virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total[a]	SVR[b]	Non-SVR	Null-response	Relapse (ETR-relapse *plus* breakthrough)	p value		
							SVR vs. non-SVR	SVR vs. null-response	SVR vs. relapse (ETR-relapse *plus* breakthrough)
NS5A	IRRDR≥6	24	18 (75)[c]	6 (25)	3 (12.5)	3 (12.5)	<0.0001	0.005	0.0006
	IRRDR≤5	44	11 (25)	33 (75)	14 (32)	19 (43)			
	Ala^{2360}	18	12 (67)	6 (33)	1 (5)	5 (28)	0.026	0.016	0.2
	Non-Ala^{2360}	50	17 (34)	33 (66)	16 (32)	17 (34)			
	ISDR≥2	18	10 (56)	8 (44)	6 (33)	2 (11)	0.27	1.0	0.048
	ISDR≤1	50	19 (38)	31 (62)	11 (22)	20 (40)			
Core	Wild-core (Arg^{70}/Leu^{91})	33	18 (55)	15 (45)	5 (15)	10 (30)	0.1	0.07	0.27
	Non-wild-core	35	11 (31)	24 (69)	12 (34)	12 (34)			
	Gln^{70}	21	5 (24)	16 (76)	8 (38)	8 (38)	0.06	0.04	0.19
	Non-Gln^{70}	47	24 (51)	23 (49)	9 (19)	14 (30)			
	Met^{91}	19	7 (37)	12 (63)	5 (26)	7 (37)	0.59	0.74	0.75
	Non-Met^{91}	49	22 (45)	27 (55)	12 (24)	15 (31)			

SVR = Sustained virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70; Met^{91} = methionine at position 91.

[a] Total number of isolates with a given factor.
[b] Number of SVR, non-SVR, null-response or relapse (ETR-relapse *plus* breakthrough) cases with a given factor.
[c] Values in parentheses are percentages.

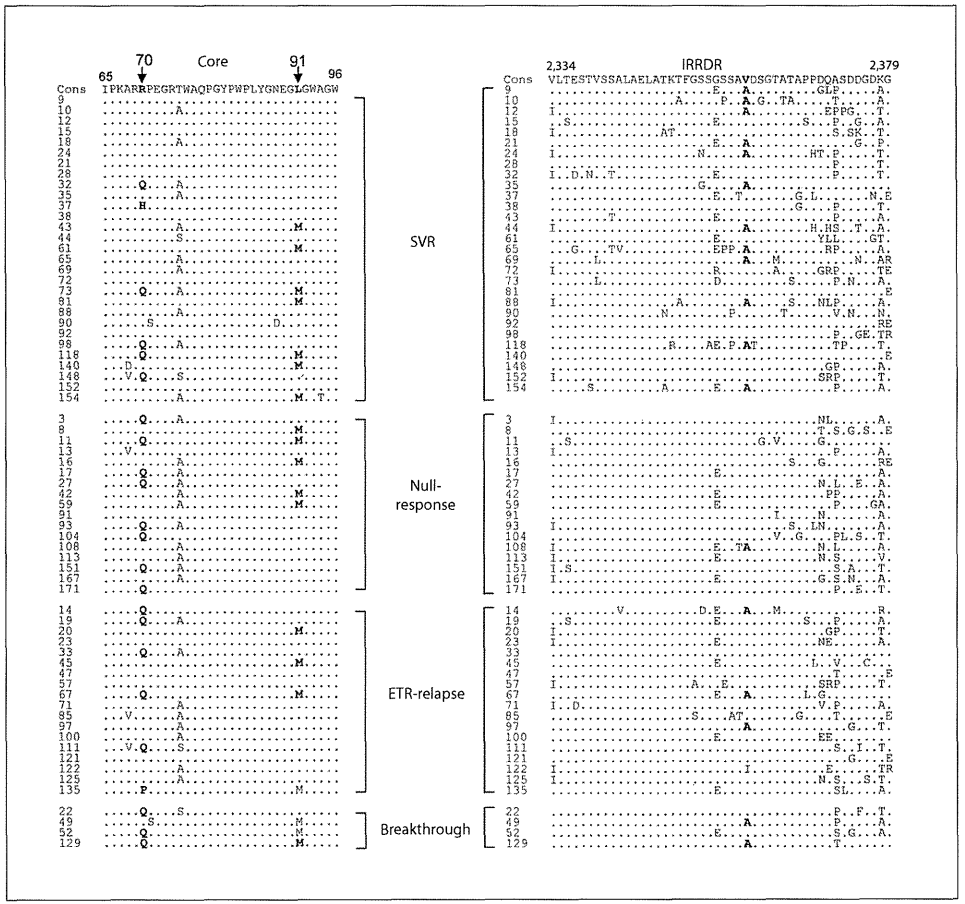

Fig. 1. Sequence alignment of the core protein (aa 65–96) and IRRDR of NS5A obtained from pretreated sera in patients infected with HCV-1b. The consensus (Cons) sequence is shown at the top. Amino acids at positions 70 and 91 of the core protein, and position 2360 of NS5A are shown in boldface.

Correlation between Core Polymorphism and Treatment Responses

Recently, it was reported that polymorphism at positions 70 and/or 91 of the core protein of HCV-1b correlates with and predicts the treatment outcome of Japanese patients treated with PEG-IFN/RBV combination therapy [8, 9]. We aimed to test the consistency of this observation among our patient cohort. The result revealed that among 33 patients infected with HCV isolates of wild-core (Arg^{70}/Leu^{91}), 18 (55%), 15 (45%), 5 (15%) and 10 (30%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3; fig. 1). On the other hand, of 35 patients infected with HCV isolates of non-wild-core, 11 (31%), 24 (69%), 12 (34%) and 12 (34%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. Thus, there was no significant correlation between wild-core and SVR or non-SVR (p = 0.1). However, a single mutation at position 70 (Gln^{70} vs. non-Gln^{70}) was significantly correlated with treatment outcome (SVR vs. null-response; p = 0.04).

As for the on-treatment responses, wild-core (Arg^{70}/Leu^{91}) was significantly correlated with EVR and ETR, whereas Gln^{70} was correlated with non-EVR and non-ETR (table 4).