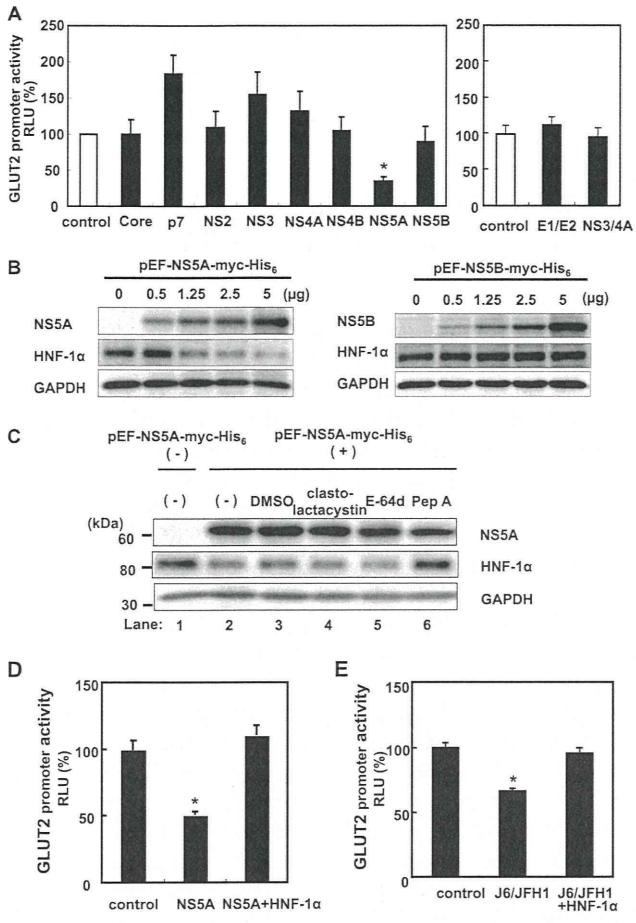

FIG 5 HCV NS5A protein is involved in suppression of GLUT2 promoter activity and lysosomal degradation of HNF-1α protein. (A) Huh-7.5 cells were plated at 1×10^5 cells/12-well plate. After cells were cultured for 12 h, cells were cotransfected with each HCV protein plasmid (0.5 μg), the human GLUT2 promoter reporter plasmid (0.5 μg), and pRL-CMV-*Renilla* (25 ng). pRL-CMV-*Renilla* was used as an internal control. At 48 h posttransfection, cells were harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. (B) Huh-7.5 cells were plated at 4×10^5 cells/six-well plate and cultured for 12 h. Cells were transfected with increasing amounts of either NS5A plasmid or NS5B plasmid as indicated. At 48 h posttransfection, cells were harvested. Whole-cell lysates were analyzed by immunoblotting with anti-HNF-1α, anti-NS5A, and anti-NS5B antibodies. The level of GAPDH served as a loading control. (C) Huh-7.5 cells (2.5×10^5 cells/six-well plate) were transfected with pEF1A-NS5A-myc-His_6. At 2 days posttransfection, proteasome inhibitor (30 μM clasto-lactacystin β-lactone) or lysosomal enzyme inhibitors (40 μM E-64d and 20 μM pepstatin A) were administered to the cells. Cells were cultured for 12 h and harvested, and the levels of endogenous HNF-1α protein were analyzed by immunoblotting with anti-HNF-1α goat PAb. The level of GAPDH served as a loading control. (D) Huh-7.5 cells (1.0×10^5 cells/12-well plate) were transfected with the human GLUT2 promoter reporter plasmid (0.5 μg) and pRL-CMV-*Renilla* (25 ng). The plasmid pEF1A/myc-His (0.5 μg) was cotransfected to the control cells. Cells were transfected with the plasmid pEF1A-NS5A-myc-His (0.5 μg) together with either empty plasmid pCMV4 (10 ng) or pCMV-HNF-1α (10 ng). At 48 h posttransfection, cells were harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. *, $P < 0.05$, compared with control. (E) Huh-7.5 cells (1.2×10^6 cells /10 cm-dish) were infected with HCV J6/JFH1 at a multiplicity of infection of 2 and cultured for 5 days. At day 5 postinfection, cells were plated at 1.0×10^5 cells/12-well plate and cultured for 12 h. Mock-infected cells were plated similarly. Cells were transfected with the human GLUT2 promoter reporter plasmid (0.5 μg) and pRL-CMV-*Renilla* (25 ng) together with either empty plasmid pCMV4 or pCMV-HNF-1α, cultured for 48 h, and harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. *, $P < 0.05$, compared with control.

pression of the GLUT2 promoter, we examined the effect of NS5A protein on the levels of endogenous HNF-1α protein. Huh-7.5 cells were transfected with increasing amounts of either an NS5A expression plasmid or NS5B expression plasmid. At 48 h posttransfection, cells were harvested, and the levels of endogenous HNF-1α protein were analyzed by immunoblot analysis. To detect endogenous HNF-1α protein, highly sensitive Western blotting detection reagents (ECL Plus Western blotting detection reagents) were used. Overexpression of NS5A (Fig. 5B, left panel) but not NS5B (Fig. 5B, right panel) significantly reduced endogenous HNF-1α protein. These results suggest that NS5A protein specifically reduces endogenous HNF-1α protein levels.

To determine if NS5A-dependent reduction of HNF-1α protein is due to lysosomal degradation, we treated the cells with lysosome protease inhibitors. Pepstatin A, but not E-64d, recovered the levels of HNF-1α protein (Fig. 5C, middle panel, lanes 5 and 6), which is consistent with the results found in HCV-infected cells. These results suggest that NS5A is responsible for HCV-induced lysosomal degradation of HNF-1α protein. Taken together, our results suggest that HCV infection suppresses GLUT2 promoter activity via NS5A-dependent lysosomal degradation of HNF-1α protein.

To verify a role of HNF-1α in the HCV-induced suppression of GLUT2 promoter activity, we examined the effects of ectopic expression of HNF-1α on GLUT2 promoter activity in NS5A-transfected cells as well as in HCV J6/JFH1-infected cells. As shown in Fig. 5D, overexpression of NS5A decreased GLUT2 promoter activity, and ectopic expression of HNF-1α restored GLUT2 promoter activity (Fig. 5D). Moreover, HCV J6/JFH1 infection significantly decreased GLUT2 promoter activity, and ectopic expression of HNF-1α restored GLUT2 promoter activity (Fig. 5E). These results are consistent with the notion that HNF-1α protein is a key regulator for HCV-induced suppression of GLUT2 promoter activity.

NS5A protein interacts with HNF-1α protein in Huh-7.5 cells and in FGR Con1 cells. It was previously reported that *in vitro* translated HNF-1 protein was pulled down with glutathione *S*-transferase (GST)-NS5A protein (32). To determine whether NS5A physically interacts with HNF-1α protein in cultured cells, Huh-7.5 cells were cotransfected with each FLAG-tagged NS5A expression plasmid together with the HNF-1α expression plasmid. Immunoprecipitation analysis revealed that HNF-1α protein was coimmunoprecipitated with FLAG-NS5A protein using anti-FLAG MAb (Fig. 6A, third blot, lane 8). No band was detected using control IgG for immunoprecipitation (Fig. 6A, third blot, lane 7). Conversely, immunoprecipitation analysis revealed that NS5A protein was coimmunoprecipitated with HNF-1α protein using anti-HNF-1α rabbit PAb (Fig. 6B, fourth blot, lane 8). Moreover, NS5A protein was coimmunoprecipitated with endogenous HNF-1α protein (Fig. 6B, fourth blot, lane 6), suggesting that NS5A protein indeed interacts with HNF-1α protein.

To confirm that HCV NS5A protein can interact with HNF-1α protein in HCV-replicating cells, we performed immunoprecipitation analysis using FGR Con1 (RCYM1) cells. NS5A protein was coimmunoprecipitated with endogenous HNF-1α protein (Fig. 6C, fourth blot, lane 2). Transfection of HNF-1α protein increased the level of coimmunoprecipitated NS5A protein (Fig. 6C, fourth blot, lane 4), suggesting that HCV NS5A protein indeed interacts with HNF-1α protein in HCV-replicating cells.

HNF-1α binds domain I of NS5A protein. To map the HNF-

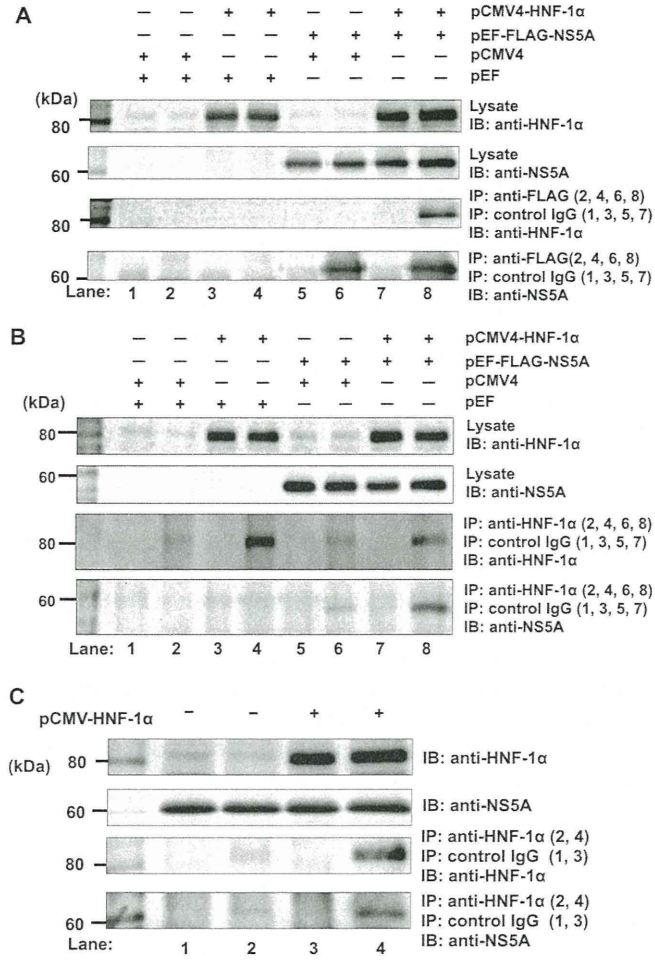

FIG 6 NS5A protein interacts with HNF-1α protein. (A) Huh-7.5 cells were plated at 1.2×10^6 cells/10-cm dish and cultured for 12 h. Cells were transfected with plasmids as indicated. At 48 h after transfection, cells were harvested. Cell lysates were immunoprecipitated with either anti-FLAG mouse MAb (lanes 2, 4, 6, and 8) or control IgG (lanes 1, 3, 5, and 7), and bound proteins were immunoblotted with anti-HNF-1α rabbit PAb (third blot) or anti-NS5A mouse MAb (fourth blot). Protein expression of HNF-1α or FLAG-NS5A was confirmed using the same cell lysates by immunoblotting with either anti-HNF-1α rabbit PAb (first blot) or anti-NS5A mouse MAb (second blot). (B) Cell lysates were immunoprecipitated with either anti-HNF-1α rabbit PAb (lanes 2, 4, 6, and 8) or control IgG (lanes 1, 3, 5, and 7), and bound proteins were immunoblotted with either anti-HNF-1α rabbit PAb (third blot) and anti-NS5A mouse MAb (fourth blot). (C) Full-genome replicon Con1 (RCYM1) cells were plated at 1.2×10^6 cells/10-cm plate and transfected with or without pCMV-HNF-1α plasmid and cultured for 48 h. Cells were harvested and assayed for immunoprecipitation with anti-HNF-1α rabbit PAb (lanes 2 and 4) or control IgG (lanes 1 and 3). Bound proteins were immunoblotted with anti-HNF-1α goat PAb (third blot) or anti-NS5A mouse MAb (fourth blot). Input samples were immunoblotted with either anti-HNF-1α PAb (first blot) or anti-NS5A MAb (second blot). IP, immunoprecipitation; IB, immunoblotting.

1α-binding site on NS5A protein, coimmunoprecipitation analyses were performed. By use of a panel of NS5A deletion mutants (Fig. 7A), FLAG-HNF-1α protein was found to coimmunoprecipitate with all of the HA-NS5A proteins except HA-NS5A consisting of aa 357 to 447 [HA-NS5A(357–447), HA-NS5A(250–447), or HA-NS5A(214–447) (Fig. 7B, lower left panel). These results suggest that domain I of NS5A consisting of aa 1 to 213 is important for HNF-1α binding. FLAG-HNF-1α protein was also found to coimmunoprecipitate with NS5A(1–126)-myc-His_6 and NS5A(1–147)-myc-His_6. These data led to the conclusion that the HNF-1α-binding domain of NS5A protein was aa 1 to 126.

DISCUSSION

In this study, we aimed to clarify molecular mechanisms of HCV-induced suppression of GLUT2 gene expression. The reporter assays of the human GLUT2 promoter suggest that the HNF-1α-binding site is crucial for HCV-induced suppression of GLUT2 promoter activity (Fig. 2). HCV infection significantly reduced the levels of HNF-1α mRNA (Fig. 3A). Moreover, HCV infection remarkably decreased HNF-1α protein levels (Fig. 4A). Our results suggest that HCV infection suppresses GLUT2 gene expression via NS5A-mediated lysosomal degradation of HNF-1α protein (Fig. 5). Immunoprecipitation analyses revealed that NS5A protein physically interacts with HNF-1α protein (Fig. 6) and that domain I of NS5A is important for HNF-1α binding (Fig. 7). Taken together, our results suggest that HCV infection suppresses GLUT2 transcription via downregulation of HNF-1α expression at both transcriptional and translational levels (Fig. 8).

We demonstrated that HNF-1α protein levels were greatly reduced compared to the reduced levels of HNF-1α mRNA. We demonstrated that pepstatin A, but not E64-d, restored the levels of HNF-1α protein, suggesting that an aspartic protease is involved in the degradation of HNF-1α protein. Pepstatin A is widely used for investigation of autophagy and lysosomal degradation. Further studies are needed to elucidate how HCV induces lysosomal degradation of HNF-1α protein and how HNF-1α protein is selectively downregulated by HCV infection. Our data suggest that the HCV NS5A protein is responsible for the HCV-induced degradation of HNF-1α protein. Using a panel of NS5A deletion mutants, we demonstrated that domain I of NS5A is important for association with HNF-1α protein. NS5A domain I is relatively conserved among HCV genotypes compared to domains II and III, suggesting that NS5A–HNF-1α interaction is common to all the HCV genotypes. Domain I coordinates a single zinc atom per protein molecule and is essential for HCV RNA replication (35). The crystal structure of NS5A domain I revealed the presence of a zinc coordination motif and a C-terminal disulfide bond (36). NS5A domain I was found to bind many host proteins, RNA, and membranes (16). It is possible that physical interaction between NS5A protein and HNF-1α protein is important for selective degradation of HNF-1α protein. One possible mechanism is that NS5A protein may recruit HNF-1α protein to the lysosome. Further study is necessary to test this possibility.

We observed that deletion of the GLUT2 transcriptional start site enhances expression of the GLUT2 reporter in FGR cells (Fig. 2B). Cha et al. (7) previously reported that deletion down to nucleotide +73 of the GLUT2 promoter resulted in a marked increase and that further deletion to nucleotide +188 caused a drastic decrease in luciferase activity, indicating the presence of negative- and positive-regulator elements in the 5′ untranslated region. The role of these elements in HCV-infected cells remains to be elucidated.

We demonstrated that HCV J6/JFH1 infection reduced the HNF-1α mRNA level and HNF-1α protein level. Our results contradict an earlier report (32) demonstrating that expression of HNF-1 mRNA was increased in subgenomic replicon Huh.8 cells (3). We observed downregulation of HNF-1α mRNA and

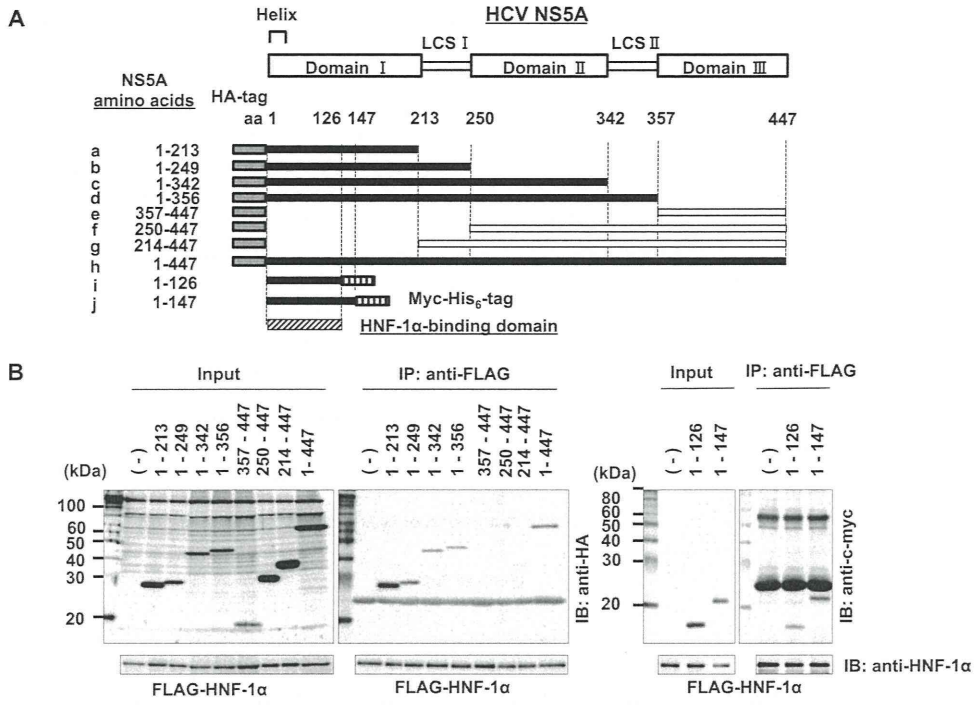

FIG 7 Mapping of the HNF-1α-binding domain for NS5A protein. (A) Schematic representation of the hepatitis C virus NS5A protein. NS5A consists of three domains (domains I, II, and III) with domains separated by low-complexity sequences (LCS I and II). The position of the amino-terminal amphipathic helix membrane anchor is shown (labeled helix). The NS5A deletion mutants (a to j) contain the NS5A amino acids indicated to the left. Each NS5A deletion mutant contains either HA tag in the N terminus (a to h) or myc-His_6 tag in the C terminus (i and j). The gray region of each represents the HA tag sequence. The lattice region of each represents the myc-His_6 tag (i and j). Closed boxes represent proteins that are bound specifically to HNF-1α protein, and open boxes represent those that are not bound. (B) Huh-7.5 cells were transfected with each NS5A mutant plasmid together with a FLAG-HNF-1α expression plasmid. At 48 h posttransfection, cells were harvested, and cell lysates were immunoprecipitated with anti-FLAG beads. Input samples and immunoprecipitated samples were immunoblotted with anti-HA MAb (two left panels, top), anti-c-myc MAb (two right panels, top), or anti-HNF-1α PAb (all panels, bottom).

HNF-1α protein in SGR cells as well as in FGR cells (data not shown). We also demonstrated that the ectopic expression of NS5A protein decreased the endogenous HNF-1α protein level. The reasons for these discrepancies remain to be elucidated.

We along with other groups previously reported that HCV NS5A protein is involved in mitochondrial reactive oxygen species (ROS) production (11, 13, 38). Mitochondrial ROS generation is known to induce the autophagy pathway (22) and lysosomal membrane permeabilization (8). Therefore, it is necessary to determine whether NS5A-induced ROS production enhances autophagic degradation or lysosomal membrane permeabilization. Several groups have reported that autophagy vesicles accumulate in HCV-infected cells and that autophagy proteins can function as proviral factors required for HCV replication (14). Autophagy degrades macromolecules and organelles. Based on the means by which cargo is delivered to the lysosomes, three different autophagy pathways are described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). At first, autophagy was considered a nonselective bulk degradation process. CMA, however, results in specific degradation of the cytosolic proteins in a molecule-by-molecule fashion. Most known substrates for CMA contain a peptide sequence biochemically related to KFERQ (12). Although the typical KFERQ peptide motif is not found in HNF-1α protein, it is possible that KFERQ-like sequences can be generated by post-translational modifications. It is also possible that HNF-1α protein possesses other degradation motifs. The molecular mechanism underlying NS5A-dependent lysosomal degradation of HNF-1α protein needs to be elucidated.

HNF-1α is a homeodomain-containing transcription factor, which is expressed in the liver, pancreatic β cells, and other tissues (1). Intriguingly, HNF-1α is known to play a crucial role in diabetes. Heterozygous germ line mutations in the gene encoding HNF-1α are responsible for an autosomal dominant form of non-insulin-dependent diabetes, MODY3 (40). Mutations in the HNF-1α gene disrupt GLUT2 function as a glucose sensor in pancreatic β cells, resulting in severe insulin secretory defects (39). It is unclear whether HNF-1α mutations in the liver affect glucose homeostasis in MODY3 patients. Two strains of HNF-1α-deficient mice have been reported. The mice of the first strain, created using standard methods for making knockout mice, are born normally, but most die postnatally around the weaning period after a progressive wasting syndrome (31). Mice of the second strain, created using the Cre-loxP recombination method, had a normal life span (20). The knockout mice of the second strain were dwarfed, diabetic, and infertile. Moreover, the knockout mice had enlarged livers and exhibited progressive liver damage.

HNF-1α was also identified as a tumor suppressor gene involved in human liver tumorigenesis since biallelic inactivating mutations of the HNF-1α gene were found in 50% of hepatocellular adenomas and, in rare cases, of well-differentiated hepatocellular carcinomas developed in the absence of cirrhosis (5).

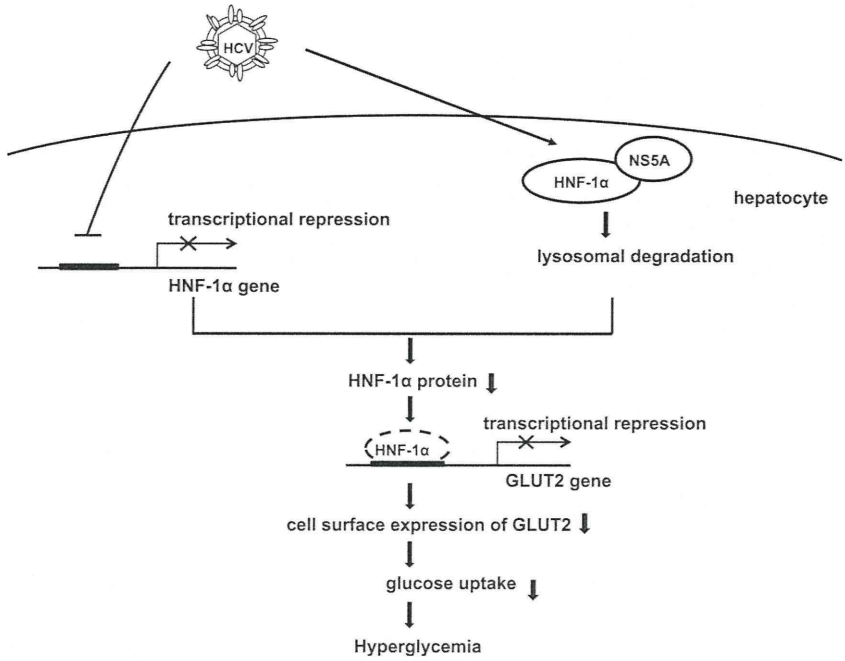

FIG 8 A proposed mechanism of the HCV-induced suppression of GLUT2 via downregulation of HNF-1α. HCV infection downregulates HNF-1α at transcriptional and posttranslational levels, resulting in suppression of GLUT2 gene transcription. HCV NS5A protein physically interacts with HNF-1α protein and enhances lysosomal degradation of HNF-1α protein.

Moreover, HNF-1α has been shown to regulate a large number of genes related to glucose, fatty acid, bile acid, cholesterol, and lipoprotein metabolisms as well as inflammation (1). Therefore, it is possible that HCV-induced downregulation of HNF-1α may play a crucial role in metabolic disorders as well as tumorigenesis.

To determine which HCV protein is involved in the suppression of the GLUT2 promoter, we examined the effects of transient expression of HCV proteins on GLUT2 promoter activity. Overexpression of NS5A suppressed GLUT2 promoter activity, whereas overexpression of p7 enhanced GLUT2 promoter activity (Fig. 5A). SGR cells express NS5A protein but lack p7 protein. FGR cells express both NS5A protein and p7 protein. However, GLUT2 promoter activity was suppressed in both SGR and FGR cells (Fig. 2B). This discrepancy between transient expression system and replicon cells may result from the differences in trafficking of p7 because it is a complex process potentially regulated by both the cleavage from its upstream signal peptides and targeting signals within the protein sequence (15).

We previously reported that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells (11). HCV infection transcriptionally upregulates the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. We demonstrated that gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of the FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with FoxO1-dependent increased gluconeogenesis. HCV-induced downregulation of GLUT2 expression and upregulation of gluconeogenesis may cooperatively contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced downregulation of GLUT2 expression and upregulation of gluconeogenesis may result in high concentrations of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentrations in the hepatocytes inhibit HCV replication (28). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

In conclusion, we provided evidence suggesting that HCV infection downregulates HNF-1α expression at both transcriptional and posttranslational levels. HCV-induced downregulation of HNF-1α may play a crucial role in glucose metabolic disorders caused by HCV infection. Strategies aimed at HCV-induced downregulation of HNF-1α protein may lead to the development of new therapeutic agents for HCV-induced diabetes.

ACKNOWLEDGMENTS

We are grateful to C. M. Rice (Rockefeller University, New York, NY) for providing Huh-7.5 cells and pFL-J6/JFH1, R. Bartenschlager (University of Heidelberg, Heidelberg, Germany) for providing an HCV subgenomic RNA replicon (pFK5B/2884Gly), and N. Kato (Okayama University, Okayama, Japan) for providing an HCV full-genome RNA replicon (pON/C-5B). We thank T. Adachi, M. Makimoto, K. Tsubaki, Y. Yasui, A. Asahi, M. Kohmoto, and Y.-H. Ide for their technical assistance. We also thank K. Hachida for secretarial work.

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 Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan. This work was also supported in part by the Japan Initiative for Global Research Network on Infectious Diseases program of MEXT, Japan. This study was also carried out as part of the Global Center of Excellence program of the Kobe University Graduate School of Medicine and the Science and Technology Research Partnership for Sustain-

able Development program of the Japan Science and Technology Agency and the Japan International Cooperation Agency.

We have no potential conflicts of interest to report.

REFERENCES

1. **Armendariz AD, Krauss RM.** 2009. Hepatic nuclear factor 1-alpha: inflammation, genetics, and atherosclerosis. Curr. Opin. Lipidol. **20**:106–111.
2. **Ban N, et al.** 2002. Hepatocyte nuclear factor-1α recruits the transcriptional co-activator p300 on the GLUT2 gene promoter. Diabetes **51**:1409–1418.
3. **Blight KJ, Kolykhalov AA, Rice CM.** 2000. Efficient initiation of HCV RNA replication in cell culture. Science **290**:1972–1974.
4. **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.
5. **Bluteau O, et al.** 2002. Bi-allelic inactivation of TCF1 in hepatic adenomas. Nat. Genet. **32**:312–315.
6. **Bungyoku Y, et al.** 2009. Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J. Gen. Virol. **90**:1681–1691.
7. **Cha JY, Kim H, Kim KS, Hur MW, Ahn Y.** 2000. Identification of transacting factors responsible for the tissue-specific expression of human glucose transporter type 2 isoform gene. Cooperative role of hepatocyte nuclear factors 1α and 3β. J. Biol. Chem. **275**:18358–18365.
8. **Denamur S, et al.** 2011. Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic. Free Radic. Biol. Med. **51**:1656–1665.
9. **Deng L, et al.** 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J. Virol. **82**:10375–10385.
10. **Deng L, et al.** 2006. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. J. Gen. Virol. **87**:1703–1713.
11. **Deng L, et al.** 2011. Hepatitis C virus infection promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway. J. Virol. **85**:8556–8568.
12. **Dice JF.** 2007. Chaperone-mediated autophagy. Autophagy **3**:295–299.
13. **Dionisio N, et al.** 2009. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. J. Hepatol. **50**:872–882.
14. **Dreux M, Chisari FV.** 2011. Impact of the autophagy machinery on hepatitis C virus infection. Viruses **3**:1342–1357.
15. **Griffin S, Clarke D, McCormick C, Rowlands D, Harris M.** 2005. Signal peptide cleavage and internal targeting signals direct the hepatitis C virus p7 protein to distinct intracellular membranes. J. Virol. **79**:15525–15536.
16. **He Y, Staschke KA, Tan SL.** 2006. HCV NS5A: a multifunctional regulator of cellular pathways and virus replication. *In* Tan SL (ed), Hepatitis C viruses: genomes and molecular biology. Horizon Bioscience, Norfolk, United Kingdom. http://www.ncbi.nlm.nih.gov/books/NBK1621/.
17. **Ikeda M, et al.** 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem. Biophys. Res. Commun. **329**:1350–1359.
18. **Inubushi S, et al.** 2008. Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein tyrosine kinase Syk. J. Gen. Virol. **89**:1231–1242.
19. **Kasai D, et al.** 2009. HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters. J. Hepatol. **50**:883–894.
20. **Lee YH, Sauer B, Gonzalez FJ.** 1998. Laron dwarfism and non-insulin-dependent diabetes mellitus in the Hnf-1α knockout mouse. Mol. Cell Biol. **18**:3059–3068.
21. **Lemon SM, Walker C, Alter MJ, Yi M.** 2007. Hepatitis C virus, p 1291–1304. *In* Knipe DM, et al (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
22. **Li ZY, Yang Y, Ming M, Liu B.** 2011. Mitochondrial ROS generation for regulation of autophagic pathways in cancer. Biochem. Biophys. Res. Commun. **414**:5–8.
23. **Lindenbach BD, et al.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
24. **Macheda ML, Rogers S, Best JD.** 2005. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J. Cell Physiol. **202**: 654–662.
25. **Malecki MT, Mlynarski W.** 2008. Monogenic diabetes: implications for therapy of rare types of disease. Diabetes Obes. Metab. **10**:607–616.
26. **Mason AL, et al.** 1999. Association of diabetes mellitus and chronic hepatitis C virus infection. Hepatology **29**:328–333.
27. **Murakami K, et al.** 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology **351**:381–392.
28. **Nakashima K, Takeuchi K, Chihara K, Hotta H, Sada K.** 2011. Inhibition of hepatitis C virus replication through adenosine monophosphate-activated protein kinase-dependent and -independent pathways. Microbiol. Immunol. **55**:774–782.
29. **Negro F.** 2011. Mechanisms of hepatitis C virus-related insulin resistance. Clin. Res. Hepatol Gastroenterol. **35**:358–363.
30. **Negro F, Alaei M.** 2009. Hepatitis C virus and type 2 diabetes. World J. Gastroenterol. **15**:1537–1547.
31. **Pontoglio M, et al.** 1996. Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. Cell **84**:575–585.
32. **Qadri I, et al.** 2004. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. Biochem. J. **378**:919–928.
33. **Shirakura M, et al.** 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. **81**:1174–1185.
34. **Takeda J, Kayano T, Fukomoto H, Bell GI.** 1993. Organization of the human GLUT2 (pancreatic beta-cell and hepatocyte) glucose transporter gene. Diabetes **42**:773–777.
35. **Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM.** 2004. The NS5A protein of hepatitis C virus is a zinc metalloprotein. J. Biol. Chem. **279**:48576–48587.
36. **Tellinghuisen TL, Marcotrigiano J, Rice CM.** 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature **435**:374–379.
37. **Wakita T, et al.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
38. **Wang AG, et al.** 2009. Non-structural 5A protein of hepatitis C virus induces a range of liver pathology in transgenic mice. J. Pathol. **219**:253–262.
39. **Wang H, Maechler P, Hagenfeldt KA, Wollheim CB.** 1998. Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line. EMBO J. **17**:6701–6713.
40. **Yamagata K, et al.** 1996. Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). Nature **384**:455–458.

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HCV NS5A Protein Containing Potential Ligands for Both Src Homology 2 and 3 Domains Enhances Autophosphorylation of Src Family Kinase Fyn in B Cells

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Abstract

Hepatitis C virus (HCV) infects B lymphocytes and induces mixed cryoglobulinemia and B cell non-Hodgkin's lymphoma. The molecular mechanism for the pathogenesis of HCV infection-mediated B cell disorders remains obscure. To identify the possible role for HCV nonstructural 5A (NS5A) protein in B cells, we generated the stable B cell lines expressing Myc-His tagged NS5A. Immunoprecipitation study in the presence or absence of pervanadate (PV) implied that NS5A was tyrosine phosphorylated by pervanadate (PV) treatment of the cells. Therefore we examined pull-down assay by using glutathione S-transferase (GST)-fusion proteins of various Src homology 2 (SH2) domains, which associates with phosphotyrosine within a specific amino acid sequence. The results showed that NS5A specifically bound to SH2 domain of Fyn from PV-treated B cells in addition to Src homology 3 (SH3) domain. Substitution of Arg^{176} to Lys in the SH2 domain of Fyn abrogated this interaction. Deletion mutational analysis demonstrated that N-terminal region of NS5A was not required for the interaction with the SH2 domain of Fyn. Tyr^{334} was identified as a tyrosine phosphorylation site in NS5A. Far-western analysis revealed that SH2 domain of Fyn directly bound to NS5A. Fyn and NS5A were colocalized in the lipid raft. These results suggest that NS5A directly binds to the SH2 domain of Fyn in a tyrosine phosphorylation-dependent manner. Lastly, we showed that the expression of NS5A in B cells increased phosphorylation of activation loop tyrosine in the kinase domain of Fyn. NS5A containing ligand for both SH2 and SH3 domains enhances an aberrant autophosphorylation and kinase activity of Fyn in B cells.

Citation: Nakashima K, Takeuchi K, Chihara K, Horiguchi T, Sun X, et al. (2012) HCV NS5A Protein Containing Potential Ligands for Both Src Homology 2 and 3 Domains Enhances Autophosphorylation of Src Family Kinase Fyn in B Cells. PLoS ONE 7(10): e46634. doi:10.1371/journal.pone.0046634

Editor: Philippe Gallay, Scripps Research Institute, United States of America

Received April 12, 2012; **Accepted** September 2, 2012; **Published** October 16, 2012

Funding: This study was supported in part by research funding from the University of Fukui, Takeda Science Foundation, Yakult Foundation, and the Grant-in-Aids from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports and Technology, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

HCV is a small enveloped positive-sense RNA virus classified within the family *Flaviviridae* [1,2]. In addition to liver cells, HCV infects B cells, leading to mixed cryoglobulinemia and B cell non-Hodgkin's lymphoma [3–5]. HCV infection in B cells enhances the expression of lymphomagenesis-related genes, such as activation-induced cytidine deaminase (AID) [6,7]. However, the molecular mechanisms of HCV infection-mediated B cell disorders remain elusive.

Non-receptor type of protein-tyrosine kinase Fyn is a member of the Src family kinases, and has regulatory roles in immune receptor signaling. Recently, Fyn has been recognized as an important mediator of mitogenic signaling and regulator of cell cycle entry, growth and proliferation. As for pathological aspects, Fyn is overexpressed in various cancers, and overexpression of Fyn in cultured cells resulted in cancer-like phenotypes [8].

The Src family kinases all share a common structure and pattern of activation. The domains of these proteins include SH2, SH3, and kinase domains followed by a short C-terminal regulatory tail. The SH2 and SH3 domains are highly conserved regions and mediate protein-protein interactions: the SH2 domain binds to phosphotyrosine residue within the specific amino acid sequence, while the SH3 domain recognizes proline rich regions. HCV NS5A was shown to interact with various SH3 domains of intracellular signaling molecules, and the kinase activity of Fyn was upregulated in liver cell lines harboring HCV replicon [9]. Binding of ligands to both the SH2 and SH3 domains disrupts autoinhibitory intramolecular interactions and leads to the opened conformation. Then autophosphorylation of the activation loop tyrosine (Tyr^{420} in Fyn) and dephosphorylation of the C-terminal tail (Tyr^{531} in Fyn) by protein-tyrosine phosphatases lead to the activation of kinase activity [10].

Previously, we reported that Syk, another non-receptor type of protein-tyrosine kinase interacts with transiently expressed NS5A in PV treated BJAB B cells [11]. This suggested that protein-tyrosine phosphorylation is required for the association of NS5A with Syk, because PV is a nonspecific inhibitor of protein-tyrosine phosphatases and treatment of cells with PV causes increase in

protein-tyrosine phosphorylation in whole cells. Recently Pfannkuche *et al.* reported that NS5A binds to the SH2 domain of Src [12]. However, molecular mechanism of their interaction and effect of NS5A on the kinase activity of Src remain unclear.

In this study, we investigated the interaction between NS5A and the SH2 domain of Fyn in B cells.

Materials and Methods

Antibodies and cDNAs

Anti-NS5A and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAbs were purchased from Millipore (Bedford, MA, USA). Anti-Myc mAb and anti-Fyn antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine (pTyr) (PY20) and human anti-IgM mAbs were from Zymed (South San Francisco, CA, USA). Anti-GST mAb was from Nacalai (Kyoto, Japan). Anti-phospho-Src family (Tyr416) antibody, which detects phosphorylated amount of Tyr^{420} in Fyn, was from Cell Signaling Technology (Danvers, MA, USA). The pEF1A-NS5A(Con1)-Myc-His plasmid and its deletion or substitution mutants were described previously [11]. Deletion of NS5A 127–146 (NS5A Δ127–146) was generated by the PCR-based method using four primers, 5′-TTGGTACCATGTCCGGCTCGTGGCTAAGAG-3′, 5′-GCTCTAGAGCAGCAGACGACGTCCTCA-3′, 5′-GGTTACGCGGGTGGGGGATCCCGAATTCTTCACAGAAGTG-3′, and 5′-CACTTCTGTGAAGAATTCGGGATCCCCCACCCGCGTAACC-3′, using NS5A cDNA as a template. Substitution of Tyr^{129} to Phe (Y129F) of NS5A 1–146 was generated by the site-directed mutagenesis using two primers, 5′-GGGGATTTCCACTTCGTGACGGGCA-3′ and 5′-TGCCCGTCACGAAGTGGAAATCCCC-3′, using NS5A 1–146 cDNA as a template. Substitutions of Tyr^{182} to Phe (Y182F), Tyr^{321} to Phe (Y321F), and Tyr^{334} to Phe (Y334F) of NS5A 147–447 were generated by the site-directed mutagenesis using two specific primers designed by QuikChange Primer Design Program (www.genomics.agilent.com), using NS5A 147–447 as a template. The resulted mutations were confirmed by the DNA sequencing.

Cell culture and transfection

B-lymphoid leukemia BJAB cells were kindly provided from Dr. Satoshi Ishido (RIKEN, Yokohama, Japan) [13] and maintained as described previously [14]. For the stable transfection of BJAB cells, 6 μg of linearized pEF1A-NS5A(Con1)-Myc-His was transfected into 5×10^6 cells/500 μl of cells by electroporation (240 V, 950 μF). Stably transfected cell lines were selected with 0.4 mg/ml of active G418 (Wako, Osaka, Japan) [15]. Cell lines were screened by level of protein expression by immunoblotting of detergent soluble lysates with anti-NS5A and anti-GAPDH mAbs as an internal control. Two positive cloned lines were selected for further analysis. For control cells, linearized empty vector was transfected by electroporation, and pooled clones resistant to 0.4 mg/ml of active G418 were utilized as control cells. COS cells were obtained from American Type Culture Collection (Manassas, VA, USA) and Ramos-T cells were kindly provided from Dr. Hamid Band (Nebraska Medical Center, NE, USA) [16]. Transient transfection of COS cells and Ramos-T cells were described previously [17]. Huh-7.5 cells were kindly provided from Dr. Charles M. Rice (The Rockefeller University, NY, USA) [18] and stably harboring an HCV replicon (pFK5B/2884 Gly) were described previously [11].

Cell activation, immunoprecipitation and immunoblotting

BJAB cells (10^8) were washed twice with serum free medium and treated with 100 μM PV or 10 μg/ml of anti-IgM mAb for 3 min at 37°C in the same medium. Either unstimulated or stimulated cells were washed twice with ice-cold PBS and then solubilized in the lysis buffer (1% Triton X-100, 50 mM Tris, pH7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin) on ice. In some experiments, 0.5% Nonidet P-40 was used instead of 1% Triton. Precleared cell lysates were incubated with the indicated antibodies prebound to protein A-agarose beads (Sigma, St. Louis, MO, USA). After rotation for 90 min at 4°C, the beads were washed 4 times with the lysis buffer, and the immunoprecipitated proteins were eluted by the heat treatment for 5 min at 100°C with 2×sampling buffer. Precipitated proteins or cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking in 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), the blots were incubated with the primary antibodies and then horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), or horseradish peroxidase-conjugated protein G (Sigma) in TBST. To enhance the signals, Immuno-enhancer Reagent A (Wako) was utilized in the reaction with anti-pTyr (pY20) mAb. Finally, proteins were visualized by the enhanced chemiluminescence (ECL) reagent (Western Lightning, PerkinElmer Life Sciences, Boston, MA) [19]. Immunoblot quantification was performed using the program Scion Image (Scion, Frederick, MD, USA).

Pull-down assay

The cDNA for Fyn-SH2 (Trp^{149}-Ala^{257}) and –SH3 (Thr^{82}-Glu^{148}) were amplified by PCR using paired primers 5′-GGAATTCATGGTACTTTGGAAAACTTGGC-3′ and 5′-CCGCTCGAGATCTTTAGCCAATCCAGAAGT-3′ for –SH2, 5′-GGAATTCAACAGGAGTGACACTGTTTGTG-3′ and 5′-CCGCTCGAGCTCTTCTGCCTGGATGGAGTC-3′ for –SH3, using mouse Fyn(T) cDNA (a gift from Dr. Yasuhiro Minami, Kobe University, Kobe, Japan) as a template. The cDNA for c-Abl-SH2 (Typ^{146}-His^{221}) and -SH3 (Leu^{84}-Val^{138}) were amplified by PCR using paired primers 5′-CGGAATTCCTGGTATCATGGCCCTGTATCT-3′ and 5′-ATAGTTTAGCGGCCGCTAGCTGGGTAGTGGAGTGTGGT-3′ for -SH2, 5′-CGGAATTCCCTTTTTGTGGCACTCTATGAT-3′ and 5′-TAGTTTAGCGGCCGCTGACGGGGGTGATGTAGTTGCT-3′ for -SH3, using mouse c-Abl cDNA (a gift from Dr. David Baltimore, California Institute of Technology, CA, USA) as a template. The cDNA for Cbl-b N-terminal region containing SH2 domain (Ala^2-Pro^{349}) was amplified by PCR using 5′-CGGAATTCCGCAAACTCAATGAATGGCAGA-3′ and 5′-CCGCTCGAGCTAAGGTGTAGGTTCACATAATCC-3′, using human Cbl-b cDNA (a gift form Dr. Stanley Lipkowitz, National Naval Cancer Center, MD, USA) as a template. Resulted PCR fragments were subcloned into pGEX-4T.3 (GE Healthcare, Piscataway, NJ, USA) to make domain in-frame with the downstream of GST and verified by DNA sequencing. The GST-rat Lyn-SH2 and Syk-SH2 (N+C) expression constructs were provided by Dr. Reuben P. Siraganian (National Institutes of Health, MD, USA). Preparation of GST-rat Vav1-SH2, mouse c-Abl SH3 domain-binding protein-2 (3BP2)-SH2, human phospholipase C (PLC)-γ2-SH2 (N+C), and rat Lyn-SH3 domain expression constructs were described elsewhere [17,20,21]. Substitution of Arg^{176} to Lys (R176K) by a point mutation of pGEX-

4T.3-Fyn-SH2 was generated by the site-directed mutagenesis using two primers 5′-TCAAAGAGAGCCAAACCACCAAAGG-3′ and 5′-TAAGAAAGGTACCTCTTGGGTTTCC-3′, using Fyn-SH2 cDNA wild type as a template. The resulted point mutation was confirmed by the DNA sequencing. All these SH2 and SH3 domains were fused downstream of GST. The GST fusion proteins were affinity-purified with glutathione Sepharose 4B beads (GE Healthcare). Extraction of GST-fusion proteins from bacteria was confirmed by the SDS-PAGE and Coomassie brilliant blue staining [22].

BJAB cells (10^8), Huh-7.5 cells stably harboring an HCV replicon (3×10^6), COS cells (10^6) or Ramos B cells expressing SV40 T antigen (Ramos-T cells) (10^7) were washed twice with serum free medium and stimulated with 100 μM PV for 3 min at 37°C. Either unstimulated or stimulated cells were solubilized in the binding buffer (1% NP-40, 50 mM Tris, pH7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin). After centrifugation, the resulted supernatants were reacted with 20 μg of GST-fusion proteins prebound to glutathione Sepharose 4B beads for 90 min at 4°C. The beads were washed 4 times with the binding buffer. Proteins interacting with GST-fusion proteins were eluted by heat treatment for 5 min at 100°C with 2×sampling buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Far-western

Anti-NS5A immunoprecipitates from BJAB cells (3×10^7) were separated by SDS-PAGE and transferred to PVDF membrane. After blocking, the membranes were incubated with 2.5 μg/ml of GST or GST-Fyn-SH2 for 1 h at 4°C. After extensive washing, membranes were reacted with anti-GST mAb, subsequently reacted with horseradish peroxidase conjugated goat anti-mouse IgG antibody, and then subjected to ECL detection [17].

Subcellular fractionation

The low density detergent-insoluble fractions were prepared by sucrose density gradient centrifugation as described [23]. BJAB cells (10^8) were solubilized in 2.5 ml of 0.05% Triton in MNEV buffer (150 mM NaCl, 25 mM Mes, pH 6.5, 5 mM EDTA, 1 mM Na_3VO_4, and protease inhibitors) and dounced 10 times. Homogenates were cleared of intact cells by centrifugation for 10 minutes at 200 *g*. The resultant supernatants (2.4 ml) were mixed with equal volumes of 80% sucrose in MNEV buffer (final, 40% sucrose and 0.025% Triton), overlayered by 4.8 ml 30% and 2.4 ml 5% sucrose in MNEV buffer, and then centrifuged for 20 hours at 200 000 *g* (P40ST rotor, Himac CP80WX, Hitachi, Tokyo, Japan). After sucrose density gradient centrifugation, 9 fractions were collected from the top of the gradient and analyzed by the immunoblotting.

Statistical analysis

Quantification of Fyn was analyzed by ImageJ software. The two-tailed Student t-test was applied to evaluate the statistical significance of differences found. A P value of <0.05 was considered statistically significant.

In vitro kinase assay

Unstimulated BJAB cells were washed twice with ice-cold PBS and then solubilized in the lysis buffer. Precleared cell lysates were incubated with anti-Fyn antibody prebound to protein A-agarose beads. After rotation for 90 min at 4°C, the beads were washed 4 times with the lysis buffer, 2 times with the kinase buffer without ATP, then incubated with 20 μl of the kinase buffer (40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 4 μM ATP, 4 μCi [γ-^{32}P] ATP) and 2.5 μg of acid-treated enolase (Sigma) for 30 min at room temperature. Reaction was terminated and proteins were eluted by the heat treatment for 5 min at 100°C with 2×sampling buffer. Proteins were separated by SDS-PAGE and gel was incubated with 1N KOH for 1 h at 56°C to remove phosphoserine and most of phosphothreonine. After fixation, the gel was dried and radiolabeled proteins were visualized by autoradiography. Immunoprecipitation of Fyn was confirmed by the immunoblotting.

Results

HCV NS5A associates with the SH2 domain of Fyn

To identify HCV NS5A-interacting protein in B cells, we generated the stable B cell lines in which Myc-His tagged NS5A protein is constitutively expressed. Since we confirmed that the parental cells did not express NS5A, we choose the clones in which the level of NS5A expression was highest (Fig. 1A). In the following experiments, two cloned lines (clone 3 and 7) were examined, although some figures present the results from only one representative cell line. For control, vector plasmid was transfected into the same parental cells and G418-resistant clones were pooled and utilized as control cells.

Next, we performed immunoprecipitation study using anti-Myc mAb and found tyrosine phosphorylated proteins (Fig. 1B). This suggests that NS5A was tyrosine phosphorylated by PV treatment or another protein with similar size that associates with NS5A (Fig. 1B). Another experiment by affinity tag purification using Nickel column which could react with His-tag (His-Accept kit, Nacalai) also showed some tyrosine phosphorylated proteins in NS5A protein complex (data not shown). These findings suggest the possible involvement of protein-tyrosine phosphorylation associating with NS5A. Therefore, we tried to identify the protein which associates with NS5A through SH2 domain, which recognizes specific phosphotyrosine-containing amino acid sequence.

Then we carried out pull-down assay using GST-fusion proteins of various SH2 domains (Fig. 1C). Among these, the SH2 domain of Fyn dramatically bound to NS5A from PV-treated B cells. The SH2 domains of PLC-γ2 weakly bound to NS5A. The SH2 domains of Lyn, Abl, Vav, 3BP2, Syk or Cbl-b interacted with NS5A at very low level (long exposure, data not shown). GST-Lyn-SH3 was utilized as positive control because it was reported to interact with NS5A [9]. Therefore, this data demonstrated that HCV NS5A selectively binds to the SH2 domains of Fyn and PLC-γ2 in B cells. GST-human Fyn-SH2 also interacted with NS5A (Fig. S1). Moreover, the NS5A interaction with GST-Fyn-SH2 was observed even in the context of HCV RNA replication (Fig. 1D). Thus, HCV NS5A selectively associates with the SH2 domain of Fyn.

NS5A binds to the SH2 domain of Fyn in a tyrosine phosphorylation-dependent manner

PV treatment of cells dramatically enhances the binding of NS5A to the SH2 domain of Fyn, but not with that of Lyn or Abl (Fig. 2A). Substitution of Arg^{176} to Lys in the SH2 domain of Fyn, which caused the loss of association with phosphotyrosine residue, abrogated the binding of the SH2 domain of Fyn to NS5A (Fig. 2B). Arg^{176} is located in the consensus sequence within the SH2 domains to interact with phosphotyrosine residue. On the other hand, the SH3 domain of these kinases associated with NS5A to a comparable level (Fig. 2C). These results suggest that

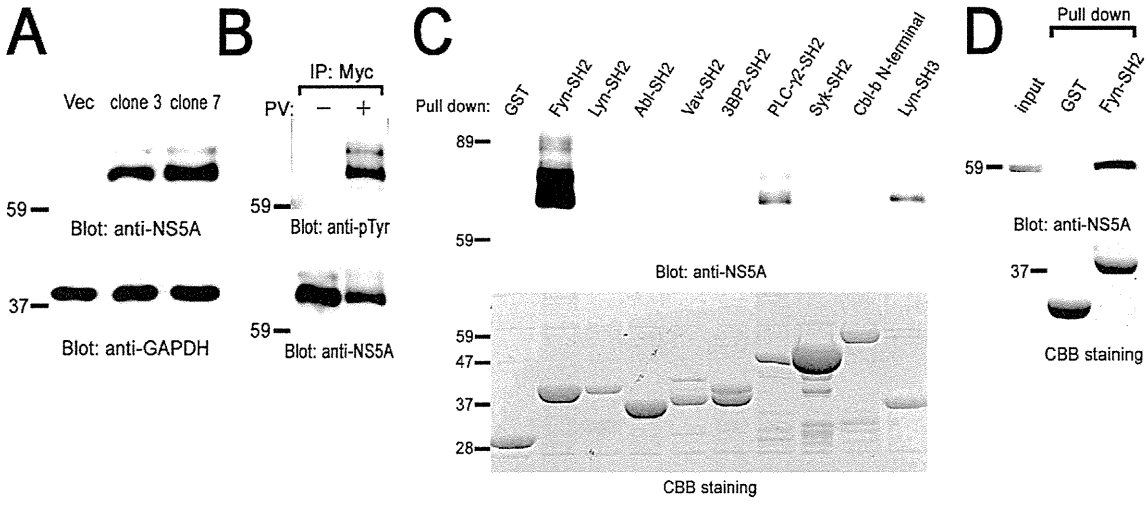

Figure 1. Identification of HCV NS5A-interacting proteins in B cells. (A) Generation of stable B cell lines expressing HCV NS5A. Detergent soluble cell lysates from vector cells (Vec) and Myc-His-NS5A expressing clones (clones 3 and 7) were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A and anti-GAPDH mAbs. (B) BJAB cells expressing Myc-His-NS5A (clone 7) were treated without (−) or with (+) PV and solubilized in the lysis buffer. Cell lysates were immunoprecipitated with anti-Myc mAb and immunoprecipitated proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-pTyr (PY20) and anti-NS5A mAbs. PV-treated cells expressing Myc-His-NS5A (clone 7) (C) or Huh-7.5 cells stably harboring an HCV subgenomic replicon (D) were solubilized in the binding buffer. Precleared lysates were reacted with the indicated GST-fusion proteins and binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A mAb. The amount of GST-fusion proteins was confirmed by Coomassie brilliant blue (CBB) staining (C and D). Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments. Similar results were obtained when another line was examined (B and C).
doi:10.1371/journal.pone.0046634.g001

increase in tyrosine phosphorylation of NS5A itself, or other associating proteins, allow the interaction with the SH2 domain of Fyn.

Central and/or C-terminal regions of NS5A binds to the SH2 domain of Fyn

To map the Fyn-SH2-binding region in NS5A, a series of deletion mutants were examined (Fig. 3). The results obtained reveals that N-terminal region (amino acids number 1–126) is not required for the interaction with the SH2 domain of Fyn in COS cells, although this region contains the region to interact with another kinase Syk (Fig. 3A) [11]. NS5A 127–146 and 147–447 could interact with the SH2 domain of Fyn. This demonstrates that deletion of the Fyn-binding region in the context of the full-length protein leads to loss of function. Similar results were obtained when HCV NS5A proteins were transiently expressed in Ramos B cells expressing SV40 T antigen (Ramos-T cells), and examined by pull-down assay (Fig. 3B). Deletion of 127–146 (NS5A Δ127–146) still allowed binding of NS5A to the SH2

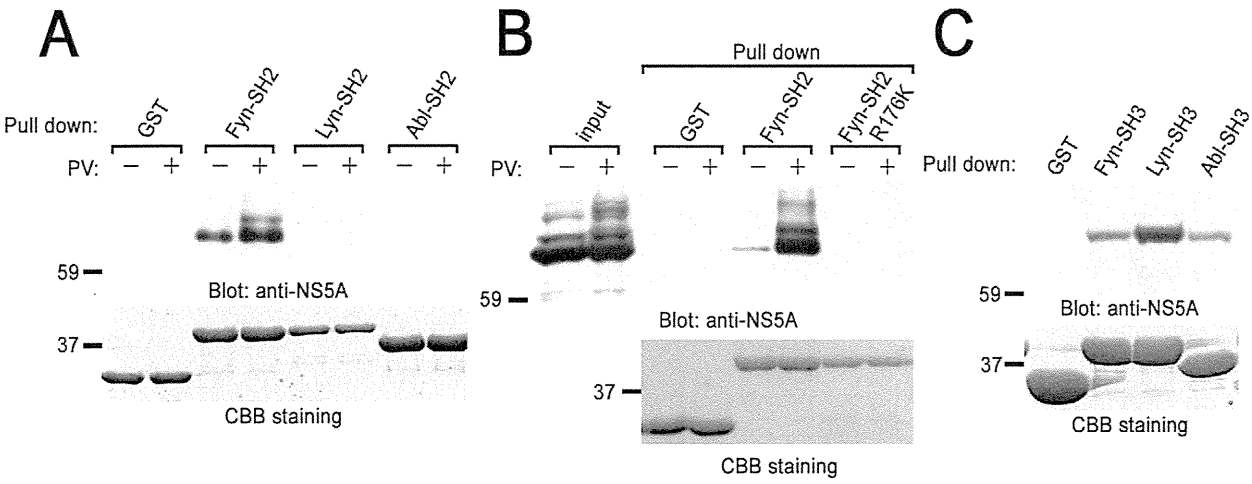

Figure 2. Pervanadate treatment of cells stimulates the binding of NS5A to the SH2 domain of Fyn in B cells. Either nontreated or PV-pretreated cells expressing Myc-His-NS5A (clone 7) were reacted with GST-fusion proteins of SH2 domains of various protein-tyrosine kinases (PTKs) (A), GST-Fyn-SH2 or GST-Fyn-SH2 R176K (B), or SH3 domains of various PTKs (C). Binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A mAb. The amount of GST-fusion proteins was confirmed by CBB staining. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments. Similar results were obtained when another line was examined.
doi:10.1371/journal.pone.0046634.g002

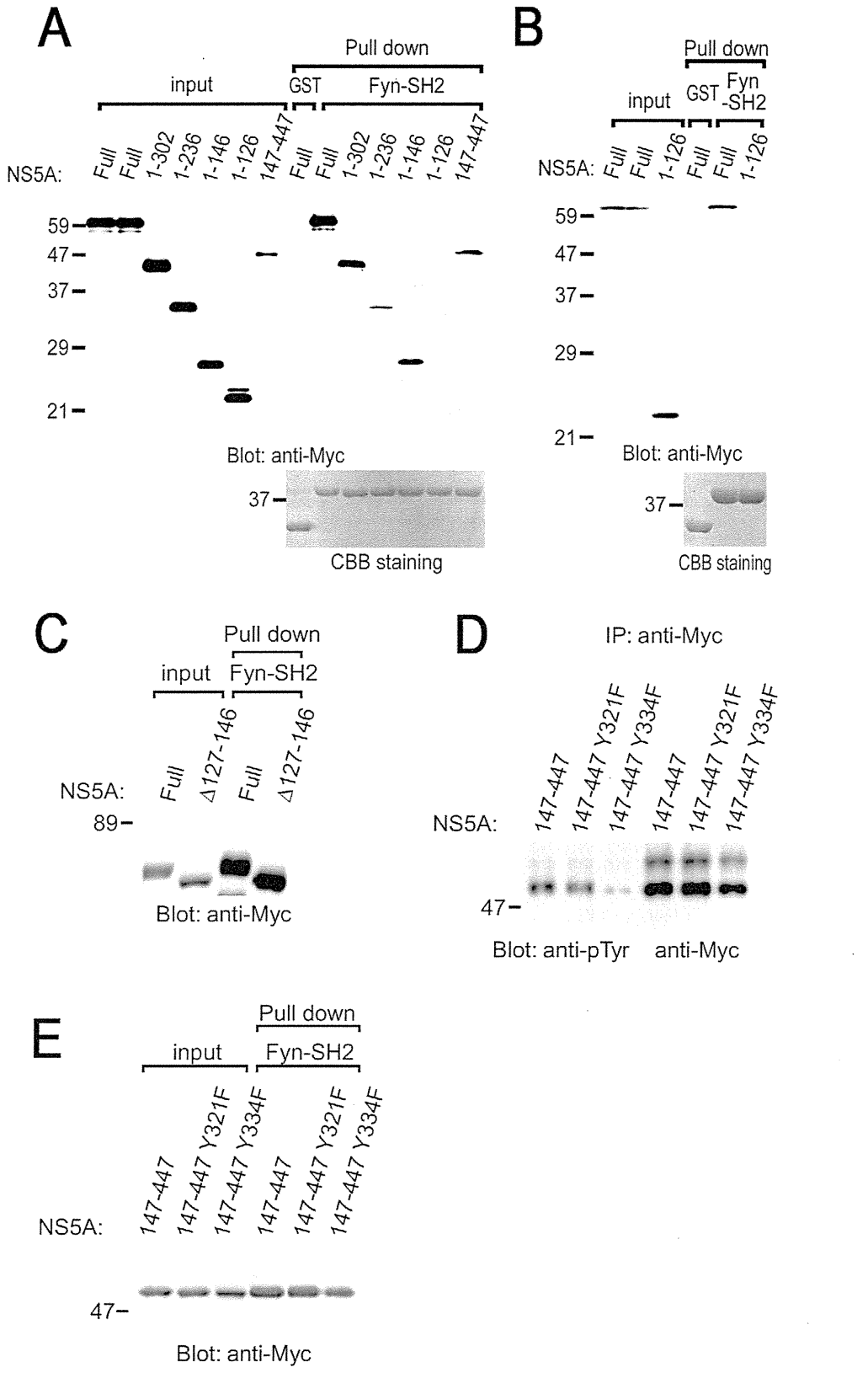

Figure 3. Structural analysis of the association of NS5A with the SH2 domain of Fyn in B cells. COS cells (A, C, E) or B cells (Ramos-T) (B) expressing different kinds of NS5A were stimulated with PV and subjected to pull-down assay using GST-Fyn-SH2. Binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-Myc mAb. The amount of GST-fusion proteins was confirmed by CBB staining. (D) COS cells expressing different kinds of NS5A mutants were stimulated with PV and subjected to immunoprecipitation. Precipitated proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-pTyr (PY20) and anti-Myc mAbs. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments.
doi:10.1371/journal.pone.0046634.g003

domain of Fyn (Fig. 3C). This suggests that NS5A Δ127–146 could interact with the SH2 domain of Fyn through NS5A 147–447.

Identification of Tyr^{334} as a tyrosine phosphorylation site in NS5A

In COS cells, full length and a series of deletion mutants of NS5A were tyrosine phosphorylated by PV treatment (Fig. S2). Because NS5A 1–126 could not bind to the SH2 domain of Fyn, we examined the possible involvement of tyrosine residue between amino acids number 127 and 146 for the binding. In this region, there is only one tyrosine residue that appears to be conserved. However, substitution of Tyr^{129} to Phe of truncated NS5A (NS5A 1–146 Y129F) still allowed tyrosine phosphorylation by PV and binding to the SH2 domain of Fyn in COS cells (Fig. S2 and S3). Thus, Tyr^{129} is not critical for the binding of NS5A to the SH2 domain of Fyn. In addition to this region (127 to 146), we examined the conserved tyrosine residues between 147 and 447 of NS5A (Tyr^{182}, Tyr^{321}, and Tyr^{334}). Among those, substitution of Tyr^{334} to Phe (Y334F) reduced tyrosine phosphorylation of NS5A 147–447, however this mutant could interact with the SH2 domain of Fyn (Fig. 3D and E). NS5A Y182F and Y321F were tyrosine phosphorylated as NS5A 147–447 (Fig. 3D and data now shown). Therefore, these results suggest that Tyr^{334} is required for tyrosine phosphorylation of NS5A, and existence of the multiple mechanisms for the binding of NS5A with Fyn including pTyr-SH2 domain interaction. Furthermore, we could not detect the increase in tyrosine phosphorylation of NS5A by *in vitro* kinase reaction with Fyn (data not shown), suggesting that some other protein-tyrosine kinases are required for phosphorylating NS5A.

The SH2 domain of Fyn directly binds to NS5A

Next we examined the mechanism of the interaction of the SH2 domain of Fyn and NS5A. Association of Fyn and NS5A in B cells were tested by the immunoprecipitation study (Fig. 4A). The result showed that NS5A was coprecipitated with anti-Fyn antibody, and vice versa. Therefore, NS5A complexes with Fyn in B cells. Far-western analysis further demonstrated that the SH2 domain of Fyn could directly bind to NS5A, suggesting that NS5A is tyrosine phosphorylated in B cells (Fig. 4B). We have shown that PV treatment of cells stimulates tyrosine phosphorylation of NS5A (Fig. 1B). These results demonstrate that NS5A could be tyrosine phosphorylated in B cells and directly associated with the SH2 domain of Fyn. In addition, we demonstrated the subcellular fractionation by sucrose density gradient centrifugation (Fig. 4C). Fractions 2–4 were regarded as low density detergent-insoluble fractions, whereas 5–9 were detergent-soluble fractions. As shown, NS5A broadly located in almost all of the fractions. In contrast, most of Fyn was located in detergent-insoluble fractions because of the lipid modification of Fyn (fractions 2–4), and some were in the detergent-soluble fractions (fractions 5–9). These results demonstrate that some of NS5A and Fyn are located in low density detergent-insoluble fractions in B cells.

Association with NS5A increases autophosphorylation and kinase activity of Fyn

Finally, we examined the effect of the expression of NS5A on the function of Fyn tyrosine kinase (Fig. 4D). Cross-linking of B cell receptor by anti-IgM mAb resulted in the increase in phosphorylation of a tyrosine residue in the activation loop of the kinase domain of Fyn, which parallels to its kinase activity [10]. Immunoprecipitation and immunoblotting experiments demonstrated that coexpression of NS5A increases phosphorylation of activation loop tyrosine and anti-IgM stimulation enhances this phenomenon. Immunoblot quantification also indicated the significant higher phosphorylation of the activation loop of Fyn in the unstimulated state in the NS5A expressing cells. In addition, we examined the biochemical kinase activity of Fyn by *in vitro* kinase assay (Fig. 4E). Coexpression of NS5A enhanced the kinase activity of Fyn as measured by both autophosphorylation and phosphorylation of exogenous substrate (enolase). This result biochemically demonstrated that association with NS5A increases tyrosine kinase activity of Fyn to phosphorylate tyrosine residues on Fyn itself and exogenous substrate. These results suggest that association of NS5A enhances an autophosphorylation and kinase activity of Fyn in B cells.

Discussion

We have demonstrated the possible tyrosine phosphorylation of NS5A in B cells and the interaction of NS5A with the SH2 domain of Fyn, in addition to SH3 domain. Previous reports demonstrated that cells harboring HCV replicon possesses the increased kinase activity of Fyn, which supports our conclusion of this study [9]. NS5A contains a highly conserved proline rich regions with Pro-X-X-Pro-X-Arg motif which is capable of the interaction with the SH3 domains of variety of cellular proteins, including Fyn [24]. Our finding reveals the second interaction site of Fyn to associate with NS5A. Therefore, NS5A could associate with both SH3 and SH2 domains. Through the two interactions, via SH3 and SH2 domains, it is predicted that NS5A could alter the conformation of Fyn to open active state. Physiological mechanism has generally been recognized that adaptor proteins with ligands of SH2 and SH3 domains bind to Src family kinases and positively regulates the kinase activity. Consistent with previous reports, our study demonstrated that NS5A protein containing potential ligands for both SH3 and SH2 domains increases autophosphorylation of Fyn in B cells.

Fyn has two tyrosine phosphorylation sites; one tyrosine in the activation loop is phosphorylated by autophosphorylation and the other in the C-terminal tail is phosphorylated by Csk to negatively regulate the kinase activity. In this manuscript, we examine the phosphorylation of tyrosine in the activation loop by using anti-phospho-Src family (Tyr416) antibody, which detects phosphorylated amount of a conserved tyrosine in the activation loop of Src family kinase (Fig. 4D). Therefore, we could conclude that tyrosine phosphorylation of Fyn was occurred in Tyr^{420} in the kinase domain.

The small interference RNA library screening study demonstrated that Csk is one of the protein-tyrosine kinases involved in the replication of HCV [25]. Csk is known to phosphorylate tyrosine residue in the C-terminal tail and negatively regulate Src family kinase, such as Fyn. Knock down of Fyn resulted in up-regulation of HCV replication [25]. This suggests that activation of Fyn suppresses HCV replication. In light of the aberrant increase in autophosphorylation of Fyn by NS5A coexpression, it is possible that NS5A negatively regulates HCV replication with activating Fyn kinase assumedly for persistent infection.

v-Src is the first discovered oncogene, and Fyn is a member of cellular Src family kinases and is also associated with cancer. Overexpression of Fyn in NIH3T3 fibroblast cells exhibited a cancer-like phonotype with increased anchorage-independent growth and prominent morphologic changes. Other studies have revealed that overexpression of Fyn results in promotion of the anti-apoptotic activity of Akt and dysregulation of anchorage-dependent cell growth. In this study, expression of NS5A enhanced autophosphorylation of Fyn in B cells, suggesting that

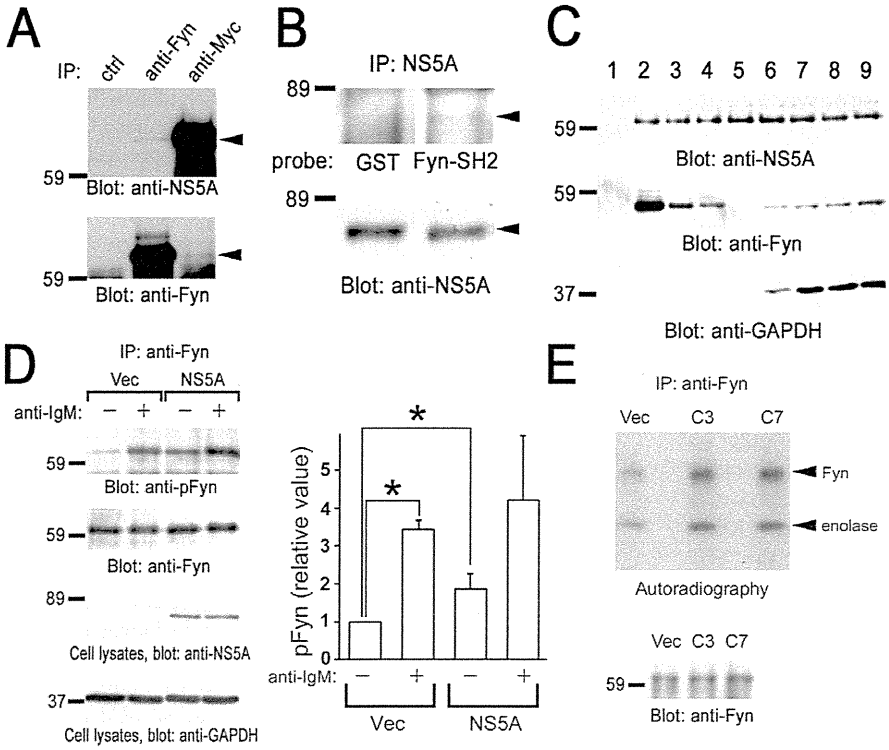

Figure 4. Association with NS5A increases the kinase activity of Fyn in B cells. (A) Endogenous interaction of Fyn with NS5A in BJAB cells. Cells were solubilized in the lysis buffer containing 0.5% Nonidet P-40. Detergent-soluble lysates from BJAB cells expressing Myc-His-NS5A (clone 7) were subjected to immunoprecipitation with anti-Fyn or anti-Myc antibodies. Protein interactions between NS5A and Fyn were analyzed by the immunoblotting with anti-NS5A mAb and anti-Fyn antibody, respectively. (B) Anti-Myc immunoprecipitates were separated by SDS-PAGE and subjected to far western analysis with GST or GST-Fyn-SH2 (GST-Fyn-SH2) (upper panel), and immunoblotting analysis with anti-NS5A mAb (lower panel). (C) Cell homogenates were fractionated by sucrose density gradient centrifugation. Proteins from these fractions were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A mAb, anti-Fyn, and anti-GAPDH antibodies. (D) Control cells (Vec) and cells expressing Myc-His-NS5A (clone 7) were unstimulated (−) or stimulated (+) with anti-IgM mAb. Anti-Fyn immunoprecipitates (IP) were separated by SDS-PAGE and analyzed by immunoblotting with anti-phospho-Src family (Tyr416) antibody recognizing autophosphorylated Fyn (pFyn) and anti-Fyn antibody. Detergent-soluble lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-NS5A and anti-GAPDH mAbs. Densitometry analysis was performed on three experiments representative of Fig. 4D. Levels of pFyn were normalized to their respective total Fyn protein. The fold changes of pFyn are shown relative to unstimulated control cells. Data represent the mean ± SD of three independent experiments. *, $P<0.05$. (E) Anti-Fyn immunoprecipitates (IP) from control cells (Vec), cells expressing Myc-His-NS5A clone 3 (C3) and clone 7 (C7) were subjected to *in vitro* kinase assay using enolase as an exogenous substrate. Radioactive proteins were separated by SDS-PAGE and visualized by autoradiography. Immunoprecipitated Fyn was analyzed by immunoblotting. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments. Similar results were obtained when another line was examined.
doi:10.1371/journal.pone.0046634.g004

HCV-mediated activation of Fyn can promote aberrant growth and anti-apoptotic status leading to B lymphomagenesis [8].

Adaptor proteins have also been recognized candidates to promote oncogenes. For example, v-Crk (CT10 regulator of tyrosine kinase)/Crk-I are adaptor proteins composed of SH2 and SH3 domains but lack negative regulatory region (phosphotyrosine and C-terminal SH3 domain). Those adaptors function as constitutively activated ones, leading to tumorgenesis. Like that, NS5A presumably works constitutive activated adaptor for Fyn kinase [26].

In conclusion, present study demonstrated that NS5A binds to the SH2 domain of Fyn in tyrosine phosphorylation-dependent manner and that NS5A containing ligand for both SH2 and SH3 domains produces an aberrant increase in autophosphorylation and kinase activity of Fyn. Further studies are needed to clarify which tyrosine residues in NS5A are phosphorylated and bind to SH2 domain of Fyn. These data, however, may contribute to our understanding of the mechanisms that HCV infection causes B lymphomagenesis.

Supporting Information

Figure S1 GST-human Fyn-SH2 could react with NS5A. The cDNA for human Fyn-SH2 (Trp^{149}-Arg^{268}) were amplified by PCR using paired primers 5′-GGAATTCATGGTACTTTG-GAAAACTTGGC-3′ and 5′-GATCAACTGCAGGGATTCT-CG -3′, using cDNA from total RNA of BJAB cells as a template. Resulted PCR fragment was subcloned into the pGEX-4T.3 (GE Healthcare) to make domain in-frame with the downstream of GST and verified by DNA sequencing. PV-treated cells expressing Myc-His-NS5A (clone 7) were solubilized in the lysis buffer. Precleared lysates were reacted with GST or GST-human Fyn-SH2 and binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A mAb. The amount of GST-fusion proteins was confirmed by CBB staining. Molecular sizing markers are indicated at left in kilodalton. The results are representative of two independent experiments.
(TIF)

Figure S2 Tyrosine phosphorylation of NS5A and its mutants in COS cells. Full length and a series of deletion

mutants of NS5A were transiently expressed in COS cells. Cells were unstimulated (−) or stimulated (+) with PV and solubilized in the lysis buffer. Cell lysates were immunoprecipitated with anti-Myc mAb and immunoprecipitated proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-pTyr (PY20) and anti-Myc mAbs. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments.
(TIF)

Figure S3 Tyr^{129} is not critical for the binding of NS5A to the SH2 domain of Fyn. Indicated mutant forms of NS5A were transiently expressed in COS cells. Cells were unstimulated (−) or stimulated (+) with PV. Cells were solubilized in the binding buffer and precleared lysates were reacted with GST-Fyn-SH2. Detergent-soluble lysates and binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-Myc mAb. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments.
(TIF)

Acknowledgments

We are grateful to Dr. R. Bartenschlager (University of Heidelberg, Germany) for providing an HCV subgenomic replicon (pFK5B/2884 Gly), to Ms. Satomi Nishibata and Ms. Kuniyo Miyagoshi for the assistance, and to Dr. Shinkou Kyo (Kobe University Graduate School of Medicine, Toyooka Public Hospital, Hyogo, Japan) and Dr. Keiko Kawauchi (Mechanobiology Institute, Singapore) for experimental assistance.

Author Contributions

Conceived and designed the experiments: KN KT HH KS. Performed the experiments: KN KT TH XS KS. Analyzed the data: KN KT KC HH KS. Contributed reagents/materials/analysis tools: KN KT KC TH XS LD IS HH KS. Wrote the paper: KN HH KS.

References

1. Moradpour D, Penin F, Rice CM (2007) Replication of hepatitis C virus. Nat Rev Microbiol 5: 453–463.
2. Suzuki T, Aizaki H, Murakami K, Shoji I, Wakita T (2007) Molecular biology of hepatitis C virus. J Gastroenterol 42: 411–423.
3. Agnello V, Chung RT, Kaplan LM (1992) A role for hepatitis C virus infection in type II cryoglobulinemia. New Engl J Med 327: 1490–1495.
4. Morsica G, Tambussi G, Sitia G, Novati R, Lazzarin A, et al. (1999) Replication of hepatitis C virus in B lymphocytes (CD19(+)). Blood 94: 1138–1139.
5. Gisbert JP, Garcia-Buey L, Pajares JM, Moreno-Otero R (2003) Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: Systematic review and meta-analysis. Gastroenterology 125: 1723–1732.
6. Machida K, Cheng KTH, Pavio N, Sung VMH, Lai MMC (2005) Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B cells. J Virol 79: 8079–8089.
7. Ito M, Murakami K, Suzuki T, Mochida K, Suzuki M, et al. (2010) Enhanced expression of lymphomagenesis-related genes in peripheral blood B cells of chronic hepatitis C patients. Clin Immunol 135: 459–465.
8. Saito YD, Jensen AR, Salgia R, Posadas EM (2010) Fyn A Novel Molecular Target in Cancer. Cancer 116: 1629–1637.
9. Macdonald A, Crowder K, Street A, McCormick C, Harris M (2004) The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. J Gen Virol 85: 721–729.
10. Bradshaw JM (2010) The Src, Syk, and Tec family kinases: Distinct types of molecular switches. Cell Signal 22: 1175–1184.
11. Inubushi S, Nagano-Fujii M, Kitayama K, Tanaka M, An C, et al. (2008) Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein tyrosine kinase Syk. J Gen Virol 89: 1231–1242.
12. Pfannkuche A, Buther K, Karthe J, Poenisch M, Bartenschlager R, et al. (2011) c-Src is required for complex formation between the hepatitis C virus-encoded proteins NS5A and NS5B: A prerequisite for replication. Hepatology 53: 1127–1136.
13. Ishido S, Choi JK, Lee BS, Wang CY, DeMaria M, et al. (2000) Inhibition of natural killer cell-mediated cytotoxicity by kaposi's sarcoma-associated herpesvirus K5 protein. Immunity 13: 365–374.
14. Ogi K, Nakashima K, Chihara K, Takeuchi K, Horiguchi T, et al. (2011) Enhancement of B-cell receptor signaling by a point mutation of adaptor protein 3BP2 identified in human inherited disease cherubism. Genes Cells 16: 951–960.
15. Sada K, Miah SM, Maeno K, Kyo S, Qu X, et al. (2002) Regulation of FcεRI-mediated degranulation by an adaptor protein 3BP2 in rat basophilic leukemia RBL-2H3 cells. Blood 100: 2138–2144.
16. Rao N, Ghosh AK, Ota S, Zhou P, Reddi AL, et al. (2001) The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. EMBO J 20: 7085–7095.
17. Shukla U, Hatani T, Nakashima K, Ogi K, Sada K (2009) Tyrosine Phosphorylation of 3BP2 Regulates B Cell Receptor-mediated Activation of NFAT. J Biol Chem 284: 33719–33728.
18. 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.
19. Nakashima K, Takeuchi K, Chihara K, Hotta H, Sada K (2011) Inhibition of hepatitis C virus replication through adenosine monophosphate-activated protein kinase-dependent and -independent pathways. Microbiol Immunol 55: 774–782.
20. Maeno K, Sada K, Kyo S, Miah SM, Kawauchi-Kamata K, et al. (2003) Adaptor protein 3BP2 is a potential ligand of Src homology 2 and 3 domains of Lyn protein-tyrosine kinase. J Biol Chem 278: 24912–24920.
21. Miah SM, Hatani T, Qu X, Yamamura H, Sada K (2004) Point mutations of 3BP2 identified in human inherited disease cherubism results in the loss of function. Genes Cells 9: 993–1004.
22. Chihara K, Nakashima K, Takeuchi K, Sada K (2011) Association of 3BP2 with SHP-1 regulates SHP-1-mediated production of TNF-α in RBL-2H3 cells. Genes Cells 16: 1133–1145.
23. Qu X, Sada K, Kyo S, Maeno K, Miah SM, et al. (2004) Negative regulation of FcεRI-mediated mast cell activation by a ubiquitin-protein ligase Cbl-b. Blood 103: 1779–1786.
24. Shelton H, Harris M (2008) Hepatitis C virus NS5A protein binds the SH3 domain of the Fyn tyrosine kinase with high affinity: mutagenic analysis of residues within the SH3 domain that contribute to the interaction. Virol J 5: 24. Available: http://www.virologyj.com/content/5/1/24.
25. Supekova L, Supek F, Lee J, Chen S, Gray N, et al. (2008) Identification of human kinases involved in hepatitis C virus replication by small interference RNA library screening. J Biol Chem 283: 29–36.
26. Birge RB, Kalodimos C, Inagaki F, Tanaka S (2009) Crk and CrkL adaptor proteins: networks for physiological and pathological signaling. Cell Commun Signal 7: 13. Available: http://www.biosignaling.com/content/7/1/13.

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

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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.

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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].

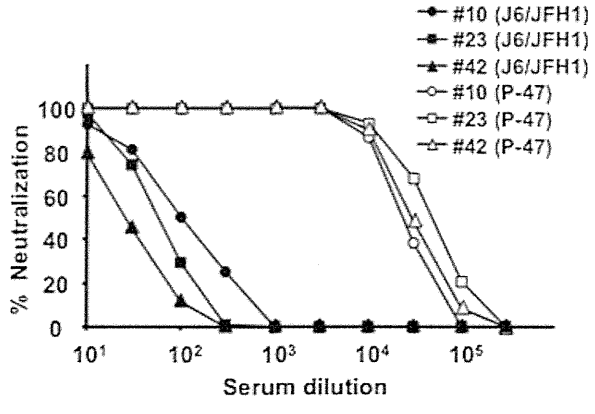

Fig. 1. Neutralization curves (NT_{50} assay) of sera obtained from HCV-infected patients against HCV J6/JFH1 and its adaptive mutant P-47. J6/JFH1 or P-47 was incubated with serial dilutions of HCV-infected patients (nos. 10, 23, and 42; all infected with HCV-1b) and tested for neutralization activities. 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

		NT_{50} titer[a] measured against	
CH/AH	Genotype	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].

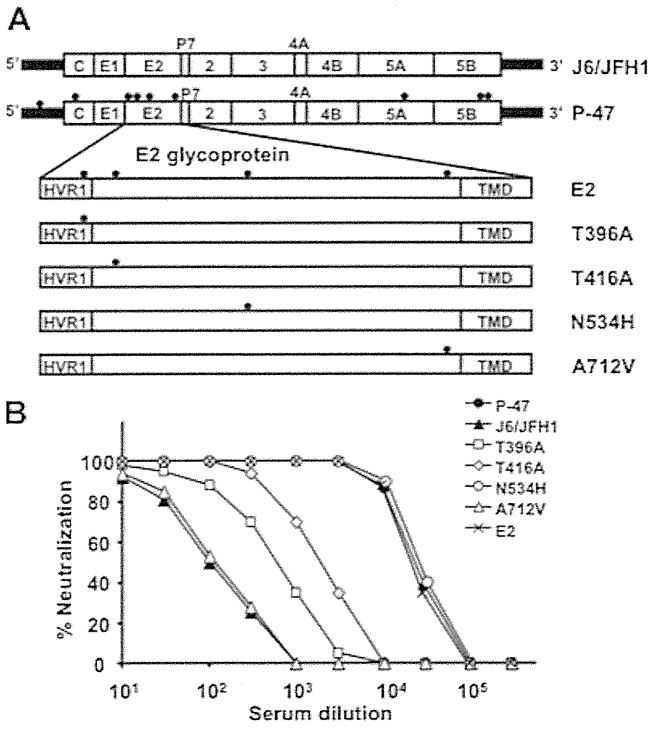

Fig. 2. Effects of amino acid mutations at positions 396, 416, 534, and 712 of the HCV E2 glycoprotein on neutralization by anti-HCV antibodies in patients' sera. **A**: A schematic diagram of the mutations seen in the adaptive mutant P-47 and recombinant viruses carrying each (T396A, T416A, N534H, and 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.

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

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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