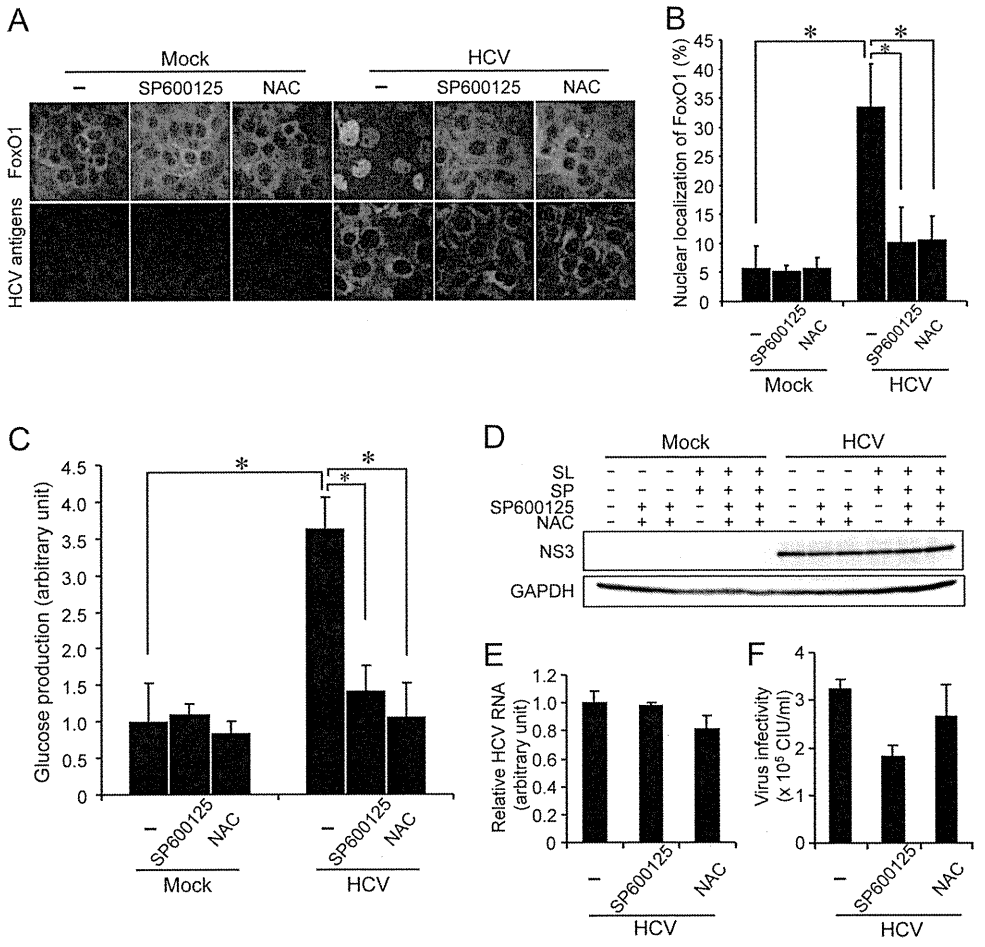

FIG. 6. HCV-induced JNK activation and ROS production are involved in FoxO1 nuclear accumulation and increased glucose production. (A) Subcellular localization of FoxO1 in HCV-infected cells and mock-infected controls with or without JNK inhibitor (SP600125 at 20 μM for 24 h) or antioxidant (NAC at 5 mM for 2 h) pretreatment at 5 dpi was examined by confocal microscopy. After fixation and permeabilization, the cells were incubated with an anti-FoxO1 rabbit monoclonal antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (top) and with serum from an HCV-infected patient followed by Alexa Fluor 594-conjugated goat anti-human IgG (bottom). (B) The percentages of cells with FoxO1 nuclear localization were determined for HCV-infected cells and mock-infected controls with or without SP600125 or NAC pretreatment. Data represent means ± SEM of data from two independent experiments. *, $P < 0.01$. (C) Extracellular glucose production was measured in HCV-infected cells and mock-infected controls with or without SP600125 or NAC pretreatment at 7 dpi and normalized to total cellular protein expression levels. Data represent means ± SEM of data from two independent experiments, and the value for the control cells was arbitrarily expressed as 1.0. *, $P < 0.01$. (D) Cellular expression levels of NS3 in HCV-infected cells and mock-infected control cells with or without sodium lactate (SL), sodium pyruvate (SP), SP600125, or NAC are shown. The amounts of GAPDH were measured as an internal control to verify equal amounts of sample loading. (E) Amounts of HCV RNA were measured by quantitative RT-PCR analysis of HCV-infected cells treated with SP600125 or NAC or left untreated at 6 dpi. The amounts were normalized to GAPDH mRNA expression levels. Data represent means ± SEM of data from two independent experiments, and the value for the nontreated HCV-infected cells was arbitrarily expressed as 1.0. (F) Virus infectivity in the culture supernatants of HCV-infected cells treated with SP600125 or NAC or left untreated at 6 dpi was measured. Data represent means ± SEM of data from two independent experiments. CIU, cell-infecting units.

hand, the nuclear accumulation of FoxO1 was clearly observed in approximately 35% of HCV-infected cells at 5 dpi. The treatment of HCV-infected cells with a JNK inhibitor (SP600125 at 20 μM for 24 h) or an antioxidant (NAC at 5 mM for 2 h) significantly inhibited HCV-induced FoxO1 nuclear accumulation.

To further verify the role played by JNK activation and ROS production in HCV-induced hepatic gluconeogenesis, the glucose production in SP600125- or NAC-treated HCV-infected cells was assessed. Treatment with SP600125 or NAC significantly impaired the HCV-induced increased glucose production at 7 dpi (Fig. 6C) but did not affect the overall abundance of the HCV NS3 protein (Fig. 6D). We also examined the possible effects of SP600125 or NAC on HCV RNA replication and infectious-virus production. The results obtained revealed that treatment with SP600125 (20 μM for 24 h) or NAC (5 mM for 2 h) barely affected HCV RNA replication (Fig. 6E). On the other hand, we noted a tendency for infectious-virus production to be only slightly suppressed by SP600125 but not by NAC (Fig. 6F). A short-term inhibition of glucose production might not sufficiently affect HCV RNA replication or virus production.

Taken together, these results indicate that ROS-mediated JNK activation plays a key role in the suppression of FoxO1

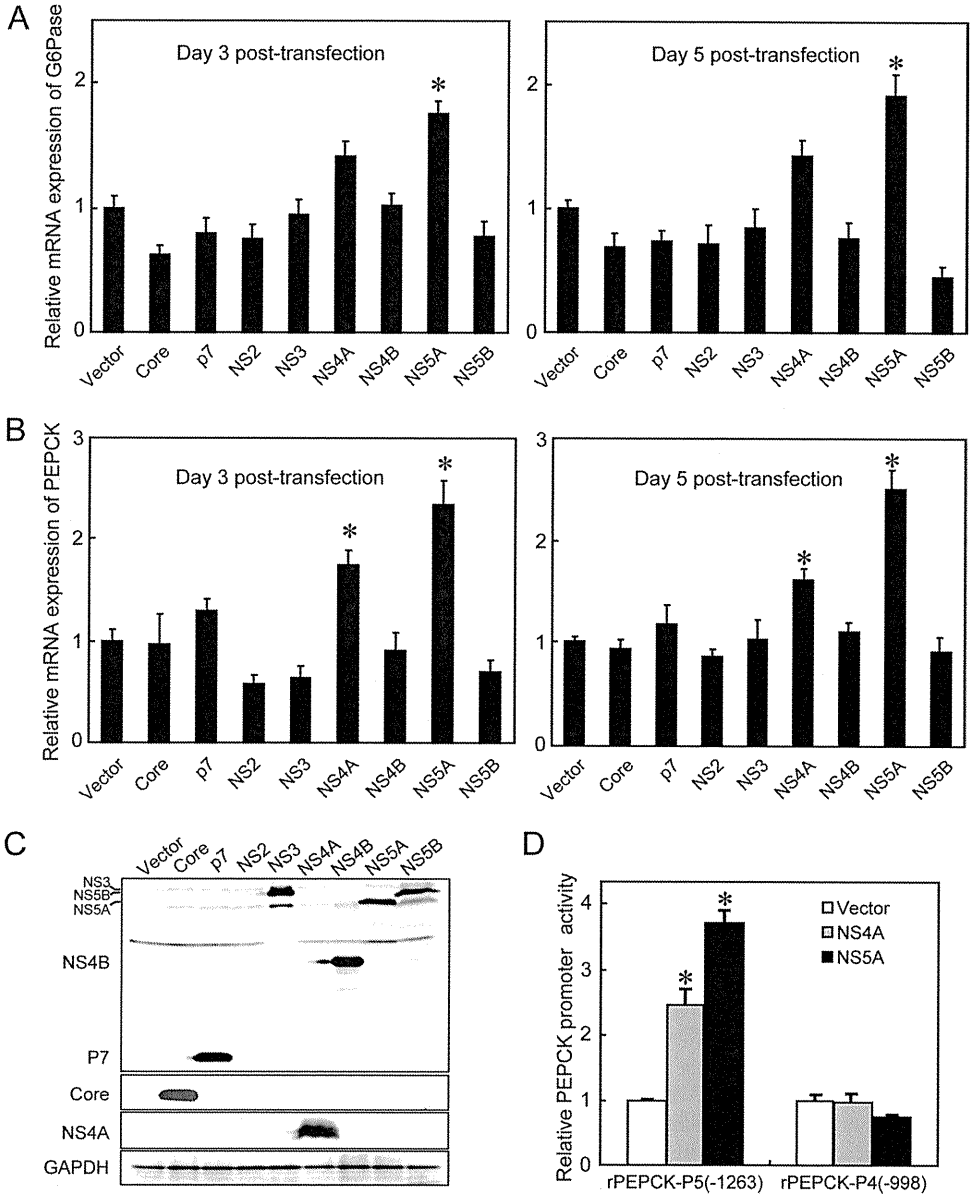

FIG. 7. HCV NS5A is involved in increased mRNA expression levels for G6Pase and PEPCK. Huh-7.5 cells were transfected with the indicated HCV viral protein expression plasmids. (A and B) At 3 and 5 days posttransfection, quantitative RT-PCR analyses of mRNA for G6Pase (A) and PEPCK (B) were conducted, and the results were normalized to β-glucuronidase mRNA expression levels. Data represent means ± SEM of data from three independent experiments, and the values for the control cells were arbitrarily expressed as 1.0. *, $P < 0.01$ compared with the control. (C) At 3 days posttransfection, the expression levels of each of the HCV proteins were examined by immunoblot analysis using antibodies against c-Myc, core, NS4A, and GAPDH. The amounts of GAPDH served as an internal control to verify equal amounts of sample loading. (D) NS5A and NS4A enhance PEPCK promoter activity. NS5A and NS4A expression plasmids were each cotransfected with rPEPCK-P5(−1263)-pGL3basic or rPEPCK-P4(−998)-pGL3basic in Huh-7.5 cells. At 48 h after transfection, the PEPCK promoter activities were measured by using a luciferase reporter assay. Data represent means ± SEM of data from three independent experiments, and the values for the control cells were arbitrarily expressed as 1.0. *, $P < 0.05$ compared with the control.

phosphorylation, the nuclear accumulation of FoxO1, and the enhancement of glucose production in HCV-infected cells.

HCV NS5A is involved in the enhancement of glucose production. To examine which HCV protein(s) is involved in the enhancement of gluconeogenesis, expression constructs of each of the HCV viral proteins were transfected into Huh-7.5 cells, and the gene expression levels of PEPCK and G6Pase were examined by real-time quantitative RT-PCR analysis. We observed that NS5A significantly promoted G6Pase gene expression (Fig. 7A). Moreover, both the NS5A and NS4A proteins significantly enhanced PEPCK gene expression at 3 and 5 days posttransfection, respectively (Fig. 7B). The expression of each of the HCV proteins except NS2 was verified by immunoblot analysis (Fig. 7C). NS2 was reported previously to be unstable and rapidly degraded by the proteasome (22).

Next, we performed a luciferase reporter assay to examine

the possible effects of NS5A and NS4A on PEPCK promoter activities. The construct rPEPCK-P5(−1263)-pGL3basic carries 1,263 bp of the PEPCK 5′-flanking region (−1263 PEPCK) and is used to monitor PEPCK promoter activity. The results demonstrated that the levels of PEPCK promoter activities were significantly higher in both NS5A- and NS4A-expressing cells than in the control cells (Fig. 7D). Interestingly, when the region of the PEPCK promoter from positions −1263 to −998 was deleted, the activation of PEPCK promoter activity in cells expressing NS5A and NS4A was abolished. These results confirmed that NS5A and NS4A activate the PEPCK promoter, leading to an increase in PEPCK mRNA expression levels. Database searches of the deleted sequence did not reveal any potential binding sequences for transcription factors (data not shown).

Recently reported data suggest that ROS production is induced in NS5A-expressing cells (17) or in hepatocytes of NS5A transgenic mice (68). We therefore sough to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. The NS5A expression plasmid was transfected into Huh-7.5 cells, and ROS production was assessed by MitoSOX at 3 days posttransfection. As shown in Fig. 8A and B, approximately 30% of NS5A-expressing cells displayed a much stronger signal than that observed for vector-transfected control cells.

We then examined whether NS5A mediated JNK/c-Jun activation and FoxO1 phosphorylation inhibition. The results obtained revealed that both the phosphorylation level at Ser63 and the total expression level of c-Jun were upregulated in NS5A-expressing cells compared to the control cells transfected with the vector plasmid or cells expressing the other HCV proteins (Fig. 8C and D, top two panels). Concomitantly, FoxO1 phosphorylation at Ser319 was clearly suppressed in NS5A- and NS4A-expressing cells compared to the control cells (Fig. 8C, compare lanes 6, 5, and 1, respectively, in the third panel). NS4A, a small protein of ca. 7 kDa, forms a stable complex with NS3 to function as a cofactor for NS3 serine protease and RNA helicase activities (51). We previously reported that NS4A caused mitochondrial damage when expressed alone but not when coexpressed with NS3 (47). We therefore speculated that the otherwise observed decrease in FoxO1 phosphorylation levels in NS4A-expressing cells might be canceled when NS4A is coexpressed with NS3. To verify this notion, we tested FoxO1 phosphorylation in cells coexpressing NS3 and NS4A. As had been expected, FoxO1 phosphorylation levels did not differ between NS3/4A-coexpressing cells and vector-transfected control cells (Fig. 8C, compare lanes 4 and 1, respectively).

Notably, we observed that the HCV core protein did not alter the phosphorylation status of c-Jun and FoxO1 (Fig. 8C, compare lanes 1 and 2), with the result being consistent with what was observed for gene expression levels of PEPCK and G6Pase in HCV core-expressing cells (Fig. 7A and B). These results imply that core is not primarily involved in HCV-induced increased gluconeogenesis under our experimental conditions. Similarly, other HCV nonstructural proteins, such as NS4B and NS5B, did not significantly influence the phosphorylation status of c-Jun and FoxO1 (Fig. 8D).

In order to further verify the effect of NS5A on the nuclear accumulation of FoxO1, we examined the subcellular localization of FoxO1 in NS5A-expressing cells by indirect immunofluorescence staining. As shown in Fig. 8E and F, the nuclear accumulation of FoxO1 was clearly observed for approximately 25% of NS5A-expressing cells but not the vector-transfected control. These results suggest that NS5A activates the JNK/c-Jun signaling pathway via increased ROS production, which results in the decreased phosphorylation and nuclear accumulation of FoxO1.

Finally, we examined the effects of NS5A and NS4A on glucose production. As shown in Fig. 9, the amounts of glucose were significantly increased in culture supernatants of NS5A- and NS4A-expressing cells, compared with the amounts of glucose in control cells, at 5 days posttransfection. Again, it is reasonable to assume that the observed increase in glucose production in NS4A-expressing cells might be canceled when NS4A is coexpressed with NS3.

These results collectively suggest that NS5A plays a role, at least to some extent, in the HCV-induced enhancement of hepatic gluconeogenesis.

DISCUSSION

Hepatocytes play an important role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. We previously reported that HCV suppresses cellular glucose uptake by downregulating the surface expression of the glucose transporters GLUT1 and GLUT2 (37). In this study, we have demonstrated that HCV promotes FoxO1-mediated hepatic gluconeogenesis, as evidenced by the increased accumulation of FoxO1 in the nucleus via the reduction of its phosphorylation status (Fig. 3 and 6A and B), which leads to increased PEPCK and G6Pase gene expression levels (Fig. 1A and B) and the subsequent upregulation of G6P and glucose production (Fig. 2). Moreover, our results indicate that HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1, leading eventually to increased glucose production (Fig. 4 to 6). Our results thus suggest that FoxO1 is a prime transcription factor in the HCV-mediated progression of hepatic gluconeogenesis through an ROS/JNK-dependent mechanism, as summarized in the schema in Fig. 10. Our results also suggest that HCV NS5A plays a role in enhanced hepatic gluconeogenesis by promoting ROS production and JNK activation (Fig. 7 to 9). In line with our observations, the NS5A-mediated induction of ROS production (68) and JNK activation (49) was reported previously by other investigators.

Increasing evidence suggests that mitochondrial dysfunction is causative of insulin resistance and type 2 diabetes. Mitochondrial dysfunction causes the upregulation of PEPCK and G6Pase, leading to increased gluconeogenesis and insulin resistance (42, 46). We previously reported that HCV causes mitochondrial damage and mitochondrion-mediated apoptosis (14, 47). Our current data further support the concept that altered mitochondrial function plays a role in the development of increased glucose production in hepatocytes.

We and other groups have reported that HCV infection increases the production of mitochondrial ROS, which plays an important role in the development and progression of inflam-

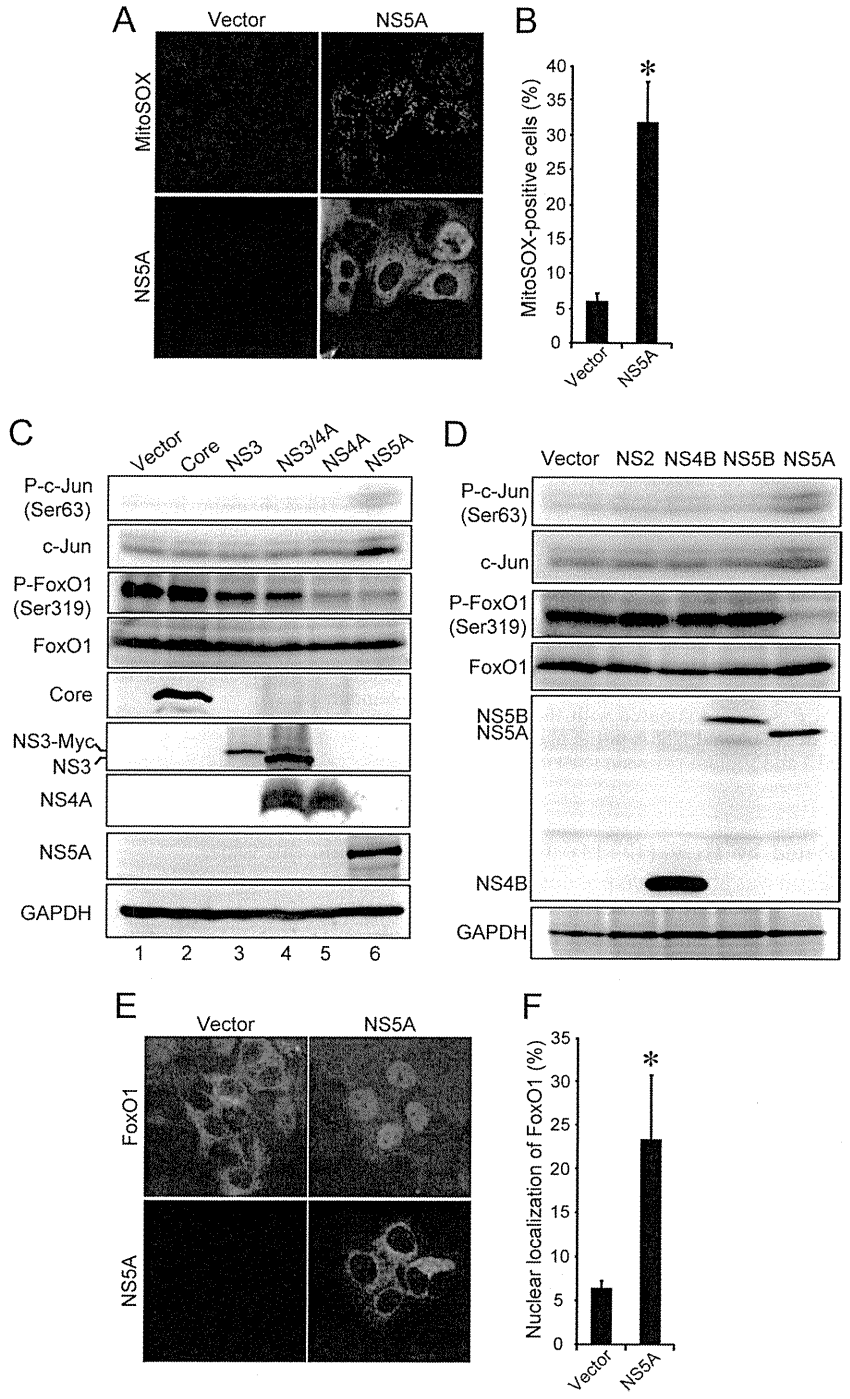

FIG. 8. HCV NS5A is involved in increased ROS production, JNK activation, FoxO1 phosphorylation suppression, and FoxO1 nuclear accumulation. (A) NS5A promotes ROS production. Huh-7.5 cells transfected with an NS5A expression plasmid or the empty control (vector) were incubated with MitoSOX (top) at 3 days posttransfection and then stained for NS5A by using anti-NS5A mouse monoclonal antibody, followed by FITC-conjugated goat anti-mouse IgG (bottom). (B) Quantification of MitoSOX-stained cells. The percentages of cells stained with MitoSOX were determined for NS5A-expressing cells and control cells. Data represent means ± SEM of data from two independent experiments. *, $P < 0.01$. (C and D) HCV NS5A activates c-Jun phosphorylation and suppresses FoxO1 phosphorylation. Huh-7.5 cells transfected with the indicated HCV viral protein expression plasmids were harvested at 3 days posttransfection, and the whole-cell lysates were subjected to immunoblot analysis using antibodies against phospho-c-Jun (Ser63), c-Jun, phospho-FoxO1 (Ser319), FoxO1, GAPDH, core, NS3, NS4A, and NS5A (C) or c-Myc (D). The amounts of GAPDH were measured as an internal control to verify equal amounts of sample loading. (E) NS5A facilitates FoxO1 nuclear accumulation. Huh-7.5 cells transfected with an NS5A expression plasmid or the empty control (vector) were fixed and permeabilized at 3 days posttransfection. The cells were incubated with an anti-FoxO1 rabbit monoclonal antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (top) or with anti-NS5A mouse monoclonal antibody followed by Alexa Fluor 594-conjugated goat anti-mouse IgG (bottom). (F) The percentages of cells with a nuclear localization of FoxO1 were determined for NS5A-expressing cells and control cells. Data represent means ± SEM of data from two independent experiments. *, $P < 0.01$.

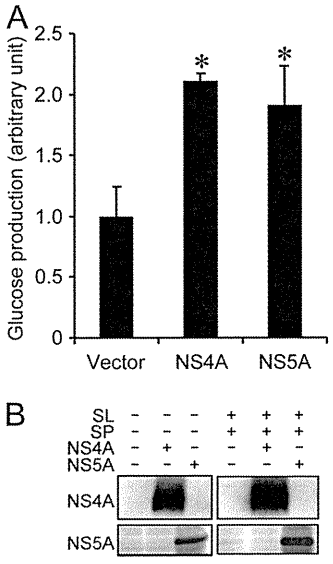

FIG. 9. HCV NS5A and NS4A enhance glucose production. (A) Huh-7.5 cells were transfected with either an NS5A or NS4A expression plasmid. At 5 days posttransfection, extracellular glucose production was measured and normalized to the total cellular protein expression level. Data represent means ± SEM of data from two independent experiments, and the values for the control cells were arbitrarily expressed as 1.0. *, $P < 0.05$ compared with the control. (B) Cellular expression levels of NS4A and NS5A in the absence and presence of sodium lactate (SL) and sodium pyruvate (SP) are shown.

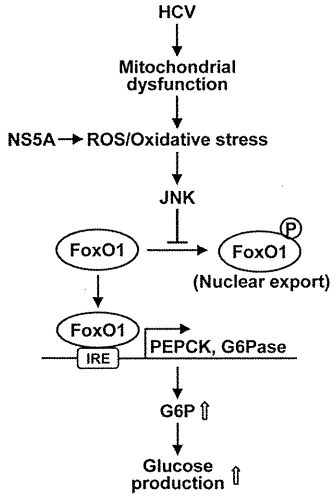

FIG. 10. Schematic representation of the HCV-dysregulated hepatic gluconeogenesis signaling pathway. HCV induces mitochondrial dysfunction (14). This results in increased ROS production and JNK activation, which induces the nuclear accumulation of FoxO1 by reducing its phosphorylation status. Consequently, PEPCK and G6Pase gene expressions are upregulated, leading to an upregulation of G6P and glucose production. NS5A plays a role in HCV-induced gluconeogenesis via the induction of ROS production. IRE, insulin response element.

matory liver disease mediated by HCV (12, 14). Increased mitochondrial ROS generation was also shown previously to be an underlying mediator of multiple forms of insulin resistance, including inflammation- or glucocorticoid-induced insulin resistance (27, 29). Moreover, a significant correlation was observed between oxidative stress and insulin resistance in patients infected with HCV genotype 1 or 2 (44). ROS have also been shown to regulate the activity of the FoxO transcription factor by posttranslational modifications, including phosphorylation (21), deacetylation (8), and ubiquitylation (67).

Although this study showed that JNK induces the nuclear accumulation of FoxO1 by reducing its phosphorylation status under oxidative stress conditions in HCV-infected cells, the precise mechanism(s) of the interplay between JNK and FoxO1 still remains to be addressed. It was reported previously that activated JNK phosphorylates IRS-1 at Ser307, which results in attenuated insulin signal transduction through the inhibition of the tyrosine phosphorylation of IRS-1 (1). Akt is a major downstream signaling protein for insulin/IRS-1 signaling and is activated through its phosphorylation on Thr308 and Ser473, the latter of which is believed to be more crucial (53). Therefore, an impairment of the insulin/IRS-1 signaling pathway should involve the downregulation of Akt phosphorylation. However, our present data showed that Akt phosphorylation on Ser473 was upregulated in HCV-infected cells at 4 and 6 dpi (Fig. 3B), suggesting that an Akt-independent pathway is involved in the JNK-mediated suppression of FoxO1 phosphorylation. Regarding this connection, it should be noted that the 14-3-3 protein, a binding partner for phosphorylated FoxO1 that mediates its nuclear export (72), is phosphorylated by JNK and that the phosphorylated 14-3-3 protein releases its binding partners, which would facilitate the nuclear accumulation of FoxO (63, 65, 70). Further studies are needed to elucidate this issue.

Another trigger that causes excessive JNK activation and insulin resistance is endoplasmic reticulum (ER) stress (28, 48). Several previous studies reported that HCV infection induces ER stress (34, 55). Under our experimental conditions, however, we did not detect significant ER stress in HCV-infected cells (14). It is thus likely that ER stress was not the primary cause of the increased gluconeogenesis in our experimental system using Huh-7.5 cells and the P-47 strain of HCV J6/JFH-1 (9, 14).

Notably, our present data showed that cells harboring the SGR or FGR and HCV-infected cells produced greater amounts of glucose than did the control cells (Fig. 2A); however, the changes in the phosphorylation status of FoxO1 and JNK in SGR- and FGR-harboring cells were not so significant compared to those in virus-infected cells (data not shown). One of the reasons for this difference is that SGR- and FGR-harboring cells were obtained through a longer cultivation in a selection medium for a month or more and that the balance of host gene induction may be somewhat different from that in virus-infected cells. Therefore, it is possible that, in addition to the JNK-FoxO1 pathway, another signaling pathway(s) is involved in the increased gluconeogenesis in SGR- and FGR-harboring cells. Studies on this issue are now under way in our laboratory.

We observed that HCV infection modulated, either positively or negatively, the transcription of the PEPCK, G6Pase, and GK genes at 3 to 5 dpi (Fig. 1). Virus infection, in general, causes dynamically changing induction and the suppression of a wide variety of host genes. For example, expression levels of certain genes, such as interferon genes, increase during an

early phase of virus infection, e.g., at 1 dpi, but return to normal levels within a few days in a cell culture system. On the other hand, the virus-infection-induced expression of other genes, such as the extracellular signal-regulated kinase (ERK) gene, remains for a prolonged period of time (data not shown). Also, some of the gene products induced in the acute phase may suppress the expression of other genes. Under these balanced conditions, it is quite possible that certain genes are induced only at a later time, e.g., 3 to 5 dpi, but not immediately after virus infection.

It was reported previously that HCV core protein-expressing transgenic mice exhibit marked insulin resistance by inhibiting IRS-1 tyrosine phosphorylation and Akt phosphorylation (45, 58). However, our present results showed that HCV NS5A, but not the core protein, was associated with increased gluconeogenesis. Moreover, it was recently reported that HCV infection significantly inhibited cellular glucose levels at 10 dpi (69), which is quite the opposite of what we observed in the present study. These results collectively suggest the possibility that multiple pathways are involved in glucose metabolism in HCV-infected cells. Also, the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle needs to be clarified.

In conclusion, our present results collectively suggest that HCV promotes hepatic gluconeogenesis, resulting in increased glucose production in hepatocytes via an NS5A-mediated, FoxO1-dependent pathway.

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ORIGINAL ARTICLE

Inhibition of hepatitis C virus replication through adenosine monophosphate-activated protein kinase-dependent and -independent pathways

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ABSTRACT

Persistent infection with hepatitis C virus (HCV) is closely correlated with type 2 diabetes. In this study, replication of HCV at different glucose concentrations was investigated by using J6/JFH1-derived cell-adapted HCV in Huh-7.5 cells and the mechanism of regulation of HCV replication by AMP-activated protein kinase (AMPK) as an energy sensor of the cell analyzed. Reducing the glucose concentration in the cell culture medium from 4.5 to 1.0 g/L resulted in suppression of HCV replication, along with activation of AMPK. Whereas treatment of cells with AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) suppressed HCV replication, compound C, a specific AMPK inhibitor, prevented AICAR's effect, suggesting that AICAR suppresses the replication of HCV by activating AMPK in Huh-7.5 cells. In contrast, compound C induced further suppression of HCV replication when the cells were cultured in low glucose concentrations or with metformin. These results suggest that low glucose concentrations and metformin have anti-HCV effects independently of AMPK activation.

Key words 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), adenosine monophosphate-activated protein kinase (AMPK), diabetes, metformin.

Hepatitis C virus, which is classified within the family *Flaviviridae*, is a small enveloped virus that possesses a positive-sense single-stranded RNA genome. HCV infection proceeds to a persistent stage at a high rate, leading to cirrhosis and hepatocellular carcinoma. Despite recent advances in the development of antiviral therapies, certain patient populations are difficult to treat (1) due to host factors such as obesity, hyperglycemia and insulin resistance (2–4).

Adenosine monophosphate-activated protein kinase is a major cellular energy sensor that is activated by cellular stresses that increase intracellular AMP (5). ZMP, which mimics AMP, also activates AMPK (6). AMPK is a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits (7). Phosphorylation of Thr^{172} in its activation loop of α subunit by upstream kinases, namely, LKB1 (8,9) and Ca^{2+}/calmodulin-dependent kinase kinase (10,11) can increase its kinase activity (12). Activated AMPK inhibits the synthesis of fatty acids, cholesterol, proteins and gluconeogenesis in hepatocytes (13–16). Phosphorylation of $Ser^{485/491}$ by protein kinase B is known to inhibit AMPK activity (17).

Hepatitis C virus infection suppresses cellular glucose uptake through down-regulation of cell surface expression

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List of Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; compound C, 6-(4-[2-piperidin-1-yl-ethoxy])-phenyl)-3-pyridin-4-yl-pyrrazolo(1,5-a)-pyrimidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; LKB1, liver kinase B1; MOI, multiplicity of infection; NS3, non-structural protein 3; PRPP, phosphoribosyl pyrophosphate; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide; ZTP, 5-aminoimidazole 4-carboxamide ribonucleoside 5-triphosphate.

of glucose transporters (18). Our preliminary experiments demonstrated that HCV infection alters the expression of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase, which catalyzes ZMP in purine nucleotide synthesis. ZMP is known to mimic the activating effects of AMP on AMPK (6). We postulated that glucose usage and/or activation of AMPK might affect the infection and replication of HCV.

In this study, we have investigated HCV replication with different glucose concentrations in the culture medium, with treatment of cells with AMPK activators (AICAR, metformin) or with the AMPK inhibitor compound C in the cell culture medium.

MATERIALS AND METHODS

Cells

The Huh-7.5 cell line used in this study, a highly HCV-susceptible subclone of Huh7 cells, was a kind gift from Dr. C. M. Rice (Center for the Study of Hepatitis C, The Rockefeller University, New York, NY, USA) (19). The cells were propagated in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS and 0.1 mM nonessential amino acids.

Viruses

The virus stock was prepared as described previously (20,21). The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1, was kindly provided by Dr. C. M. Rice. The HCV RNA genome was transcribed *in vitro* from pFL-J6/JFH1 and transfected to Huh-7.5 cells. The supernatant was harvested as a virus stock. In this study we used an adapted strain of the virus obtained by passaging the HCV genotype 2a, J6/JFH1, infected cells 47 times (20,22). Virus infection was performed at a MOI of three. Culture supernatants of uninfected cells were used as controls (mock preparation). Virus infectivity was measured by indirect immunofluorescence analysis as described previously (20).

Reagents

5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside and uridine were purchased from Sigma (St. Louis, MO, USA), compound C from Chemdea (Ridgewood, NJ, USA), metformin from Enzo Life Sciences (Plymouth Meeting, PA, USA) and Hoechst 33258 from Wako (Osaka, Japan).

Immunoblotting

Immunoblotting was essentially as described previously (23). Cells were solubilized in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 mM NaF, 0.1 mM Na_3VO_4, 10 mM EDTA, 1% Triton-X, and protease inhibitor cocktail [Sigma]). Cell debris was removed by centrifugation and resulted supernatants were diluted 1:2 (v/v) with 3×sampling buffer. Protein quantification was carried out using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of soluble proteins were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride transfer membrane (Millipore, Billerica, MA, USA). After blocking in 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), the blots were reacted with the respective primary antibodies. The primary antibodies used were anti-phospho-AMPKα (Thr172) monoclonal antibody (clone D79.5E, Cell Signaling Technology, Danvers, MA, USA), anti-AMPKα antibody (Cell Signaling Technology), anti-HCV core monoclonal antibody (clone C7–50, Thermo Fisher Scientific), anti-AMPKα antibody (Phospho-Ser$^{485/491}$) (anti-pAMPK [Ser485/491]) (GenScript, Piscataway, NJ, USA), and anti-HCV NS3 monoclonal antibody (Millipore). Horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies. The respective protein bands were visualized by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA) (24,25). Protein loading was normalized by probing with anti-GAPDH monoclonal antibody (clone 6C5, Millipore).

Indirect immunofluorescence

Cells seeded in 96-well plates were infected with HCV at a MOI of 3.0 for 4 hr or left uninfected. The cells were incubated for 30 hr and fixed with cold methanol for 10 min at room temperature. After being washed with PBS twice, the cells were stained with anti-HCV core monoclonal antibody and visualized by using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA) and the tyramide signal amplification cyanine 3 system (Perkin Elmer). Stained cell samples were examined by fluorescence microscopy (Olympus IX70 microscope system, Tokyo, Japan).

Statistical analysis

The one-tailed Student *t*-test was applied to evaluate the statistical significance of differences found. A P value of <0.05 was considered statistically significant.

RESULTS

Glucose shortage in the culture medium suppresses the replication of hepatitis C virus along with activation of adenosine monophosphate-activated protein kinase

Any virus requires an energy source for replication. We surmised that glucose shortage in the cell culture medium would have a harmful effect on energy metabolism in HCV-infected Huh-7.5 cells. We used Huh-7.5 cells and a J6/JFH1-derived, cell-adapted strain of HCV throughout this study. First, we examined the effect of alterations in glucose concentration in the cell culture medium on the replication of HCV. Reducing the glucose concentration from 4.5 to 1.0 g/L resulted in a significant decrease in HCV replication, as demonstrated by decreased virus infectivity in culture supernatants (Fig. 1a), and decreased production of HCV core protein (Fig. 1b, third panel). In order to estimate the intracellular energy status, we examined the kinase activity of AMPK by the immunoblotting of phosphorylated Thr^{172} in AMPK. Reducing the glucose concentration from 4.5 to 2.0 g/L resulted in a dramatic increase in phosphorylation of Thr^{172} in AMPK, suggesting that Huh-7.5 cells sensed poor nutrition when cultured with 2.0 g/L of glucose in the medium, irrespective of infection with HCV (Fig. 1b). Phosphorylation of $Ser^{485/491}$ of AMPK was not affected by infection with HCV (Fig. 1c). Phosphorylation of AMPK was not affected by infection with HCV, although almost all of the cells were infected in this experiment (Fig. 1d). These results demonstrate that glucose shortage in the cell culture medium suppresses replication of HCV along with activation of AMPK in Huh-7.5 cells. Glucose shortage activates AMPK regardless of HCV infection.

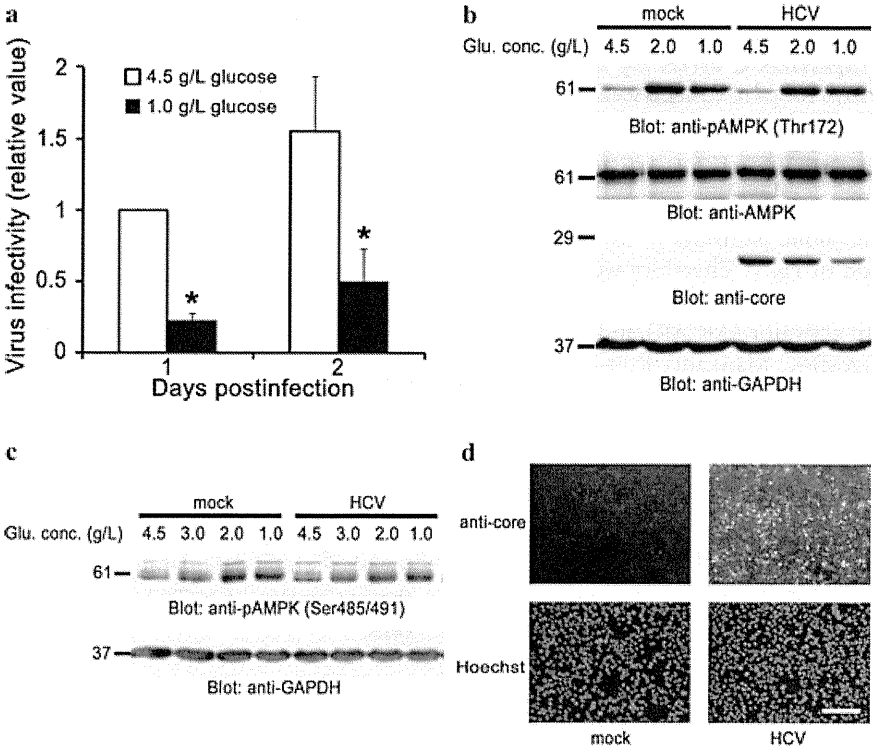

Fig. 1. Glucose shortage suppresses HCV replication and activates AMPK. (a) Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr and then incubated at the indicated concentration of glucose in serum-free DMEM. Infectivity titer in culture supernatants of HCV-infected cells cultured in medium containing 4.5 g/L glucose at day 1 postinfection was arbitrarily expressed as 1.0. Data are expressed as means ± standard deviations (SD) of three independent experiments. *, $P < 0.05$, compared with the control. (b and c) Huh-7.5 cells were mock infected or infected with HCV at a MOI of 3.0 for 4 hr and then incubated at the indicated concentrations of glucose in serum-free DMEM for 30 hr. (b) Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-phospho-AMPKα (Thr172) (anti-pAMPK (Thr172)), anti-AMPK, anti-HCV core, anti-GAPDH antibodies as indicated. (c) Cell lysates were analyzed by immunoblotting with anti-AMPKα antibody (anti-pAMPK (Ser485/491)) and anti-GAPDH monoclonal antibody. (d) Huh-7.5 cells mock infected or infected with HCV at a MOI of 3.0 for 4 hr were subjected to indirect immunofluorescence analysis by anti-HCV core antibody. Nuclei were stained with Hoechst 33258. Scale bar, 200 μm. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments. Conc., concentration; glu., glucose.

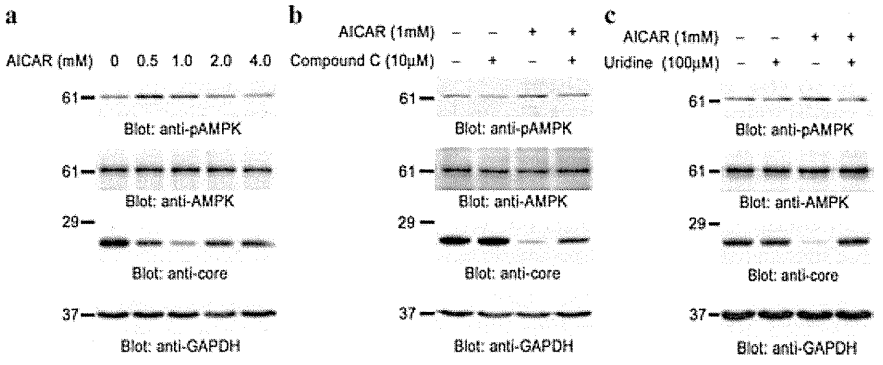

Fig. 2. AICAR suppresses HCV replication by activating AMPK. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. (a) The cells were treated with the indicated concentrations of AICAR for 20 hr. (b) 10 μM compound C was added to the cells 30 min prior to the addition of AICAR and was present in the medium during the entire 20 hr of incubation with AICAR. (c) The cells were treated with 1 mM AICAR for 20 hr with or without supplementation with 100 μM uridine. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as Figure 1. The results are representative of three independent experiments.

5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside suppresses the replication of hepatitis C virus by activating adenosine monophosphate-activated protein kinase in Huh-7.5 cells

Activated AMPK inhibits the synthesis of fatty acids, cholesterol, proteins and gluconeogenesis (13–16). Imbalance of these metabolic pathways in liver cells might affect the replication of HCV. Therefore we next examined whether activated AMPK suppresses the replication of HCV by using an activator (AICAR) and an inhibitor (compound C) of AMPK. Mankauri J. *et al.* have previously reported that treatment of Huh-7 parental cells with AICAR suppresses the replication of JFH-1 (26). In this study, we adopted a more efficient HCV replication system, Huh-7.5 cells and a J6/JFH1-derived, cell-adapted strain of HCV. Similar to the previous finding, activation of AMPK by AICAR suppressed the expression of HCV core protein in Huh-7.5 cells (Fig. 2a, lanes 1–3). Activation of AMPK by AICAR was observed when the cells were treated with relatively low concentrations (0.5 or 1.0 mM), but not with higher concentrations (2.0 or 4.0 mM). Possible reasons for the latter effect are that higher concentrations of AICAR could suppress the synthesis of purine nucleotides and/or increase the concentration of ZTP, thus inhibiting AMPK (6,27).

To examine whether the inhibitory effect of AICAR on HCV replication is mediated by activation of AMPK, we tested an AMPK inhibitor (compound C) in this experiment. We found that pretreatment of cells with 10 μM compound C attenuates AICAR-mediated suppression of HCV core protein expression (Fig. 2b, lane 4). This suggests that AICAR-mediated suppression of HCV replication is mediated by activation of AMPK. Addition of compound C to the cell culture medium without AICAR did not affect the expression of HCV core protein, suggesting that this inhibitor does not affect the replication of HCV under nutritious condition in which AMPK is inactive (Fig. 2b, lane 2).

In the presence of AICAR, the amounts of uridine triphosphate and cytidine triphosphate are decreased in the cultured cells as a result of PRPP depletion (27). PRPP is an important precursor for pyrimidine nucleotide synthesis. PRPP-derived pyrophosphate can increase ZTP/ZMP which are then no longer able to activate AMPK (6,28). To complement the pyrimidine shortage in Huh-7.5 cells treated with AICAR, the cells were co-incubated with 100 μM uridine in the presence of 1 mM AICAR. This resulted in the complete prevention of AICAR-mediated activation of AMPK and the resulting suppression of HCV (Fig. 2c, lane 4). Taken together, these data demonstrate that AICAR suppresses the replication of HCV by activating AMPK in Huh-7.5 cells.

Glucose shortage and metformin have an anti-hepatitis C virus effect independently of adenosine monophosphate-activated protein kinase activation

It is important to note that glucose shortage activates AMPK because of cellular energy limitations, whereas AICAR can activate AMPK regardless of cellular energy status. Therefore we tried another AMPK activator, metformin, which activates AMPK by impairing complex 1 of the mitochondrial respiratory chain (29,30). In addition, in mice metformin has a LKB1/AMPK-independent

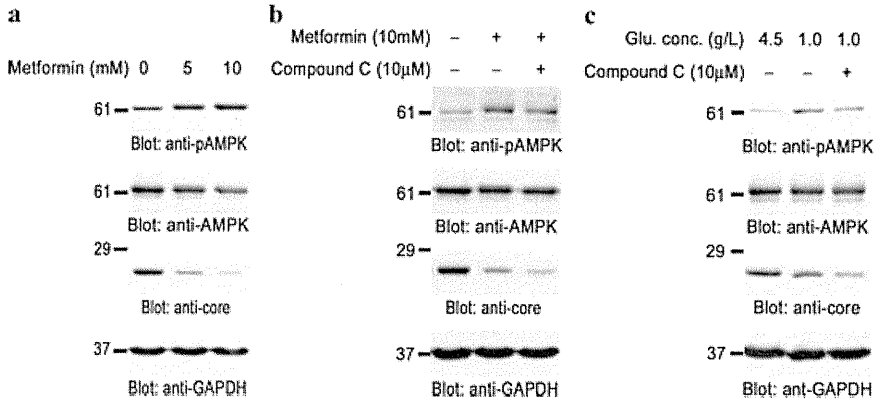

Fig. 3. Compound C, an AMPK inhibitor, stimulates the anti-HCV effects of glucose shortage or metformin. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. (a) The cells were treated with the indicated concentration of metformin for 20 hr. (b) 10 μM compound C was added to the cells 30 min prior to the addition of metformin and present in the medium during the entire 20 hr of incubation with metformin. (c) The cells were incubated with or without 10 μM compound C at the indicated glucose concentrations in serum-free DMEM for 15 hr. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as Figure 1. The results are representative of three independent experiments. Conc., concentration; glu., glucose.

inhibitory role on gluconeogenesis by decreasing the hepatic energy state (31). Treatment of cells with metformin activated AMPK and suppressed replication of HCV in a concentration-dependent manner in Huh-7.5 cells (Fig. 3a). However, co-incubation of cells with compound C, an inhibitor of AMPK, did not prevent metformin-mediated suppression of HCV replication (Fig. 3b, lane 3). Relatively speaking, compound C enhances the suppression of HCV replication induced by metformin. Likewise, compound C promoted suppression of HCV replication when the cells were cultured under conditions of glucose shortage (Fig. 3c, lane 3). These results demonstrate that glucose shortage and metformin inhibit HCV replication independently of AMPK activation.

The effects of adenosine monophosphate-activated protein kinase activators/inhibitor on hepatitis C virus non-structural protein 3

Finally, we tested the effects of AMPK activators/inhibitor on the expression of other HCV protein besides core protein (Fig. 4). As shown, treatment of cells with AICAR, metformin or glucose shortage suppressed the expression of NS3 protein. In addition, compound C attenuated the anti-HCV effect of AICAR, whereas it enhanced the anti-HCV effect of metformin or glucose shortage. These results support the conclusion that AMPK activators/inhibitor affect the replication of HCV, as demonstrated by the expression of HCV core protein (Figs. 1–3).

DISCUSSION

Previous reports have suggested that HCV infection directly causes insulin resistance, resulting in the progression of diabetes (32,33). Moreover it has been reported that insulin resistance is a negative predictor of the response to antiviral therapy in chronic hepatitis C patients treated with peginterferon plus ribavirin (4). However, the association between virus proliferation and hyperglycemia due to insulin resistance remains elusive. In this study, we have demonstrated that HCV proliferation is promoted in Huh-7.5 cells cultured at 4.5 g/L glucose, the equivalent of the blood glucose concentrations of diabetes patients (Fig. 1). This result suggests that intensive control of glucose concentrations would aid antiviral therapy in hepatitis C patients with diabetes.

We have demonstrated that activation of AMPK suppresses HCV replication (Fig. 2). This result suggests that AMPK as a potential target for the treatment of chronic hepatitis C. Therapeutic interest has recently been increased by the findings that hepatic AMPK is activated by adiponectin (34) and by thiazolidinedione-type antidiabetic drugs (35). Pharmacological activation of AMPK may provide a new strategy for both the management of chronic hepatitis C itself and metabolic hepatic disorders linked to HCV infection.

Adenosine monophosphate-activated protein kinase, a major energy sensor, is activated by energy depletion. AMPK is directly activated by AICAR, being metabolized to ZMP in the cell, regardless of the cellular energy status (6). We have demonstrated that AICAR-mediated suppression of HCV proliferation is AMPK-dependent

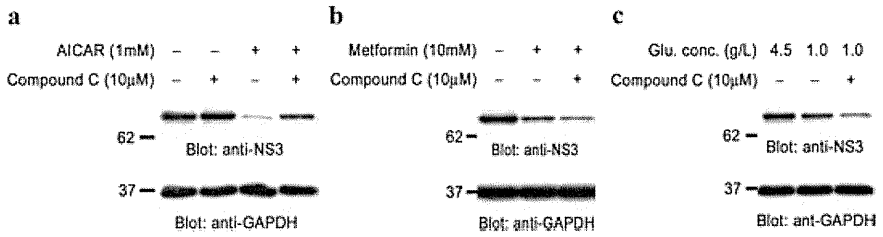

Fig. 4. AMPK activators/inhibitors' effect on the expression of HCV NS3 protein. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. The cells were treated with the indicated reagents. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-HCV NS3 and anti-GAPDH antibodies. The results are representative of three independent experiments.

(Fig. 2). In terms of phosphorylation of AMPK, the most effective concentration of AICAR was 0.5 mM, whereas the most effective concentration of AICAR for suppression of HCV replication was clearly 1.0 mM. One of the possible explanations of this discrepancy is that the former immunoblot shows the state of AMPK phosphorylation at the endpoint of the experiment, whereas the latter immunoblot reflects the accumulation of the expression of core protein during the whole period of the experiment. Compound C, a specific AMPK inhibitor that competes with ATP (36), could inhibit the effect of AICAR on HCV proliferation (Fig. 2b). Compound C did not completely reverse the suppressive effect of AICAR treatment on HCV replication. In general, inhibitors do not completely suppress the effect of reagents or enzymes; however it is still possible that the suppression of HCV replication by AICAR cannot be explained purely by activation of AMPK. Uridine as a source of pyrimidine could also prevent the effect of AICAR (Fig. 2c). Moreover, we focused on another mechanism of AICAR-mediated inhibition of HCV replication. Activated AMPK causes inhibitions of fatty acids and cholesterol synthesis. Recent reports have shown a crucial involvement of fatty acids, cholesterol and lipid droplets in infectious virion production (37–40). Therefore, we predicted that AICAR-mediated inhibition of HCV replication might be due to lipid depletion. To investigate this possibility, we added mevalonolactone and/or oleic acid in the presence of AICAR to the cell culture medium, and then examined the replication of HCV. However, the addition of lipids had almost no effect on AICAR-mediated suppression of HCV (Fig. S1). This suggests that HCV replication does not require additional lipids when the cells are treated with AICAR, which shifts cellular metabolism from energy expenditure to energy production by activating AMPK.

Cell confluency is known to activate AMPK, and LKB1 is a major kinase that activates AMPK. Replication of the HCV replicon is known to be inhibited in confluent Huh-7 cells (41). Replication of HCV replicon in HeLa cells, a known LKB1-deficient cell line, is not affected by their confluence (42). These data suggest that confluence-mediated suppression of HCV replication requires the LKB1-AMPK pathway. Our experiments demonstrated that confluence of cells can activate AMPK and suppress replication of HCV in Huh-7.5 cells (Fig. S2). It is still not clear whether this anti-HCV effect is due to relative undernutrition resulting from increased cell numbers or the activation of AMPK by the confluence itself.

Culturing cells under a shortage of glucose or with metformin can activate cellular AMPK and suppress replication of HCV in the cells (Fig. 1 and 3). Under such low energy conditions, compound C, a specific AMPK inhibitor, can induce further suppression of HCV replication. The explanation of this phenomenon is as follows: AMPK is activated in order to restore energy status. In the presence of glucose depletion or energy limitations by metformin, compound C-induced AMPK inhibition may lead to failure to maintain ATP concentrations. Various compensatory mechanisms may maintain intracellular ATP concentrations. In other words, under energy limitations the breakdown of the fuel gauge, AMPK, may proceed to imbalance in metabolism leading to poor replication of HCV. Recent reports have shown that metformin therapy is associated with a reduced hepatocarcinogenesis risk in type 2 diabetes patients (43) and an improvement of sustained virological response in chronic hepatitis C patients (44). The present study provides evidence for the possibility that not only metformin monotherapy, but also AMPK inhibitor and metformin combination therapy, may be helpful in the treatment of chronic hepatitis C.

In a previous study using JFH-1 strain of HCV and Huh-7 cells, it was reported that HCV-infection causes $Ser^{485/491}$ phosphorylation of AMPK and inhibits the kinase activity of AMPK. Inhibition of AMPK facilitates HCV replication (26). In the present study using Huh-7.5 cells and a J6/JFH1-derived, cell-adapted strain of HCV, inhibition of AMPK by HCV replication was not observed. Moreover, phosphorylation of $Ser^{485/491}$ was not affected by HCV-infection (Fig. 1c). Since this experimental system using Huh-7.5 and the cell-adapted HCV strain produces

infectious HCV particles efficiently, inhibition of AMPK by HCV replication may play a minor role in efficient HCV replication. In addition, a previous study having shown that AMPK activators suppress HCV replication, we further investigated the mechanisms of AMPK involvement in HCV replication by using a specific AMPK inhibitor. AICAR-induced AMPK activation plays a critical role in the suppression of HCV (Fig. 2), meanwhile AMPK inhibitor rather potentiates the anti-HCV effects of metformin or glucose shortage (Fig. 3). These data suggest that AMPK activation does not simply lead to an anti-HCV effect.

In conclusion, we have shown the replication of HCV by AMPK-dependent and -independent mechanisms in Huh-7.5 cells. HCV does not replicate efficiently under the low energy conditions that activate AMPK. Hence, correction of hyperglycemia in hepatitis C patients should have a beneficial effect on anti-HCV therapy and the clinical course of hepatitis C. We suggest that AMPK is a therapeutic target for the treatment of chronic hepatitis C patients.

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DISCLOSURE

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Fig. S1. Exogenous lipid has no effect on AICAR-mediated suppression of HCV. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. The cells were

treated with 1 mM AICAR for 20 hr with or without supplementation with100 μM mevalonolactone (Sigma) and 100 μM oleic acid (Sigma) (Lipid). Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as shown in Figure 1b. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments.

Fig. S2. Cell confluence activates AMPK and suppresses HCV replication. 10^5 (sub-confluent) or 4×10^5 Huh-7.5 cells (confluent) were seeded in 24-well plates overnight. 4×10^5 Huh-7.5 cells resulted in a 100% confluent monolayer of cells in the culture plates. The cells were infected with HCV at a MOI of 3.0 for 4 hr and incubated for 20 hr. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as shown in Figure 1b. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments.

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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

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

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

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

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

MATERIALS AND METHODS

Cells and Viruses

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

Human Sera and Anti-HCV Neutralization Test

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

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

Statistical Analysis

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

RESULTS

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

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

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

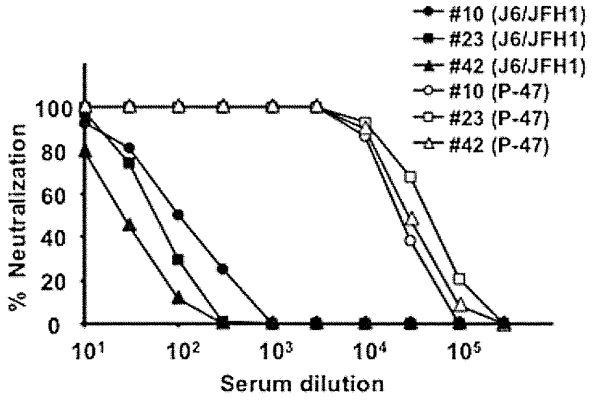

Fig. 1. Neutralization curves (NT_{50} assay) of sera obtained from HCV-infected patients against HCV J6/JFH1 and its adaptive mutant P-47. J6/JFH1 or P-47 was incubated with serial dilutions of HCV-infected patients (nos. 10, 23, and 42; all infected with HCV-1b) and tested for neutralization activities.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

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

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

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

DISCUSSION

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

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

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

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

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

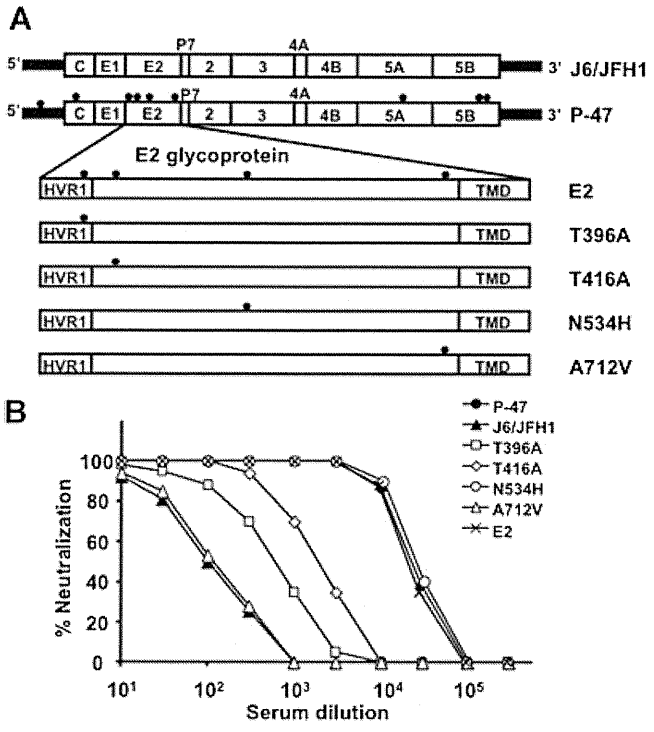

Fig. 2. Effects of amino acid mutations at positions 396, 416, 534, and 712 of the HCV E2 glycoprotein on neutralization by anti-HCV antibodies in patients' sera. **A**: A schematic diagram of the mutations seen in the adaptive mutant P-47 and recombinant viruses carrying each (T396A, T416A, N534H, and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

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

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