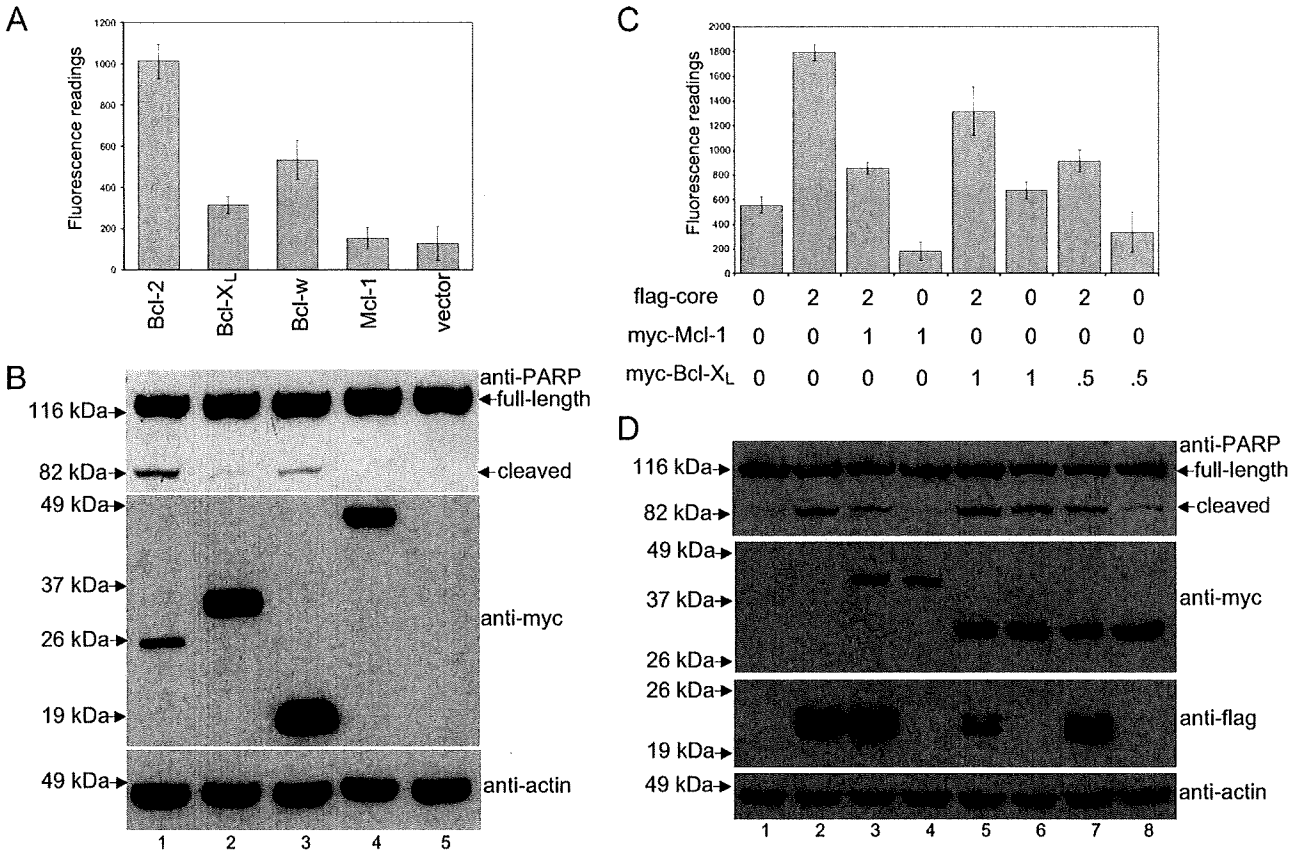

FIG. 4. Effects of Mcl-1 and Bcl-X_L overexpression on the proapoptotic property of the core protein. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were transfected with Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, or vector only. All experiments were performed in triplicate, and the average values with standard deviations are plotted. (B) Western blot analysis also was performed to determine the cleavage of endogenous PARP (top) and expression levels of the myc-tagged prosurvival members of the Bcl-2 family (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). (C) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were singly transfected with vector, the wild-type core protein, Mcl-1, or Bcl-X_L, or that were cotransfected with wild-type core protein and Mcl-1 or Bcl-X_L. The amounts of flag-core and myc-Mcl-1 or myc-Bcl-X_L DNAs used in each of the transfections are indicated in micrograms. In each transfection, the total amount of DNA was normalized to 3 μg with the addition of empty vector if necessary. All experiments were performed in triplicate, and the average values with standard deviations are plotted. (D) Western blot analysis also was performed to determine the cleavage of endogenous PARP (top) and expression levels of myc-tagged Mcl-1 and Bcl-X_L and flag-tagged core protein (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

with A completely abolishes the proapoptotic property of the core protein. On the other hand, the replacement of the highly conserved D124 residue with A seems to increase the proapoptotic property of the core protein slightly. The levels of activated caspase-3 induced by the wild-type core protein and the D124A substitution mutant in six independent experiments were compared using the two-tailed Student's *t* test, and the difference was found to be statistically significant (Fig. 6C). This phenomenon has not been reported for other BH3-only proteins, but there are a few known functional BH3 domains that do not contain D at this position (54, 55).

Furthermore, coimmunoprecipitation experiments showed that the L119A, V122A, and L126A substitution mutants have reduced binding to Mcl-1 (Fig. 7A). Similar results were obtained in four independent experiments, and the percentages of binding compared to that of the wild-type core protein were estimated by using an imaging densitometer to measure the intensity of the core protein signals after coimmunoprecipitation. For each experiment, three different autoradiographs (with different exposure times) were used, and the average values are shown in Table S1 in the supplemental material. The average percentages in binding of Mcl-1 to the L119A, V122A, and L126A mutants are 33, 62, and 9% of the binding to the wild-type core protein, respectively. For all three mutants, the reduced interactions with Mcl-1 compared to those of the wild-type core protein are statistically significant (see Table S1 in the supplemental material). As the D124A substitution mutant induced a slightly higher level of apoptosis than the wild-type core protein (Fig. 6), a coimmunoprecipitation experiment also was performed to determine if this mutant can bind Mcl-1. Two different amounts of flag-tagged plasmids (0.5 and 1.0 μg) were used, and the results show that the D124A substitution mutant binds Mcl-1 to an extent similar to that of the wild-type core protein under both conditions (Fig. 7B).

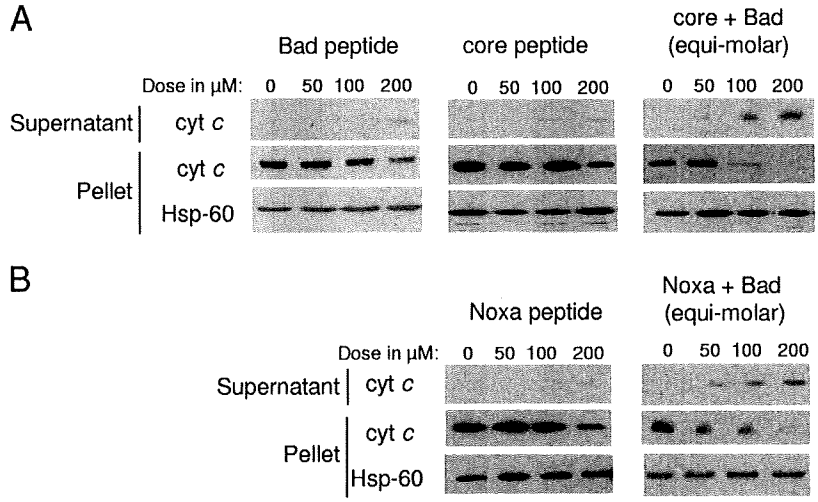

FIG. 5. Release of cytochrome *c* from isolated mitochondria by a combination of the core-BH3 and Bad-BH3 peptides or Noxa-BH3 and Bad-BH3 peptides. (A) Mitochondria isolated from 293T cells were incubated with either Bad peptide, core protein peptide, or a combination of the two peptides in equimolar concentrations. The total amount of peptide used in each experiment is indicated. Following centrifugation, the supernatants and pellets were subjected to Western blot analysis with anti-cytochrome *c* (cyt *c*) or anti-Hsp-60 antibodies. (B) The same experiment was repeated with Noxa peptide or a combination of Noxa and Bad peptides in equimolar concentrations.

While the V122 and L126 residues, at the h3 and h4 positions, respectively, of the core protein are highly conserved in different genotypes of HCV, the core proteins from genotype 2a strains typically have V instead of L at the h2 position (Fig. 1B). Replacing the L119 of the genotype 1b core protein with V reduced the proapoptotic property of the core protein dramatically (Fig. 8). Interestingly, in all known BH3-only proteins, this position is usually an L residue that is essential for the proapoptotic properties of these proteins (Fig. 1A). The reverse experiment was performed by determining if the core protein of a genotype 2a strain (JFH-1 strain) can induce apoptosis. The overexpression of the genotype 2a core protein induced much less apoptosis than the genotype 1b core protein (Fig. 8). However, replacing the V119 of the genotype 2a core protein with L resulted in a significant increase in apoptosis induction, such that the level was similar to that induced by the genotype 1b core protein (Fig. 8).

A single substitution from V to L at residue 119 in the core protein of the HCV J6/JFH-1 strain is associated with increased abilities to induce apoptosis. The pFL-J6/JFH-1 plasmid encoding the entire viral genome of a chimeric strain of HCV genotype 2a (J6/JFH-1) can be used to generate infectious HCV (37). In the J6/JFH-1 clone, the core protein contains V at residue 119, just like the JFH-1 clone. A mutant virus, J6/JFH-1(V119L), was generated successfully by replacing the V119 residue with L. Parental J6/JFH-1 and mutant J6/JFH-1(V119L) viruses then were used to infect naïve Huh7.5 cells, and cell viabilities were measured at different time points after infection (Fig. 9A). From day 2 p.i., cells infected by either virus have lower viabilities than mock-infected cells, indicating that the viruses have induced cytopathic effects (CPE). This is consistent with recent observations by us and other researchers (17, 41). Results from days 6 and 8 p.i. show that the J6/JFH-1(V119L) virus induced higher levels of CPE and, therefore, lower levels of cell viability compared to those of the parental J6/JFH-1 virus (Fig. 9A), which is in agreement with the overexpression studies shown in Fig. 8. The CPE is mediated primarily through apoptosis, as indicated by the activation of caspase-3 (Fig. 9B) and DNA fragmentation (Fig. 9C). The production of cell-free infectious virus particles by the J6/JFH-1(V119L) virus also was significantly higher than that produced by the parental J6/JFH-1 virus (Fig. 9D). On the other hand, there was no significant difference in the percentage of HCV-infected cells in the cultures (Fig. 9E) or HCV RNA replication in the cells between the two viruses (Fig. 9F). We next analyzed the possible interaction between endogenous Mcl-1 and the core proteins of either J6/JFH-1 or J6/JFH-1(V119L) in virus-infected cells. As shown in Fig. 9G, the core protein of J6/JFH-1(V119L) was coimmunoprecipitated with Mcl-1 (lane 6). On the other hand, the Mcl-1 interaction of the core protein of J6/JFH-1 was barely detected under the same experimental conditions (lane 4). These results collectively imply the possibility that the V119L mutation of the core protein promotes its interaction with Mcl-1 and is responsible for the increased ability of the virus to induce apoptosis, which favors a higher degree of infectious progeny virus release from the host cell at the late time points of infection compared to that of the parental J6/JFH-1 virus.

DISCUSSION

Besides playing important roles in maintaining homeostasis in healthy cells through the regulation of apoptosis, members of the Bcl-2 family also are involved in viral infections. Indeed, several viruses have been shown to encode homologs of prosurvival Bcl-2 proteins, and these viral proteins act to inhibit apoptosis in infected cells and prevent the premature death of these cells (see reviews in references 14, 26, 51, and 70). Other viral proteins, which can be proapoptotic, prosurvival, or both, do not share any sequence homology with members of the Bcl-2 family but also can modulate apoptosis in the host cells

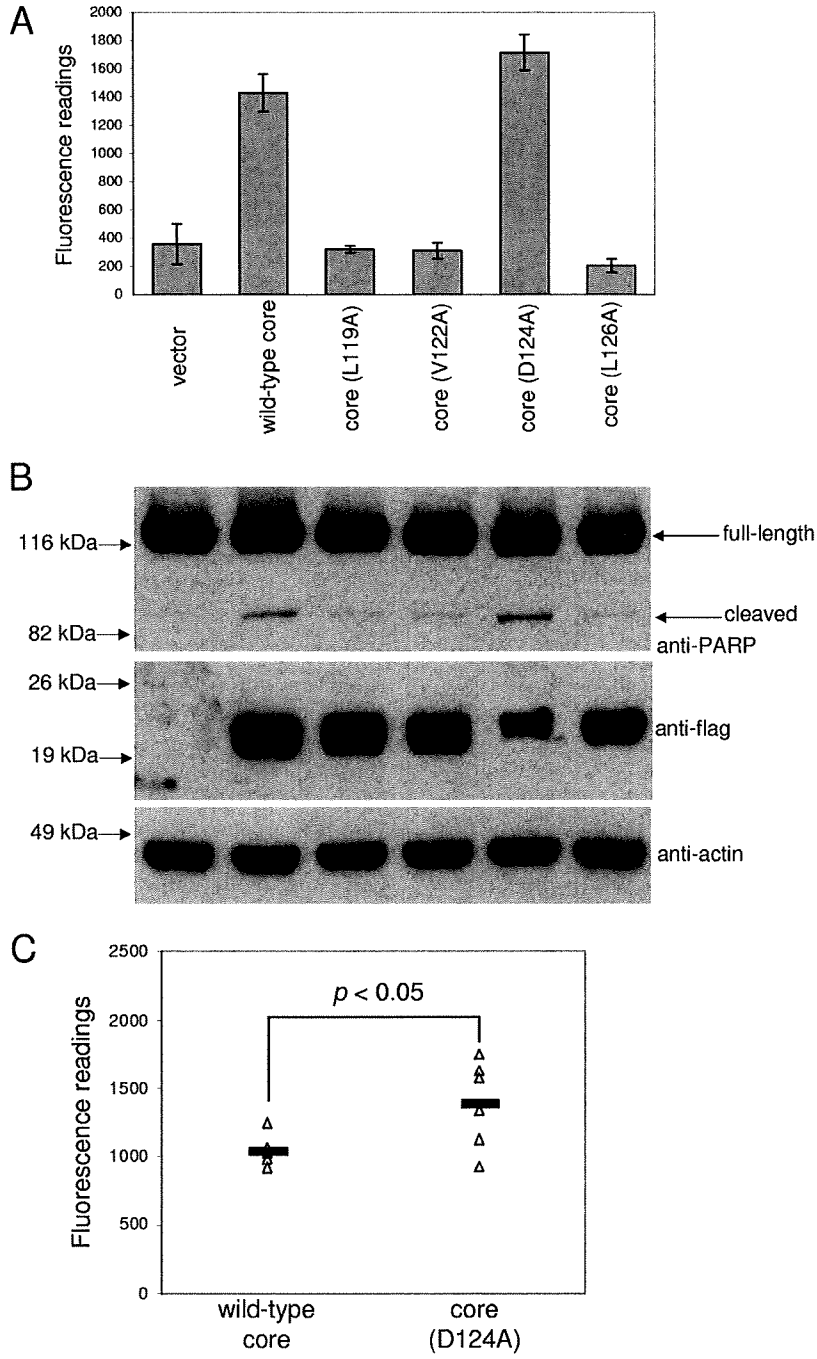

FIG. 6. Effects of alanine substitutions on the proapoptotic property of the core protein. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, wild-type core, or alanine substitution mutants. All experiments were performed in triplicate, and the average values with standard deviations are plotted. (B) Western blot analysis also was performed to determine the cleavage of endogenous PARP (top) and expression levels of the core proteins (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). (C) The levels of activated caspase-3 induced by the wild-type core protein and the D124A mutant in six independent experiments were compared using the two-tailed Student's t test, and the difference was found to be statistically significant ($P < 0.05$). The values from each of the experiments are plotted as open triangles, and the average values are plotted as solid lines.

by interfering at different apoptotic checkpoints (see reviews in references 8, 23, 27, and 43).

Unlike the multi-BH domain members, the BH3-only members of the Bcl-2 family contain a single BH3 domain. Although all BH3-only proteins can bind to the hydrophobic grooves on the surfaces of the prosurvival members, recent quantitative measurements have revealed that the affinities of association between different pairs of BH3-only and prosur-

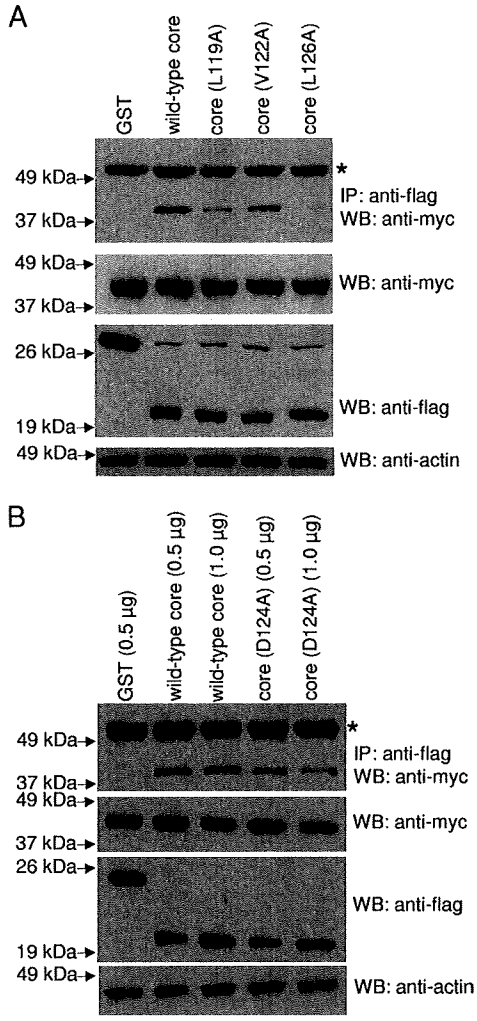

FIG. 7. Effects of alanine substitutions on the binding of the core protein to Mcl-1. (A) Huh7 cells were transfected with cDNA constructs (1.0 μg) for expressing flag-GST (negative control), flag-tagged wild-type core protein, or single-alanine-substitution mutants (L119A, V122A, and L126A). All cells were cotransfected with myc-tagged Mcl-1 (1.5 μg). (B) Huh7 cells were transfected with cDNA constructs for expressing flag-GST (negative control, 0.5 μg), flag-tagged wild-type core protein (0.5 or 1.0 μg), or single-alanine-substitution mutant D124A (0.5 or 1.0 μg). All cells were cotransfected with myc-tagged Mcl-1 (1.5 μg). Coimmunoprecipitation then was performed as described in the legend to Fig. 3A. The amount of myc-tagged proteins that coimmunoprecipitated (IP) with the flag-tagged proteins was determined by Western blot analysis (WB) with an anti-myc rabbit polyclonal antibody (top). The amounts of myc-tagged and flag-tagged proteins in the lysates before IP were determined by subjecting aliquots of the lysates to Western blot analysis (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). The protein marked with an asterisk represents the heavy chain of the antibody used for IP (top). Similar results were obtained in four independent experiments, and a representative set of data is presented.

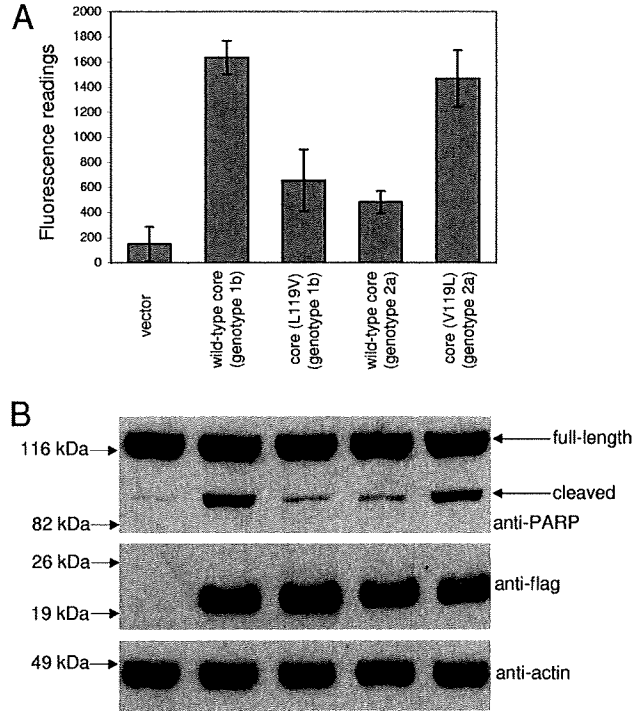

FIG. 8. Comparison of the proapoptotic properties of the core proteins of genotypes 1b and 2a. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, wild-type core of genotype 1b or 2a, or their substitution mutants. All experiments were performed in triplicate, and the average values with standard deviations are plotted. (B) Western blot analysis also was performed to determine the cleavage of endogenous PARP (top) and expression levels of the core proteins (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

vival members vary greatly (11, 32). For example, Bim and Puma bind all prosurvival members tested, while Noxa binds strongly only to Mcl-1 and A1. On the other hand, Bad binds much more strongly to Bcl-2, Bcl-X_L, and Bcl-w than Mcl-1. Taken together with results from successive studies, it becomes clear that the BH3-only members can be classified into subclasses (see reviews in references 21, 24, 58, and 71). In this study, we demonstrate that the HCV core protein is a BH3-only viral homologue of the Bcl-2 family, and its BH3 domain is essential for the induction of apoptosis (Fig. 1 and 2). In coimmunoprecipitation experiments, the core protein interacted specifically with the prosurvival Mcl-1 protein but not with prosurvival proteins Bcl-X_L and Bcl-w (Fig. 3), suggesting that its property is most similar to that of Noxa (11). Consistently, the overexpression of Mcl-1 protects against core protein-induced apoptosis (Fig. 4). However, the overexpression of Bcl-X_L also protects against core protein-induced apoptosis (Fig. 4). This may be due to the ability of a high level of Bcl-X_L to prevent the complementation between the core protein and endogenous Bad protein, which binds strongly to Bcl-X_L (11), as we have observed that a combination of the core protein and Bad peptide mimetics caused efficient cytochrome *c* release from the mitochondria (Fig. 5). The complementation between Bad and the core protein is similar to that observed between Bad and Noxa, which act in combination to neutralize the two classes of prosurvival proteins, one comprised of Bcl-2, Bcl-X_L, and Bcl-w and the other of Mcl-1 and A1 (11). In overexpres-

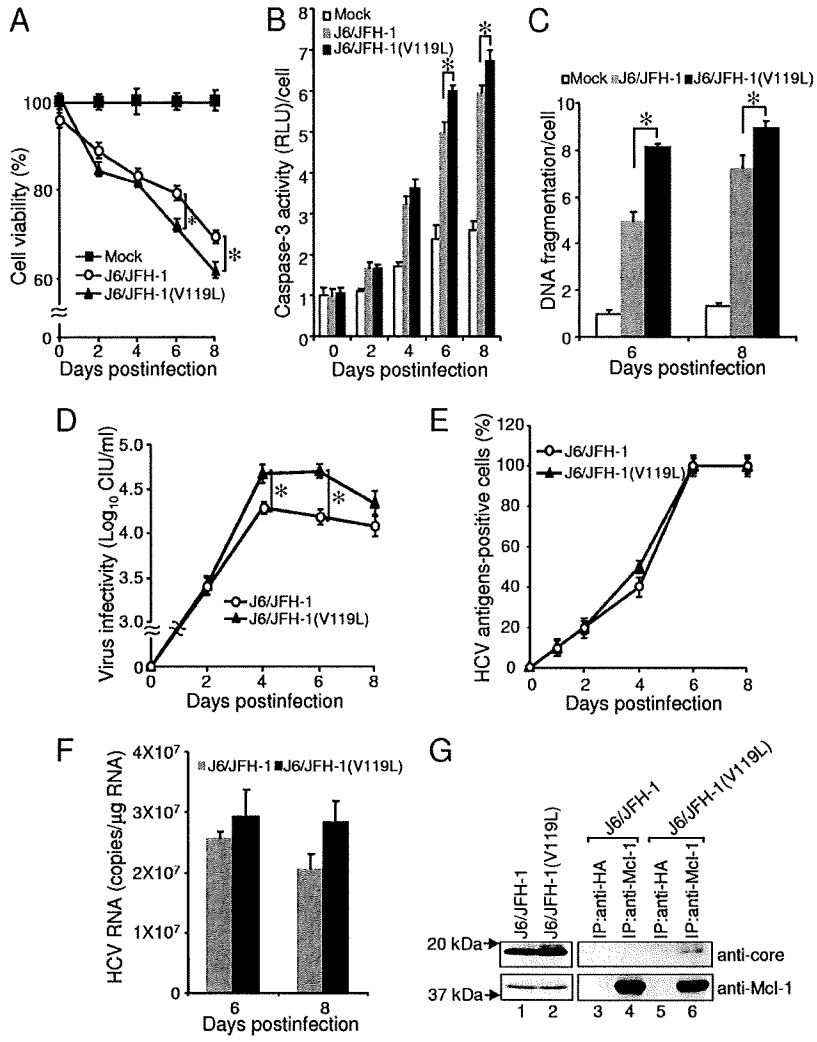

FIG. 9. Comparison of parental J6/JFH-1 and mutant J6/JFH-1(V119L) recombinant viruses. Huh7.5 cells were infected with recombinant HCV at a multiplicity of infection of 0.1 CIU/cell or with a mock preparation, and various assays were performed at different days after infection. (A) Cell viabilities were determined. (B) Caspase-3 activity per cell was determined. (C) The amount of DNA fragmentation per cell was determined. (D) The production of cell-free infectious virus particles was determined. (E) Virus spread in the culture was quantitated. (F) HCV RNA replication was determined by quantitative real-time PCR analysis. (G) Interaction of the core protein with Mcl-1 was determined by coimmunoprecipitation experiments at 3 days p.i. IP was performed using anti-Mcl-1 or anti-HA rabbit polyclonal antibodies and protein A agarose beads. The amounts of the core protein in the lysates before (lanes 1 and 2) and after IP (lanes 3 to 6) were determined by Western blot analysis with an anti-core monoclonal antibody (top). Similarly, the amounts of endogenous Mcl-1 in the samples were determined using an anti-Mcl-1 monoclonal antibody (bottom). Statistical analysis was performed using the one-way analysis of variance to determine if the differences between parental and mutant viruses were statistically significant, and those with P values of <0.05 (marked by asterisks) are considered statistically significant. Data were obtained from three independent experiments, each with triplicate cultures.

sion studies, the core protein and Noxa also induced comparable levels of apoptosis (see Fig. S3 and S5 in the supplemental material). Taken together, these findings suggest that core can mimic Noxa and interfere directly with the prosurvival function of Mcl-1.

A comparison of the BH3 domain of the core protein to the corresponding domains of other BH3-containing proteins (Fig. 1A) revealed that it contains three out of the four hydrophobic residues that can be accommodated within the hydrophobic pockets of previously described BH3 binding grooves (see reviews in references 50 and 69). Alanine substitution experiments revealed that all three hydrophobic residues in the BH3 domain of the core protein are essential for apoptosis induction (Fig. 6). In coimmunoprecipitation experiments, these alanine substitution mutants also bound Mcl-1 to a lesser extent than the wild-type core protein (Fig. 7A). Since these alanine substitution mutants still can bind Mcl-1, albeit at a lower level than that of the wild-type core protein, it appears that the interactions between these mutants and Mcl-1 are not sufficient to induce apoptosis. In several mutagenesis studies, the interaction between Bcl-2 family members and apoptosis regulation have been observed to be discordant. For example, two mutants of the BH3-only protein Bik, Bik-(43-94) and Bik-(43-120), heterodimerized with prosurvival Bcl-2 and Bcl-X_L but

were unable to induce efficient cell death (19). A Bad mutant containing an alteration of a critical residue within its BH3 domain, E113 to K, also was found to have significantly reduced apoptotic activity compared to that of wild-type Bad, despite binding to Bcl-2 and/or Bcl-X_L to the same extent as wild-type Bad (35). Therefore, the induction of apoptosis by the core protein may be controlled by a critical threshold affinity of binding between the core protein and Mcl-1, or there are contributions from a yet-to-be characterized pathway(s). Two of these residues (V122 and L126) are conserved in the major genotypes of HCV, but residue 119 is a V in genotype 2a (Fig. 1B). When L119 of the genotype 1b core protein was replaced with V, its ability to induce apoptosis was greatly reduced (Fig. 8). Conversely, when V119 of the genotype 2a core protein was replaced with L, its ability to induce apoptosis was greatly enhanced. Thus, the results suggest that the genotype 1b core protein induces apoptosis efficiently via a BH3 domain, while genotype 2a core protein is comparatively less efficient. Another highly conserved residue in the BH3 domain of the core protein is D124. However, the replacement of D124 with A did not reduce the proapoptotic function of the core protein (Fig. 6). Thus far, there are only a few known functional BH3 domains that do not contain D at this position (61, 62). Unlike most BH3-only proteins, the core protein has a charged residue (R115) in the h1 position (Fig. 1A). Interestingly, the second BH3 domain of mouse Noxa (mNoxaB) also has a charged residue (E74) in this position. Indeed, the nuclear magnetic resonance structure of the complex between mouse Mcl-1 and a peptide mimetic of mNoxaB shows that E74 is tolerated at the h1 position because its charged carboxyl group is coordinated by another charged residue, K215, in mouse Mcl-1 (15). However, R115 of the core protein is basic instead of acidic, and how this residue can be accommodated in the hydrophobic groove of Mcl-1 is unclear. Interestingly, replacing the residue at the h1 position (I58) of a novel BimBH3 variant, Bim_S2A, with A also has little effect on its interaction with Mcl-1 (34). Thus, it appears that the residue in the h1 position is not always involved in the interaction between BH3-only proteins and Mcl-1, but further biophysical and biochemical studies are required to delineate the precise structure-function relationship for the interaction between core and Mcl-1.

To determine if the results from overexpression studies are relevant to the modulation of apoptosis in host cells during HCV infection, the J6/JFH-1-based (genotype 2a) system was used to generate HCV carrying a substitution at residue 119 of the core protein. While the parental wild-type and mutant viruses replicated efficiently in Huh7.5 cells, the J6/JFH-1(V119L) virus (which expresses the core protein with L at the h2 position of the BH3 domain) caused a significantly higher level of apoptosis in the infected cells than the parental J6/JFH-1 virus (which expresses the core protein with V at the h2 position of the BH3 domain) (Fig. 9). This is in good agreement with the overexpression studies and indicates that the BH3 domain of the core protein contributes to the induction of apoptosis in HCV-infected cells. Thus, it appears that core protein-mediated apoptosis during infection by HCV of genotype 2a is less efficient than that of the other genotypes having L at residue 119 of the core protein (Fig. 1B). Coimmunoprecipitation experiments revealed that the core protein of J6/JFH-1(V119L), but not that of J6/JFH-1, interacted with Mcl-1 in virus-infected cells (Fig. 9). This result is consistent with the overexpression studies and suggests the possibility that the core protein induces apoptosis, at least partly, through the interaction with Mcl-1 in HCV-infected cells. Interestingly, more progeny virus is released from cells infected with the J6/JFH-1(V119L) virus than by those infected with the parental J6/JFH-1, while there is no difference in the efficiency of infection or amount of HCV replication inside the cells (Fig. 9).

However, it also is apparent that the parental J6/JFH-1 virus still caused a high level of apoptosis in the infected cells, and for the early time points there was no significant difference in the levels of apoptosis induced by the parental J6/JFH-1 virus and the J6/JFH-1(V119L) mutant virus (Fig. 9). This implies that there are other viral factors that contribute to the induction of apoptosis during HCV infection. For example, several nonstructural HCV proteins, like NS3, NS4A, NS5A, and NS5B, can induce apoptosis when they are overexpressed in certain types of cells (see recent reviews in references 20 and 28). In addition, other domains in the core protein have been shown to bind host proteins and may contribute to apoptosis regulation by interfering with different cellular pathways (see reviews in references 33, 42, and 52). For example, the N-terminal domain (aa 1 to 75) of the core protein interacts with Hsp60, leading to the production of reactive oxygen species and enhancement of tumor necrosis factor alpha-mediated apoptosis (30), while a C-terminal domain (aa 153 to 192) facilitates Fas oligomerization and is required for apoptosis induction in Jurkat cells (46). However, the relative contribution of these various factors to apoptosis induction during HCV infection remains to be determined.

We further examined the importance of residue 119 of the core protein in HCV replication. In multiple independent transfection experiments, we observed that the J6/JFH-1 mutant possessing A at position 119 [J6/JFH-1(V119A)] barely replicated in the cells and did not produce any infectious virus particles in the culture supernatants (data not shown). This result suggests the possibility that this single point mutation impairs the interaction of the core protein with other viral and/or cellular protein(s) that is required for HCV RNA replication and infectious virion production. Similarly, the J6/JFH-1 mutants each possessing A at positions 122 [J6/JFH-1(V122A)], 124 [J6/JFH-1(D124A)], or 126 [J6/JFH-1(L126A)] barely replicated in the cells and did not produce any infectious virion in the culture supernatants (data not shown), with the results suggesting an important role(s) for these residues as well as for position 119. In this connection, the essential role for the HCV core protein in infectious virion production recently has been confirmed, and numerous residues required for this role have been identified (47).

By using the JFH-1 infectious clone, recent studies have revealed that the association of the core protein with the lipid droplet (LD) is critical for the production of infectious virus particles (6, 45). Boulant and coworkers reported that there are two amphipathic α-helices in the so-called D2 domain of the core protein (~118 to 179 aa) (5, 7), and the hydrophobic residues within this domain are critical for the efficient attachment of the core protein to LD (5). Our results showed that residues L119, V122, and L126 of the core protein are essential

for the induction of apoptosis, and these residues are found on the hydrophobic face of the first α-helix of the D2 domain. Interestingly, the replacement of L119 with E did not affect LD association, while the replacement of L126 with E significantly reduced LD association (5). The contribution of V122 to LD association was not investigated. Consistently, the J6/JFH-1(V119L) virus, but not the J6/JFH-1(L126A) virus, replicated efficiently to produce infectious virus particles. Since L119 of the genotype 1b core protein, which occupies the crucial h2 position in the BH3 domain, is essential for its proapoptotic property but not for its association with LD, it is clear that the BH3 domain of the core protein is an independent motif that partially overlaps with the LD association domain.

Recently, Makes caterpillars floppy 1 (Mcf1), a bacterial toxin, was reported to contain a BH3-like domain (18). In addition, HBSP, a spliced hepatitis B viral protein, also contains a BH3-like domain (39). Here, we show that the HCV core protein is another BH3-like viral homologue, and it contributes directly to the induction of apoptosis during HCV infection. Our results also reveal that it is a bona fide BH3-only protein that appears to interfere with the prosurvival property of Mcl-1 in a manner similar to that of Noxa. Our observation that the enhanced apoptotic activity of the J6/JFH-1(V119L) virus is correlated with an increase in infectious progeny HCV release seems to be counterintuitive, as many viruses adopted strategies to prevent apoptosis in the infected cells so as to allow viral replication and the packaging of progeny genomes within the cells (14, 26, 51, 70). However, enhanced releases of virus from infected cells that are undergoing apoptosis also have been reported for other viruses, like the infectious bursal disease virus, adenovirus, and Aleutian mink disease parvovirus (4, 44, 73), indicating that apoptosis can be advantageous for viral spreading at the late stages of infection. Future studies to define the precise manner by which the BH3 domain of the core protein modulates apoptosis during infection will provide important insights into HCV replication as well as pathogenesis.

Besides the genotype 1b core protein, the properties of the genotype 1a core protein also have been examined in various studies. The apoptotic property of the genotype 1a core protein has yet to be studied using the JFH-1-based infectious clone system, although previous studies have attributed both prosurvival and proapoptotic properties to it (25, 30, 46, 57). Similar observations also have been described in overexpression studies using the genotype 1b core protein and appear to be dependent on the death stimuli and types of cells used (3, 9, 10, 36, 49, 53, 56, 60, 76). Several studies have identified domains or regions within the core protein that interfere with specific apoptosis pathways. For instance, the interaction of the N-terminal domain (residues 1 to 75) of the genotype 1a core protein with Hsp60 enhanced tumor necrosis factor alpha-mediated apoptosis, while its C-terminal region (residues 153 to 192) is required for Fas ligand-independent apoptosis (30, 46). The genotype 1b core protein (residues 1 to 153) binds to the death domain of FADD, resulting in enhanced apoptosis (76). However, an overlapping domain spanning the first 46 aa of the core protein is involved in ASPP2 interaction, which leads to the inhibition of p53-mediated apoptosis (9). These findings suggest that multiple domains present in the core protein contribute to the modulation of apoptosis via diverse pathways. Therefore, the net apoptotic effect of the core protein may be dependent on the relative strength of its prosurvival and proapoptotic properties. Unlike the genotype 2a core protein, the BH3 domains of the genotype 1b core protein and the genotype 1a core protein share an identical sequence (Fig. 1B) and are expected to function in a similar manner. However, we cannot rule out that there may be differences in the manner in which the core proteins of genotypes 1a and 1b modulate apoptosis during infection. For example, they may be involved in different virus-virus or virus-host interactions. Thus, more studies are needed to understand the contributions of genotype-dependent factors to the regulation of apoptosis during HCV infection.

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REFERENCES

1. **Adams, J. M., and S. Cory.** 2007. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr. Opin. Immunol. **19**:488–496.
2. **Bartenschlager, R., and V. Lohmann.** 2000. Replication of hepatitis C virus. J. Gen. Virol. **81**:1631–1648.
3. **Benali-Furet, N. L., M. Chami, L. Houel, F. De Giorgi, F. Vernejoul, D. Lagorce, L. Buscail, R. Bartenschlager, F. Ichas, R. Rizzuto, and P. Paterlini-Brechot.** 2005. Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. Oncogene **24**:4921–4933.
4. **Best, S. M., J. B. Wolfinbarger, and M. E. Bloom.** 2002. Caspase activation is required for permissive replication of Aleutian mink disease parvovirus in vitro. Virology **292**:224–234.
5. **Boulant, S., R. Montserret, R. G. Hope, M. Ratinier, P. Targett-Adams, J. P. Lavergne, F. Penin, and J. McLauchlan.** 2006. Structural determinants that target the hepatitis C virus core protein to lipid droplets. J. Biol. Chem. **281**:22236–22247.
6. **Boulant, S., P. Targett-Adams, and J. McLauchlan.** 2007. Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. J. Gen. Virol. **88**:2204–2213.
7. **Boulant, S., C. Vanbelle, C. Ebel, F. Penin, and J. P. Lavergne.** 2005. Hepatitis C virus core protein is a dimeric alpha-helical protein exhibiting membrane protein features. J. Virol. **79**:11353–11365.
8. **Boya, P., A. L. Pauleau, D. Poncet, R. A. Gonzalez-Polo, N. Zamzami, and G. Kroemer.** 2004. Viral proteins targeting mitochondria: controlling cell death. Biochim. Biophys. Acta **1659**:178–189.
9. **Cao, Y., T. Hamada, T. Matsui, T. Date, and K. Iwabuchi.** 2004. Hepatitis C virus core protein interacts with p53-binding protein, 53BP2/Bbp/ASPP2, and inhibits p53-mediated apoptosis. Biochem. Biophys. Res. Commun. **315**:788–795.
10. **Chang, M. L., J. C. Chen, M. Y. Chang, C. T. Yeh, W. P. Lin, C. K. Liang, S. F. Huang, K. N. Dang, C. T. Chiu, and D. Y. Lin.** 2008. Acute expression of hepatitis C core protein in adult mouse liver: mitochondrial stress and apoptosis. Scand. J. Gastroenterol. **43**:747–755.
11. **Chen, L., S. N. Willis, A. Wei, B. J. Smith, J. I. Fletcher, M. G. Hinds, P. M. Colman, C. L. Day, J. M. Adams, and D. C. Huang.** 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol. Cell **17**:393–403.
12. **Cheng, E. H., D. G. Kirsch, R. J. Clem, R. Ravi, M. B. Kastan, A. Bedi, K. Ueno, and J. M. Hardwick.** 1997. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science **278**:1966–1968.
13. **Chou, A. H., H. F. Tsai, Y. Y. Wu, C. Y. Hu, L. H. Hwang, P. I. Hsu, and P. N. Hsu.** 2005. Hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of mitochondria apoptosis signaling pathway. J. Immunol. **174**:2160–2166.
14. **Cuconati, A., and E. White.** 2002. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev. **16**:2465–2478.

15. **Czabotar, P. E., E. F. Lee, M. F. van Delft, C. L. Day, B. J. Smith, D. C. Huang, W. D. Fairlie, M. G. Hinds, and P. M. Colman.** 2007. Structural insights into the degradation of Mcl-1 induced by BH3 domains. Proc. Natl. Acad. Sci. USA **104**:6217–6222.
16. **Danial, N. N.** 2007. BCL-2 family proteins: critical checkpoints of apoptotic cell death. Clin. Cancer Res. **13**:7254–7263.
17. **Deng, L., T. Adachi, K. Kitayama, Y. Bungyoku, S. Kitazawa, S. Ishido, I. Shoji, and H. Hotta.** 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondria-mediated, caspase-3-dependent pathway. J. Virol. **82**:10375–10385.
18. **Dowling, A. J., N. R. Waterfield, M. C. Hares, G. Le Goff, C. H. Streuli, and R. H. ffrench-Constant.** 2007. The Mcf1 toxin induces apoptosis via the mitochondrial pathway and apoptosis is attenuated by mutation of the BH3-like domain. Cell Microbiol. **9**:2470–2484.
19. **Elangovan, B., and G. Chinnadurai.** 1997. Functional dissection of the pro-apoptotic protein Bik. Heterodimerization with anti-apoptosis proteins is insufficient for induction of cell death. J. Biol. Chem. **272**:24494–24498.
20. **Fischer, R., T. Baumert, and H. E. Blum.** 2007. Hepatitis C virus infection and apoptosis. World J. Gastroenterol. **13**:4865–4872.
21. **Fletcher, J. I., and D. C. Huang.** 2008. Controlling the cell death mediators Bax and Bak: puzzles and conundrums. Cell Cycle **7**:39–44.
22. **Fu, N. Y., S. K. Sukumaran, and V. C. Yu.** 2007. Inhibition of ubiquitin-mediated degradation of MOAP-1 by apoptotic stimuli promotes Bax function in mitochondria. Proc. Natl. Acad. Sci. USA **104**:10051–10056.
23. **Galluzzi, L., C. Brenner, E. Morselli, Z. Touat, and G. Kroemer.** 2008. Viral control of mitochondrial apoptosis. PLoS Pathog. **4**:e1000018.
24. **Häcker, G., and A. Weber.** 2007. BH3-only proteins trigger cytochrome *c* release, but how? Arch. Biochem. Biophys. **462**:150–155.
25. **Hahn, C. S., Y. G. Cho, B. S. Kang, I. M. Lester, and Y. S. Hahn.** 2000. The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. Virology **276**:127–137.
26. **Hardwick, J. M., and D. S. Bellows.** 2003. Viral versus cellular BCL-2 proteins. Cell Death Differ. **10**(Suppl. 1):S68–S76.
27. **Hay, S., and G. Kannourakis.** 2002. A time to kill: viral manipulation of the cell death program. J. Gen. Virol. **83**:1547–1564.
28. **Herzer, K., M. F. Sprinzl, and P. R. Galle.** 2007. Hepatitis viruses: live and let die. Liver Int. **27**:293–301.
29. **Hope, R. G., and J. McLauchlan.** 2000. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J. Gen. Virol. **81**:1913–1925.
30. **Kang, S. M., S. J. Kim, J. H. Kim, W. Lee, G. W. Kim, K. H. Lee, K. Y. Choi, and J. W. Oh.** 2009. Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-alpha-mediated apoptosis. Cancer Lett. **279**:230–237.
31. **Kozopas, K. M., T. Yang, H. L. Buchan, P. Zhou, and R. W. Craig.** 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc. Natl. Acad. Sci. USA **90**:3516–3520.
32. **Kuwana, T., L. Bouchier-Hayes, J. E. Chipuk, C. Bonzon, B. A. Sullivan, D. R. Green, and D. D. Newmeyer.** 2005. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol. Cell **17**:525–535.
33. **Lai, M. M., and C. F. Ware.** 2000. Hepatitis C virus core protein: possible roles in viral pathogenesis. Curr. Top. Microbiol. Immunol. **242**:117–134.
34. **Lee, E. F., P. E. Czabotar, M. F. van Delft, E. M. Michalak, M. J. Boyle, S. N. Willis, H. Puthalakath, P. Bouillet, P. M. Colman, D. C. Huang, and W. D. Fairlie.** 2008. A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation. J. Cell Biol. **180**:341–355.
35. **Lee, J. W., Y. H. Soung, S. Y. Kim, S. W. Nam, C. J. Kim, Y. G. Cho, J. H. Lee, H. S. Kim, W. S. Park, S. H. Kim, J. Y. Lee, N. J. Yoo, and S. H. Lee.** 2004. Inactivating mutations of proapoptotic Bad gene in human colon cancers. Carcinogenesis **25**:1371–1376.
36. **Lee, S. K., S. O. Park, C. O. Joe, and Y. S. Kim.** 2007. Interaction of HCV core protein with 14-3-3epsilon protein releases Bax to activate apoptosis. Biochem. Biophys. Res. Commun. **352**:756–762.
37. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
38. **Liu, Q., C. Tackney, R. A. Bhat, A. M. Prince, and P. Zhang.** 1997. Regulated processing of hepatitis C virus core protein is linked to subcellular localization. J. Virol. **71**:657–662.
39. **Lu, Y. W., T. L. Tan, V. Chan, and W. N. Chen.** 2006. The HBSP gene is expressed during HBV replication, and its coded BH3-containing spliced viral protein induces apoptosis in HepG2 cells. Biochem. Biophys. Res. Commun. **351**:64–70.
40. **Marusawa, H., M. Hijikata, T. Chiba, and K. Shimotohno.** 1999. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-κB activation. J. Virol. **73**:4713–4720.
41. **Mateu, G., R. O. Donis, T. Wakita, J. Bukh, and A. Grakoui.** 2008. Intragenotypic JFH1 based recombinant hepatitis C virus produces high levels of infectious particles but causes increased cell death. Virology **376**:397–407.
42. **McLauchlan, J.** 2000. Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. J. Viral Hepat. **7**:2–14.
43. **McLean, J. E., A. Ruck, A. Shirazian, F. Pooyaei-Mehr, and Z. F. Zakeri.** 2008. Viral manipulation of cell death. Curr. Pharm. Des. **14**:198–220.
44. **Mi, J., Z. Y. Li, S. Ni, D. Steinwaerder, and A. Lieber.** 2001. Induced apoptosis supports spread of adenovirus vectors in tumors. Hum. Gene Ther. **12**:1343–1352.
45. **Miyanari, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno.** 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. **9**:1089–1097.
46. **Moorman, J. P., D. Prayther, D. McVay, Y. S. Hahn, and C. S. Hahn.** 2003. The C-terminal region of hepatitis C core protein is required for Fas-ligand independent apoptosis in Jurkat cells by facilitating Fas oligomerization. Virology **312**:320–329.
47. **Murray, C. L., C. T. Jones, J. Tassello, and C. M. Rice.** 2007. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J. Virol. **81**:10220–10231.
48. **Nomura-Takigawa, Y., M. Nagano-Fujii, L. Deng, S. Kitazawa, S. Ishido, K. Sada, and H. Hotta.** 2006. Non-structural protein 4A of hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. J. Gen. Virol. **87**:1935–1945.
49. **Otsuka, M., N. Kato, H. Taniguchi, H. Yoshida, T. Goto, Y. Shiratori, and M. Omata.** 2002. Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. Virology **296**:84–93.
50. **Petros, A. M., E. T. Olejniczak, and S. W. Fesik.** 2004. Structural biology of the Bcl-2 family of proteins. Biochim. Biophys. Acta **1644**:83–94.
51. **Polster, B. M., J. Pevsner, and J. M. Hardwick.** 2004. Viral Bcl-2 homologs and their role in virus replication and associated diseases. Biochim. Biophys. Acta **1644**:211–227.
52. **Ray, R. B., and R. Ray.** 2001. Hepatitis C virus core protein: intriguing properties and functional relevance. FEMS Microbiol. Lett. **202**:149–156.
53. **Realdon, S., M. Gerotto, F. Dal Pero, O. Marin, A. Granato, G. Basso, M. Muraca, and A. Alberti.** 2004. Proapoptotic effect of hepatitis C virus CORE protein in transiently transfected cells is enhanced by nuclear localization and is dependent on PKR activation. J. Hepatol. **40**:77–85.
54. **Ruggieri, A., T. Harada, Y. Matsuura, and T. Miyamura.** 1997. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology **229**:68–76.
55. **Sabile, A., G. Perlemuter, F. Bono, K. Kohara, F. Demaugre, M. Kohara, Y. Matsuura, T. Miyamura, C. Brechot, and G. Barba.** 1999. Hepatitis C virus core protein binds to apolipoprotein AII and its secretion is modulated by fibrates. Hepatology **30**:1064–1076.
56. **Sacco, R., T. Tsutsumi, R. Suzuki, M. Otsuka, H. Aizaki, S. Sakamoto, M. Matsuda, N. Seki, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2003. Anti-apoptotic regulation by hepatitis C virus core protein through up-regulation of inhibitor of caspase-activated DNase. Virology **317**:24–35.
57. **Saito, K., K. Meyer, R. Warner, A. Basu, R. B. Ray, and R. Ray.** 2006. Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. J. Virol. **80**:4372–4379.
58. **Shibue, T., and T. Taniguchi.** 2006. BH3-only proteins: integrated control point of apoptosis. Int. J. Cancer. **119**:2036–2043.
59. **Soo, H. M., A. Garzino-Demo, W. Hong, Y. H. Tan, Y. J. Tan, P. Y. Goh, S. G. Lim, and S. P. Lim.** 2002. Expression of a full-length hepatitis C virus cDNA up-regulates the expression of CC chemokines MCP-1 and RANTES. Virology **303**:253–277.
60. **Takamatsu, M., T. Fujita, and H. Hotta.** 2001. Suppression of serum starvation-induced apoptosis by hepatitis C virus core protein. Kobe J. Med. Sci. **47**:97–112.
61. **Tan, K. O., K. M. Tan, S. L. Chan, K. S. Yee, M. Bevort, K. C. Ang, and V. C. Yu.** 2001. MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. J. Biol. Chem. **276**:2802–2807.
62. **Tan, K. O., K. M. Tan, and V. C. Yu.** 1999. A novel BH3-like domain in BID is required for intramolecular interaction and autoinhibition of pro-apoptotic activity. J. Biol. Chem. **274**:23687–23690.
63. **Tan, Y. J., B. C. Fielding, P. Y. Goh, S. Shen, T. H. Tan, S. G. Lim, and W. Hong.** 2004. Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis via a caspase-dependent pathway. J. Virol. **78**:14043–14047.
64. **Tan, Y. J., E. Teng, S. Shen, T. H. Tan, P. Y. Goh, B. C. Fielding, E. E. Ooi, H. C. Tan, S. G. Lim, and W. Hong.** 2004. A novel severe acute respiratory syndrome coronavirus protein, U274, is transported to the cell surface and undergoes endocytosis. J. Virol. **78**:6723–6734.
65. **Targett-Adams, P., G. Hope, S. Boulant, and J. McLauchlan.** 2008. Maturation of hepatitis C virus core protein by signal peptide peptidase is required for virus production. J. Biol. Chem. **283**:16850–16859.
66. **Uhlmann, E. J., T. Subramanian, C. A. Vater, R. Lutz, and G. Chinnadurai.** 1998. A potent cell death activity associated with transient high level expression of BCL-2. J. Biol. Chem. **273**:17926–17932.

67. **van Delft, M. F., and D. C. Huang.** 2006. How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res. **16**:203–213.
68. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
69. **Walensky, L. D.** 2006. BCL-2 in the crosshairs: tipping the balance of life and death. Cell Death Differ. **13**:1339–1350.
70. **White, E.** 2006. Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis. Cell Death Differ. **13**:1371–1377.
71. **Willis, S. N., and J. M. Adams.** 2005. Life in the balance: how BH3-only proteins induce apoptosis. Curr. Opin. Cell Biol. **17**:617–625.
72. **Yamanaka, T., M. Uchida, and T. Doi.** 2002. Innate form of HCV core protein plays an important role in the localization and the function of HCV core protein. Biochem. Biophys. Res. Commun. **294**:521–527.
73. **Yao, K., and V. N. Vakharia.** 2001. Induction of apoptosis in vitro by the 17-kDa nonstructural protein of infectious bursal disease virus: possible role in viral pathogenesis. Virology **285**:50–58.
74. **Yasui, K., T. Wakita, K. Tsukiyama-Kohara, S. I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J. R. Wands, and M. Kohara.** 1998. The native form and maturation process of hepatitis C virus core protein. J. Virol. **72**:6048–6055.
75. **Youle, R. J., and A. Strasser.** 2008. The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. **9**:47–59.
76. **Zhu, N., C. F. Ware, and M. M. Lai.** 2001. Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. Virology **283**:178–187.

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HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters☆

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***Background/Aims*: Persistent infection with hepatitis C virus (HCV) causes extrahepatic diseases, including diabetes. We investigated the possible effect(s) of HCV replication on cellular glucose uptake and expression of the facilitative glucose transporter (GLUT) 2 and 1.**

***Methods*: We used Huh-7.5 cells harboring either an HCV subgenomic RNA replicon (SGR) or an HCV full-genomic RNA replicon (FGR), HCV-infected cells, and the respective cells treated with interferon (IFN). We also used liver tissue samples obtained from patients with or without HCV infection.**

***Results*: Glucose uptake and surface expression of GLUT2 and GLUT1 were suppressed in SGR, FGR and HCV-infected cells compared to the control cells. Expression levels of GLUT2 mRNA, but not GLUT1 mRNA, were lower in SGR, FGR and HCV-infected cells than in the control. Luciferase reporter assay demonstrated decreased GLUT2 promoter activities in SGR, FGR and HCV-infected cells. IFN treatment restored glucose uptake, GLUT2 surface expression, GLUT2 mRNA expression and GLUT2 promoter activities. Also, GLUT2 expression was reduced in hepatocytes of liver tissues obtained from HCV-infected patients.**

***Conclusions*: HCV replication down-regulates cell surface expression of GLUT2 partly at the transcriptional level, and possibly at the intracellular trafficking level as suggested for GLUT1, thereby lowering glucose uptake by hepatocytes.**

***Keywords*: Diabetes mellitus; Down-regulation; Glucose uptake; GLUT1; GLUT2; Hepatitis C virus; Hepatocyte; Interferon; Replicon**

1. Introduction

Hepatitis C virus (HCV) is a small, enveloped RNA virus, which belongs to the genus *Hepacivirus* within the family *Flaviviridae*. The viral genome consists of single-stranded, positive-sense RNA of 9.6 kb that encodes a polyprotein of about 3000 amino acids. There are six major genotypes of HCV worldwide, with each genotype being further classified into a number of subtypes, such as HCV-1a and -1b [1,2]. The polyprotein is processed by host cellular and viral proteases to yield at least 10 structural and nonstructural (NS) proteins, such

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Abbreviations: FGR, full-genome RNA replicon; GLUT, glucose transporter; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; SGR, subgenomic RNA replicon.

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as core protein, envelope glycoproteins (E1 and E2), p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [3,4].

HCV prevails in most parts of the world with an estimated number of about 170 million carriers and, hence, HCV infection is a major global healthcare problem [5]. Persistent infection with HCV causes not only liver diseases, including hepatitis, but also extrahepatic manifestations, such as type 2 diabetes [6–8]. While it has been known that liver cirrhosis impairs the glucose metabolism of the liver, there are some reports showing that HCV-infected patients over 40 years old have an increased risk for type 2 diabetes – three times higher than that for patients without HCV infection [9,10]. These reports imply the possibility that HCV infection directly predisposes the host towards type 2 diabetes. However, the precise mechanism(s) is poorly understood.

Glucose is transported into the cell via various isoforms of the facilitative glucose transporter (GLUT) that are present in most cells. Currently, a total of 14 isoforms have been identified in the GLUT family [11–13]. GLUT2 is expressed tissue-specifically in the liver, pancreatic β-cells, hypothalamic glial cells, retina and enterocytes [14]. On the other hand, GLUT1 is expressed at high levels in all fetal tissues and, in adults, it is widely expressed but most abundant in erythrocytes, endothelial cells of the blood–brain barrier, renal tubules of the kidney, and any kind of malignant cells including hepatocellular carcinoma [13].

In the present study, we demonstrated that HCV infection suppressed hepatocytic glucose uptake through down-regulation of surface expression of GLUT in a human hepatocellular carcinoma-derived cell line Huh-7.5. We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was lower than in those from patients without HCV infection. We propose that HCV replication decreases glucose uptake and cell surface expression of GLUT, which would eventually lead to glucose metabolism disorder.

2. Materials and methods

2.1. Cell culture, HCV RNA replication, HCV infection and IFN treatment

A human hepatoma-derived cell line, Huh-7.5, which is highly permissive to HCV RNA replication [15], was kindly provided by Dr. C.M. Rice (The Rockefeller University, New York, NY, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

Huh-7.5 cells stably harboring an HCV-1b subgenomic RNA replicon (referred to as SGR cells, hereafter) were prepared as describe previously [16–18], using pFK5B/2884Gly (a kind gift from Dr. R. Bartenschlager, University of Heidelberg, Heidelberg, Germany). In SGR cells, the HCV subgenomic RNA replicon autonomously replicates to express NS3 to NS5B of HCV (Fig. 1). Cells harboring a full-length HCV-1b RNA replicon derived from pON/C-5B (referred to as FGR cells, hereafter) were described previously [19,20]. In FGR cells, the genome-size HCV RNA replicon autonomously replicates to express all the HCV proteins (the core protein, E1, E2, p7, NS2, NS3 to NS5B).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 [21], 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 virus produced in the culture supernatant was used for infection experiments at multiplicities of infection of 1.0 and cultured for 5 days after virus infection.

In some experiments, SGR and FGR cells, as well as HCV-infected cells at 5 days after virus infection, were treated with 1000 IU/ml of IFN (Sigma, St. Louis, MI, USA) for 10 days to eliminate HCV replication.

2.2. Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde and incubated with mouse monoclonal antibody against HCV NS5A (Chemicon International, Inc., Temecula, CA, USA) or HCV core (Abcam, Tokyo, Japan). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (MBL Co. Ltd., Nagoya, Japan), and observed under a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

2.3. Immunoblotting

Cells were solubilized in lysis buffer as reported previously [22]. The cell lysates were electrophoresed subjected to 8% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA). The membranes were incubated with mouse monoclonal antibodies against HCV NS5A or NS3 (Chemicon), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (MBL). The positive bands were visualized by using ECL detection system (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.4. Uptake of 2-deoxy-D-glucose and thymidine

Cells cultured in 12-well plates were deprived of serum by incubation in serum-free medium for 12 h. The cells were then pre-incubated for 20 min in 450 μl of KRH (25 mM Hepes, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.3 mM KH_2PO_4 and 0.1% BSA, pH 7.4). Glucose uptake assay was performed as describe previously [23]. In brief, glucose uptake was initiated by addition of 50 μl of reaction solution (KRH containing 0.5 mM, 0.25 μCi 2-deoxy-D-[1,2-^{3}H]glucose) to each well. As a negative control, 100 μM phloretin was added to reaction solution. After 10 min, transport was terminated by washing the cells with ice-cold KRH buffer containing 100 μM phloretin. The cells were solubilized by 0.1% sodium dodecyl sulfate, and the incorporated radioactivity was measured by liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA). In some experiments, GLUT1 and GLUT2 were ectopically expressed by using the pCAGGS expression vector [24] and glucose uptake was measured as described above.

2.5. Flow cytometry

To examine cell surface expression of GLUT1 and GLUT2, cells harvested in PBS containing 0.2% EDTA were incubated with rabbit polyclonal antibodies against GLUT1 or GLUT2 (1:200; Alpha Diagnostic International, San Antonio, TX, USA) on ice for 1 h. After being washed, the cells were incubated with FITC-labeled goat anti-rabbit IgG (1:200; BD Pharmingen, Franklin Lakes, NJ, USA) on ice for another 1 h. Analysis was carried out using flow cytometer and a total of 10,000 live cell events were measured. Results were displayed graphically as overlaying histograms demonstrating the shift of the mean FITC staining value.

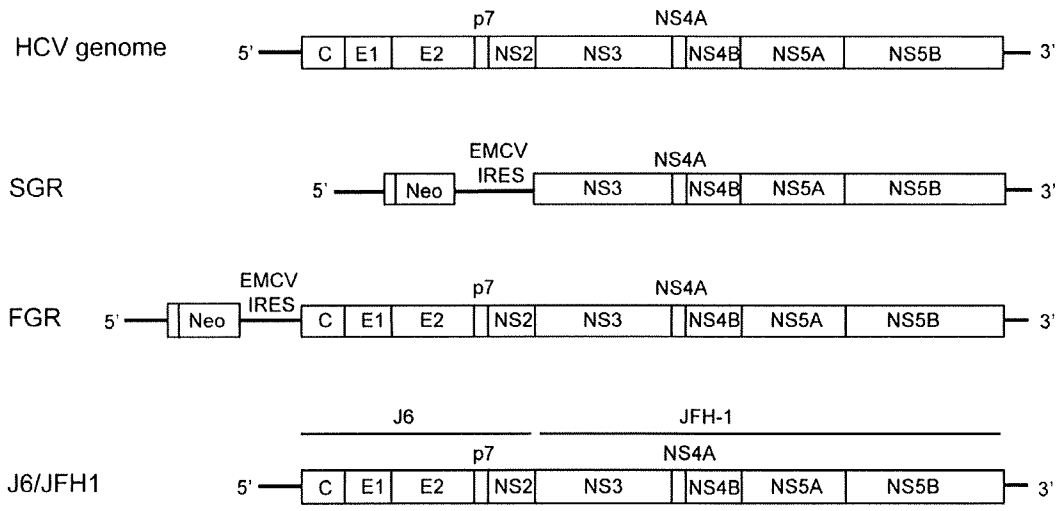

Fig. 1. The HCV genome and HCV RNA replicons. Schematic diagram of the HCV genome, SGR, FGR and the chimeric HCV J6/JFH1 genome are shown. EMCV IRES, encephalomyocarditis virus internal ribosome entry site; Neo, neomycin-resistance gene.

2.6. Real-time quantitative RT-PCR

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and cDNA was generated using QuantiTect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR *Premix Ex Taq* (Takara Bio, Kyoto, Japan) using SYBR green chemistry in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). β-Glucuronidase was used as an internal control. The primers used are shown in Table 1.

2.7. Luciferase reporter assay

We constructed the human GLUT2 promoter-luciferase reporter gene (pGLUT2-1291Luc) by cloning a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter (−1291 to +308) [14] into the pGL4 vector plasmid (Promega, Madison, WI, USA). pGLUT2-1291Luc thus contains a 1291-bp fragment of the human GLUT2 promoter upstream of the minimal promoter and the coding sequence of the *Photinus pyralis* (firefly) luciferase. pRL-CMV-*Renilla* (Promega) was used as an internal control. Cells were transfected with pGLUT2-1291Luc (1 μg) and pRL-CMV-*Renilla* (10 ng). After 24 h, a luciferase assay was performed by using Dual-luciferase reporter assay system (Promega). Firefly and *Renilla* luciferase activities were measured by Lumat LB 9501 (Berthold, Bad Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

2.8. Immunohistochemistry

Human adult liver autopsy materials and surgically removed liver tissues of patients with HCV- or HBV-associated hepatocellular carcinoma, and those with metastatic liver cancer were obtained with written informed consent. The tissues were fixed with 10% buffered formalin (pH 7.0), embedded in paraffin and sectioned at intervals of 4 μm. Immunohistochemical staining was performed with a DAKO ENVISION+ Kit (Dako, Glostrup, Denmark). In brief, fixed sections were treated with 3% hydrogen peroxide, and were autoclaved at 121 °C for 20 min. Then, the sections were incubated with a blocking solution and then with either anti-GLUT2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or normal rabbit IgG (Santa Cruz Biotechnology) as a control. The sections were incubated with horseradish peroxidase-labeled polymer-conjugated goat anti-rabbit IgG, followed by incubation in a chromogenic solution. The sections were then counterstained with hematoxylin and examined with a light microscope. GLUT2 expression levels were arbitrarily determined by two examiners, including a pathologist, in a blinded manner.

2.9. Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was evaluated by ANOVA, and statistical significance was defined as $P < 0.05$.

Table 1
Sequences and positions of the primers used in this study.

Gene name (GenBank ID)	Primer	Position	PCR product (bp)
GLUT2	5′-TGGGCTGAGGAAGAGACTGT-3′	279–298	461
(J03810)	5′-AGAGACTGAAGGATGGCTCG-3′	739–720	
GLUT1	5′-TGAACCTGCTGGCCTTC-3′	437–453	399
(AK292791)	5′-GCAGCTTCTTTAGCACA-3′	835–819	
HCV NS5B	5′-ACCAAGCTCAAACTCACTCCA-3′	9191–9211	119
(AJ238799)	5′-AGCGGGGTCGGGCACGAGACA-3′	9309–9289	
β-glucuronidase	5′-ATCAAAAACGCAGAAAATACG-3′	1747–1767	238
(M15182)	5′-ACGCAGGTGGTATCAGTCTTG-3′	1984–1964	

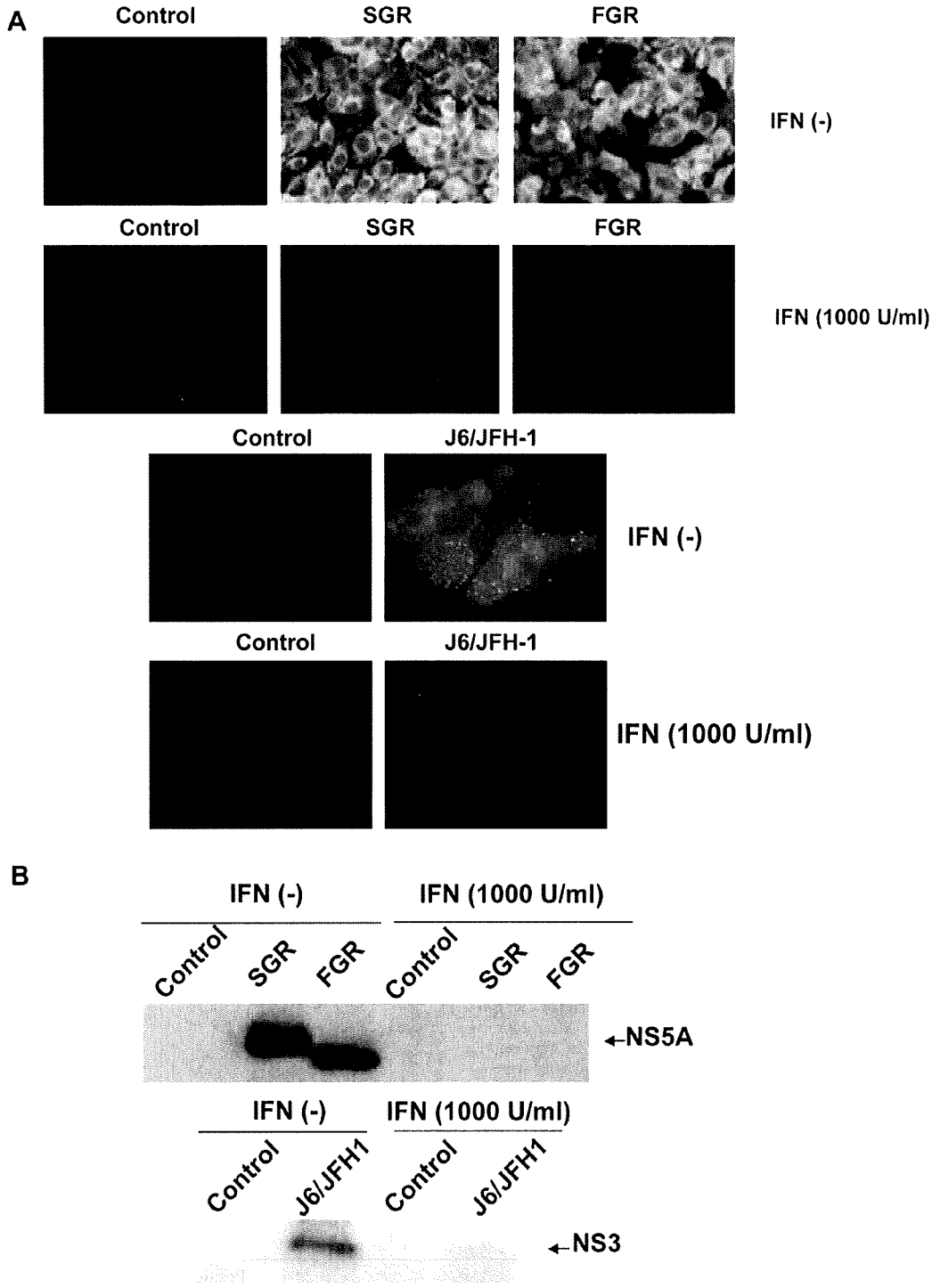

Fig. 2. Expression of HCV proteins in SGR, FGR, HCV-infected cells and the respective cells treated with IFN. (A) Cells were immunostained with anti-NS5A antibody (for SGR, FGR and the control cells) or anti-core antibody (for HCV-infected cells and the control). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to immunostaining. (B) Cells were analyzed by immunoblotting with anti-NS5A antibody (upper panel) or anti-NS3 antibody (lower panel). In parallel, cells were treated with IFN (1,000 IU/ml) for 10 days to eliminate HCV replication before being subjected to immunoblotting.

3. Results

3.1. HCV protein expression in SGR, FGR, HCV-infected cells and those treated with IFN

Immunofluorescence analysis revealed that almost all the cells in SGR and FGR cultures, and >90% of the cells in the HCV J6/JFH1-infected culture were positive for HCV antigens (Fig. 2A). Western blot analysis also confirmed HCV protein expression in SGR, FGR and HCV-infected cells (Fig. 2B). In some experiments, HCV replication in SGR, FGR and HCV-infected cells was eliminated by IFN treatment for 10 days (Fig. 2A and B).

3.2. Selective suppression of cellular glucose uptake by HCV replication

2-Deoxygluose uptake levels in SGR, FGR and HCV-infected cells were significantly suppressed by about 50–60%, compared with the control Huh-7.5 cells (Fig. 3A and B). On the other hand, thymidine uptake, which was used as a control, did not significantly differ among all the cells tested (data not shown). Moreover, glucose uptake levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 3A and B). These results strongly suggest that cellular glucose uptake is selectively suppressed by HCV RNA replication.

3.3. Down-regulation of cell surface expression of GLUT2 and GLUT1 by HCV replication

GLUT2 is the principal glucose transporter of hepatocytes *in vivo* while GLUT1 is expressed in a wide variety of cultured cells. We therefore examined cell surface expression of GLUT2 and GLUT1 by flow cytometry analysis. As shown in Fig. 4A, cell surface expression of GLUT2 and GLUT1 was markedly down-regulated in SGR and FGR cells, compared with the control. On the other hand, cell surface expression of transferrin receptor was not significantly suppressed in SGR or FGR, compared with the control, with the result ensuring the specificity of the down-regulation of GLUT2 and GLUT1 cell surface expression in SGR and FGR (Fig. 4A). Moreover, treatment of SGR and FGR cells with IFN restored the surface expression of GLUT2 and GLUT1 (Fig. 4A). These results suggest that HCV RNA replication specifically mediates down-regulation of GLUT2 and GLUT1.

Down-regulation of GLUT2 surface expression was observed also in HCV-infected cells (Fig. 4B). On the other hand, down-regulation of GLUT1 surface expression was only marginal and, compared to that of GLUT2, less evidently observed in HCV-infected cells. As a control, cell surface expression of transferrin receptor did not differ at all between HCV-infected cells and the control. Again, treatment of HCV-infected cells with IFN restored surface expression of GLUT2 (Fig. 4B).

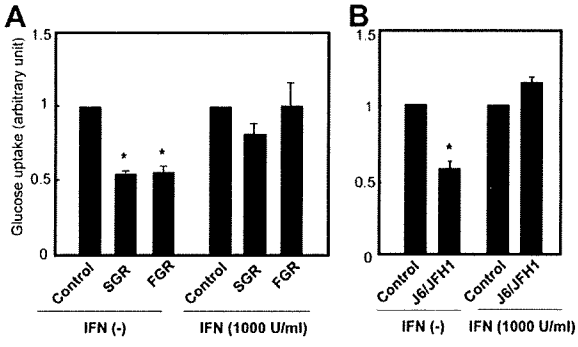

Fig. 3. Selective suppression of cellular glucose uptake by HCV replication. (A) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in SGR, FGR and HCV-negative control. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis. Data represent mean ± SEM of four independent experiments and the values for the control cells were arbitrarily expressed as 1.0. *$P < 0.01$, compared with the control. (B) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in J6/JFH-1-infected cells and the uninfected control. In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis.

3.4. Proteasomal degradation is not involved in the down-regulation of GLUT2 or GLUT1

Some viruses down-regulate cell surface molecules, such as immunoreceptors and intercellular adhesion molecules, through ubiquitination and proteasomal degradation of the target proteins [25]. To test this possibility, we treated SGR and FGR cells with lactacystin, a potent proteasome inhibitor. While lactacystin treatment enhanced cell surface expression of transferrin receptor, the same treatment did not increase cell surface expression of GLUT2 or GLUT1 in SGR or FGR cells (Fig. 5). This result suggested that down-regulation of cell surface expression of GLUT2 or GLUT1 in HCV-replicating cells was not due to increased degradation through the ubiquitin–proteasome system. The result rather implied the possible involvement of another mechanism(s), e.g., transcriptional suppression and/or impaired intracellular trafficking.

3.5. Transcriptional suppression of GLUT2, but not GLUT1, by HCV replication

To examine whether HCV RNA replication suppresses GLUT2 and GLUT1 expression at the transcriptional level, we measured mRNA expression levels by quantitative RT-PCR. The results obtained revealed that GLUT2 mRNA levels were reduced significantly in SGR, FGR and HCV-infected cells, compared to the control (Fig. 6A). It should be noted that the degree of GLUT2 mRNA suppression was greater in FGR

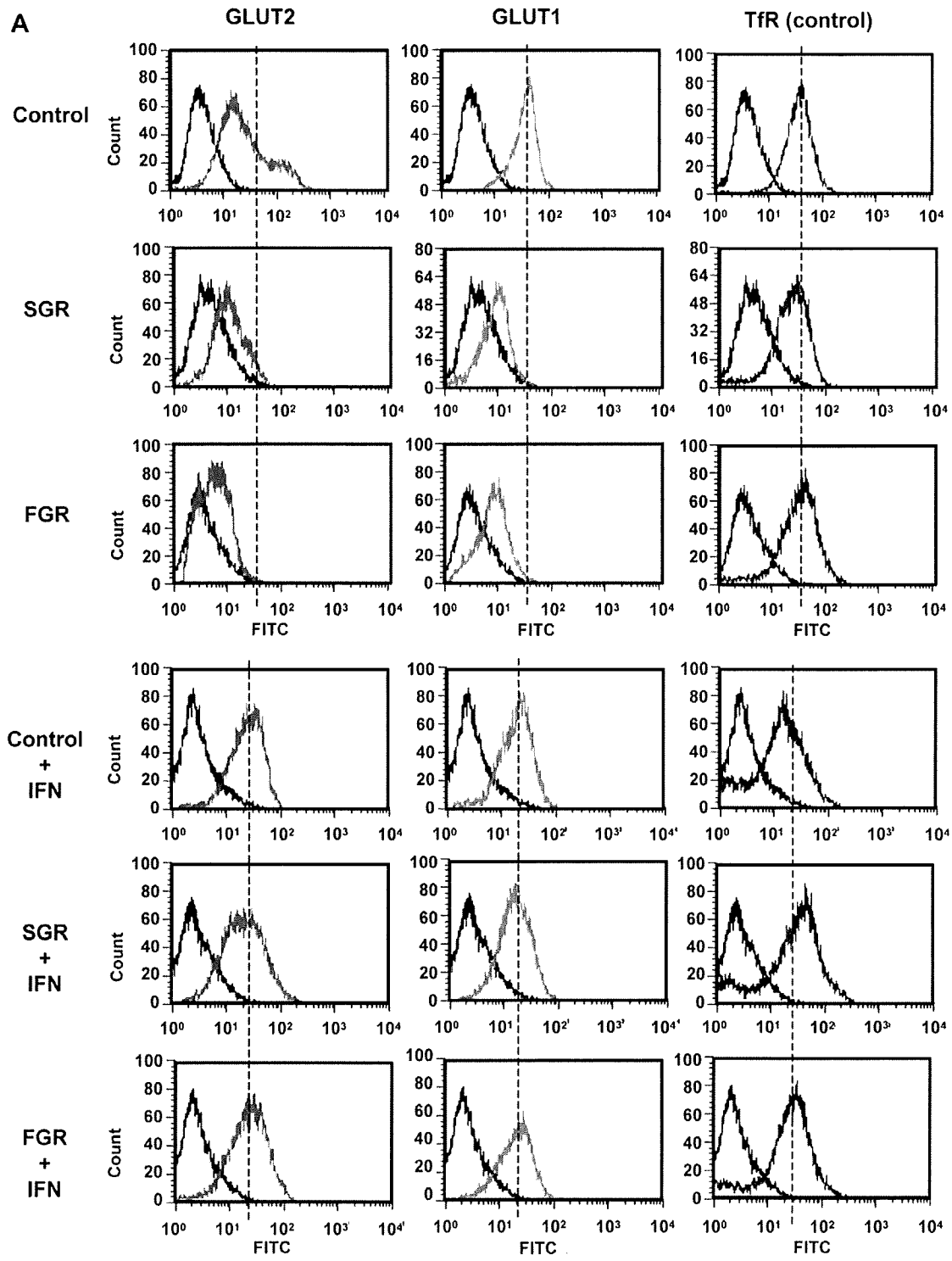

Fig. 4. Down-regulation of cell surface expressions of GLUT2 and GLUT1 by HCV replication. (A) SGR, FGR, the HCV-negative control cells were stained with specific antibodies, followed by FITC-conjugated second antibody (GLUT2, red line; GLUT1, green line) or stained with FITC-conjugated antibody alone (black line). Transferrin receptor (TfR) served as a control (blue line). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry. (B) HCV-infected cells and the uninfected control were analyzed by flow cytometry as in (A). In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry analysis.

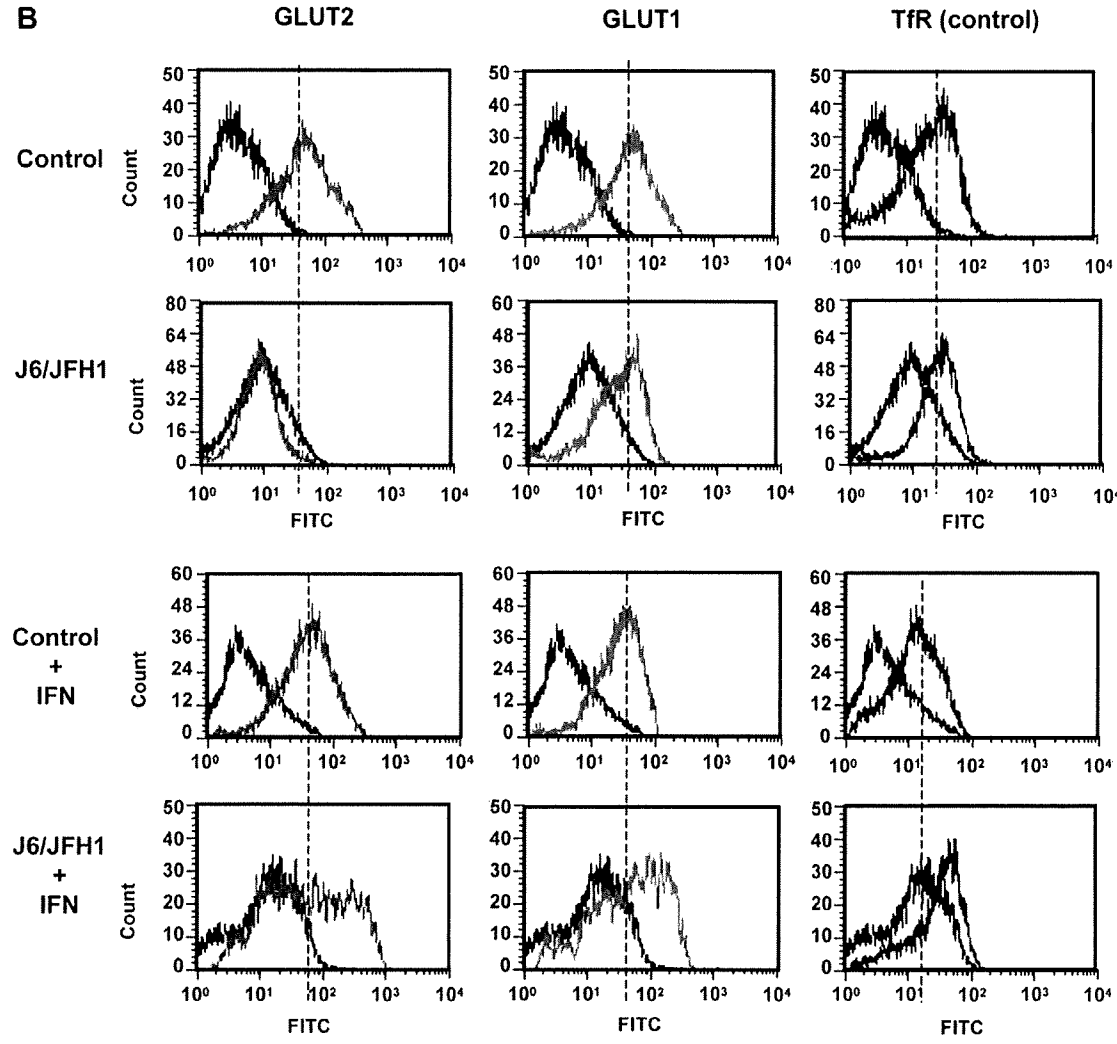

Fig. 4 (*continued*)

than in SGR cells. On the other hand, GLUT1 mRNA levels were not affected by HCV RNA replication (SGR and FGR) or HCV infection (Fig. 6B).

We also confirmed that GLUT2 mRNA expression levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 6A).

3.6. Suppression of GLUT2 promoter activity by HCV replication

Next, we performed luciferase reporter assay to examine the possible effect of HCV replication on GLUT2 promoter activities. The result obtained demonstrated that GLUT2 promoter activities were significantly suppressed in SGR, FGR and HCV-infected cells, compared to the control cells (Fig. 6C). Furthermore, GLUT2 promoter activities in SGR, FGR and HCV-infected cells were restored by IFN treatment. It is thus likely that HCV replication suppresses GLUT2 promoter activity, thereby decreasing GLUT2 mRNA levels.

3.7. Ectopically expressed GLUT1 or GLUT2 mediates increased glucose uptake in SGR, FGR and HCV-infected cells

We examined the possible effects of ectopically expressed GLUT1 and GLUT2 on glucose uptake in SGR, FGR and HCV-infected cells. Glucose uptake was significantly increased by ectopically expressed GLUT1 or GLUT2 in SGR, FGR and HCV-infected cells as well as in the control Huh-7.5 cells (Fig. 6D). It should be noted that, in this series of transient transfection experiments, only ca. 20% of the cells were ectopically overexpressing GLUT1 or GLUT2. These results collectively suggest the possibility that down-regulation

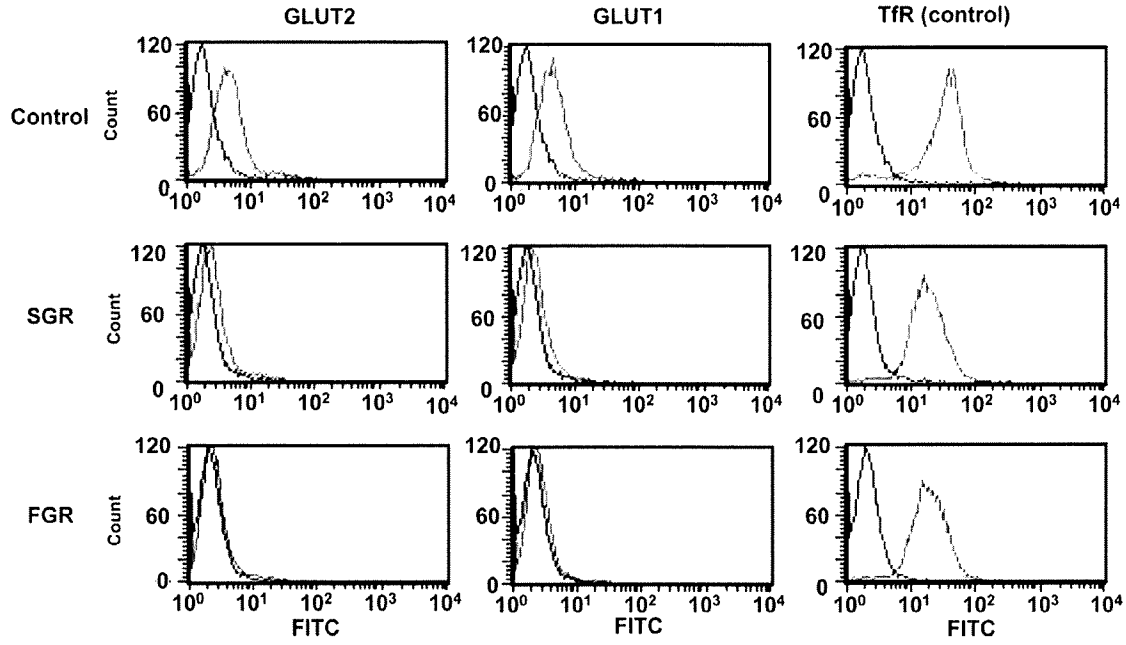

Fig. 5. Effects of lactacystin treatment on cell surface expression of GLUT2, GLUT1 and transferrin receptor (TfR). Cells were treated with lactacystin (10 μM) overnight to inhibit proteasomal degradation, and analyzed by flow cytometry. Cells treated with lactacystin are shown in red line and those left untreated in blue line. The negative controls stained with FITC-conjugated antibody alone are shown in black line.

of GLUT1 and GLUT2 expression is primarily involved in the decreased glucose uptake in SGR, FGR and HCV-infected cells.

3.8. Decreased GLUT2 expression in hepatocytes obtained from HCV-infected patients

GLUT2 is the principal glucose transporter expressed in hepatocytes *in vivo*. As shown in Fig. 7B, practically all hepatocytes obtained from patients without HCV infection showed positive staining for GLUT2, which was most evidently observed near the plasma membrane. On the other hand, hepatocytes obtained from HCV-infected patients showed markedly reduced GLUT2 staining in most, if not the entire, areas of the section, compared with the uninfected control (Fig. 7D). This heterogeneous staining pattern might reflect concomitant presence of areas comprising either virus-infected or uninfected hepatocytes in a tissue sample. Whereas all the sections obtained from 8 patients without HCV infection showed evenly positive staining for GLUT2, sections from 8 (89%) of 9 HCV-infected patients showed moderately to markedly reduced GLUT2 staining (Table 2). Reduced GLUT2 staining was observed also with hepatocytes in the liver tissues obtained from HBV-infected patients. However, the areas of reduced GLUT2 staining appeared to be more restricted in sections obtained from HBV-infected patients than in those from HCV-infected ones.

4. Discussion

HCV infection is known as an initiation and precipitating factor of type 2 diabetes [7–10,26,27]. Progression of liver fibrosis induced by persistent viral infection may induce diabetes [28]. Furthermore, it has been reported that the prevalence of diabetes is higher among patients with HCV-associated liver cirrhosis than in those with HBV-associated cirrhosis [7]. It is likely, therefore, that HCV infection itself is a risk factor of diabetes. Previous reports suggest that HCV infection directly causes insulin resistance that would cause the progression of diabetes [29–31]. However, the underlying mechanism(s) is not yet completely elucidated. In this study, we analyzed the effect of HCV infection on cellular glucose uptake and expression of glucose transporters.

We observed that glucose uptake was suppressed in cells harboring HCV RNA replicons (SGR and FGR) and those infected with HCV than in the control cells (Fig. 3). It has been reported that glucose disposal *in vivo* occurs through both insulin-dependent and insulin-independent mechanism [32]. We observed that treatment of SGR, FGR and the control Huh-7.5 cells with insulin (10^{-4} M to 10^{-9} M) increased glucose uptake by only about 50% from their basal levels (data not shown). Nevertheless, decreased glucose uptake by HCV-infected hepatocytes is a potential cause of hyperglycemia as the liver is a big organ accounting for 2% of the total body weight.

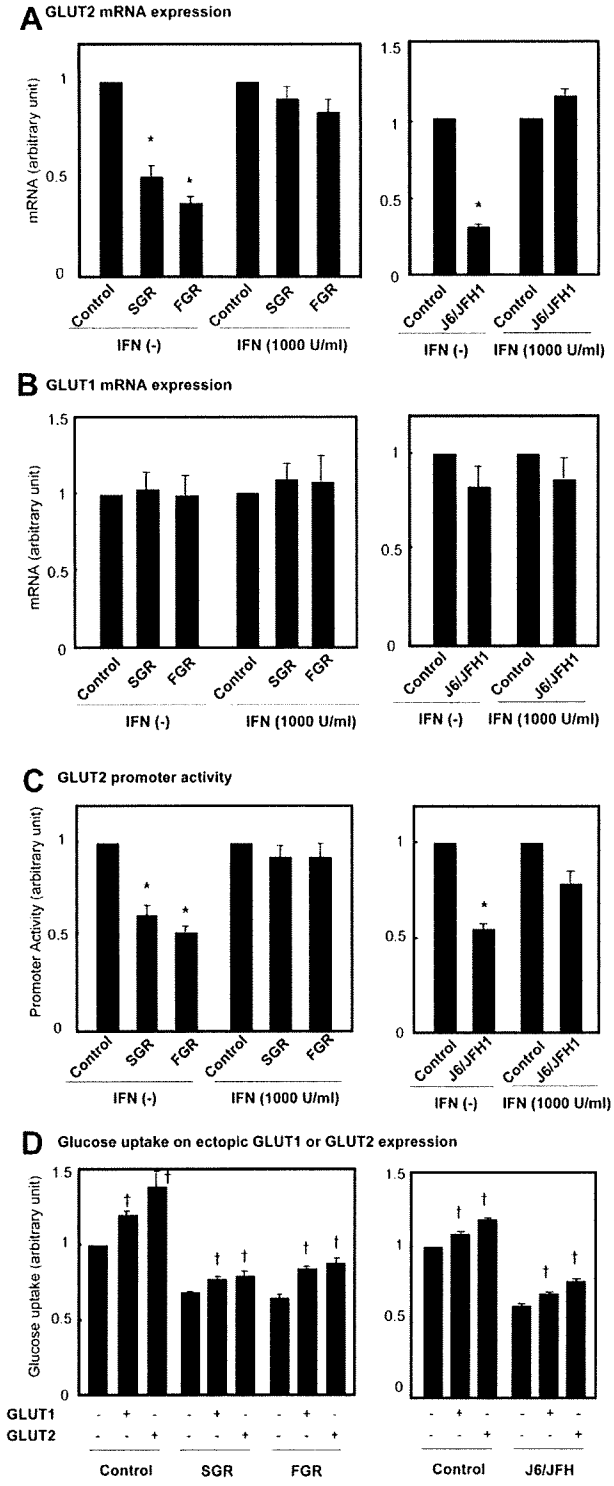

Fig. 6. Differential suppression of GLUT2 and GLUT1 mRNAs by HCV replication. (A and B) Quantitative RT-PCR analysis of mRNA for GLUT2 (A) and GLUT1 (B). mRNA expression levels of GLUT2 and GLUT1 in SGR, FGR and HCV-infected cells were determined and normalized with β-glucuronidase mRNA levels. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to quantitative RT-PCR analysis. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.01$, compared with the control. (C) GLUT2 promoter activities in SGR and FGR, HCV-infected cells were analyzed using luciferase reporter assay. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to luciferase reporter assay. Data represent mean ± SEM of five independent experiments. $^{*}P < 0.01$, compared with the control. (D) Glucose uptake in cells ectopically expressing GLUT1 or GLUT2. Data represent mean ± SEM of two independent experiments. $^{\dagger}P < 0.01$, compared with mock transfected control.

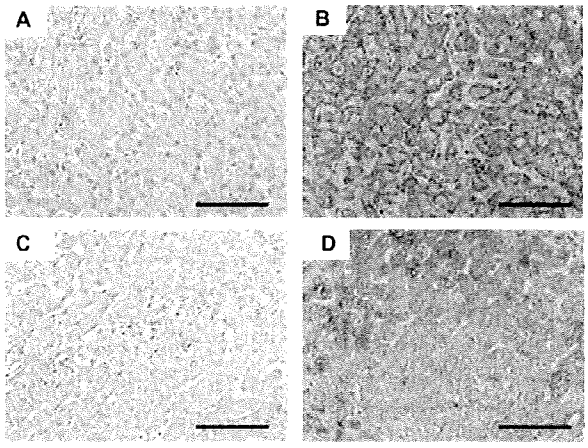

Fig. 7. Down-regulation of GLUT2 expression in HCV-infected human liver tissues *in vivo*. Normal human adult liver tissues (A and B) and HCV-infected, non-cancerous liver tissues (C and D) were fixed with formalin, sectioned and stained with normal rabbit IgG (A and C) or polyclonal anti-GLUT2 antibody (B and D). Scale bar = 100 μm.

Any proliferating cell requires energy sources, including glucose, and GLUTs play an important role in glucose uptake into the cell. In the liver, GLUT2 is the predominant glucose transporter, which regulates glucose metabolism by mediating a bidirectional transport, both entry and exit, of glucose into and from hepatocytes [13]. GLUT1, on the other hand, is known to be expressed in malignant cells including hepatocellular carcinoma [12,13] and a wide variety of cultured cells. In the present study we found that cell surface expression of GLUT2 and GLUT1 was markedly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 4A and B).

Table 2
Reduction of GLUT2 expression in hepatocytes of HCV-infected and HBV-infected human liver tissues.

Liver tissues	Sample No.	Reduction of GLUT2 expression
Uninfected	1	– *
	2	–
	3	–
	4	–
	5	–
	6	–
	7	–
	8	–
HCV-infected	9	1+ (Focal)[a]
	10	1+ (Focal)
	11	3+ (Diffuse)
	12	3+ (Diffuse)
	13	3+ (Diffuse)
	14	3+ (Focal)
	15	–
	16	2+ (Focal)
	17	3+ (Diffuse)
HBV-infected	18	–
	19	3+ (Diffuse)
	20	1+ (Focal)
	21	–
	22	2+ (Focal)
	23	1+ (Focal)
	24	2+ (Focal)

* -, no reduction; 1+, weak reduction; 2+, moderate reduction; 3+, strong reduction.

[a] Parentheses indicate either focal or diffuse appearance of the areas with reduced GLUT2 expression in each liver tissue sample.

GLUT2 expression is regulated at the transcriptional level, at least partly, by glucose [33]. It has been reported that hyperglycemia increases the GLUT2 mRNA and protein expression in an *in vivo* study [34]. Our present study demonstrated that GLUT2 mRNA expression was significantly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 6A). Consistent with this result, GLUT2 promoter activities, as measured by luciferase reporter assay, were suppressed in SGR, FGR and HCV-infected cells (Fig. 6C). In this connection, it was reported that GLUT2 promoter activities were up-regulated by sterol response element-binding protein (SREBP)-1c [35,36]. We confirmed in our study that GLUT2 promoter activities were up-regulated by over-expression of human SREBP-1c, and that the SREBP-1c-mediated GLUT2 promoter activities were suppressed significantly in SGR, FGR and HCV-infected cells (data not shown).

Unlike GLUT2 mRNA, GLUT1 mRNA was not suppressed by HCV RNA replication or HCV infection (Fig. 6B). Nevertheless, cell surface expression of GLUT1 was markedly down-regulated in SGR and FGR cells (Fig. 4A). As GLUT1 surface expression was not restored by treatment with lactacystin, a potent proteasome inhibitor (Fig. 5), it was unlikely that HCV-mediated suppression of GLUT1 surface expression was mediated through increased degradation by the ubiquitin-proteasome system. We assume that intracellular trafficking of GLUT1 (and possibly GLUT2 as well) is impaired by HCV RNA replication although we could not precisely prove it due mainly to the lack of an appropriate antibody that enables us to monitor GLUT1 trafficking. Further study is needed to elucidate the issue.

By means of immunohistochemical analysis, we confirmed that GLUT2 was strongly expressed in hepatocytes of the liver tissues obtained from all of 8 individuals without HCV infection (Fig. 7B and Table 2). More importantly, we demonstrated that GLUT2 expression was significantly down-regulated in hepatocytes obtained from 8 of 9 HCV-infected patients (Fig. 7D and Table 2). Interestingly, the areas where GLUT2 down-regulation was observed appeared to be scattered across the liver tissue sections. This may reflect the general observation that a group of hepatocytes in limited areas of the hepatic lobules, but not all the hepatocytes, are infected with HCV *in vivo*. By means of real-time quantitative PCR analysis, we found a tendency that levels of GLUT2 mRNA expression in liver tissues obtained from HCV-infected patients were lower than that obtained from uninfected controls although the dif-

ference was not statistically significant (data not shown). As stated above, not all the hepatocytes in the liver were infected with HCV and, therefore, the possible reduction of GLUT2 mRNA expression in HCV-infected hepatocytes might have been masked by the normal levels of expression in uninfected hepatocytes concomitantly present in the same tissue samples.

It should also be noted that GLUT2 staining was also reduced in hepatocytes obtained from HBV-infected patients, though to a lesser extent than that from HCV-infected ones (Table 2). We assume that inflammatory responses in the liver may trigger some intracellular event that leads to decreased GLUT2 expression in hepatocytes *in vivo*.

In conclusion, we have demonstrated for the first time that HCV replication inhibits cellular glucose uptake through down-regulation of cell surface expression of GLUT2 and possibly GLUT1. It is conceivable that the decreased glucose uptake by hepatocytes causes impaired glucose metabolism, leading eventually to the initiation and progression of diabetes mellitus during a prolonged period of HCV persistence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.029.

References

[1] Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 2005;42:962–973.

[2] Lu L, Li C, Fu Y, Thaikruea L, Thongswat S, Maneekarn N, et al. Complete genomes for hepatitis C virus subtypes 6f, 6i, 6j and 6m: viral genetic diversity among Thai blood donors and infected spouses. J Gen Virol 2007;88:1505–1518.

[3] Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature 2005;436:933–938.

[4] Appel N, Schaller T, Penin F, Bartenschlager R. From structure to function: new insights into hepatitis C virus RNA replication. J Biol Chem 2006;281:9833–9836.

[5] Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 2005;5:558–567.

[6] Galossi A, Guarisco R, Bellis L, Puoti C. Extrahepatic manifestations of chronic HCV infection. J Gastrointestin Liver Dis 2007;16:65–73.

[7] Caronia S, Taylor K, Pagliaro L, Carr C, Palazzo U, Petrik J, et al. Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999;30:1059–1063.

[8] Mason AL, Lau JY, Hoang N, Qian K, Alexander GJ, Xu L, et al. Association of diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999;29:328–333.

[9] Mehta S, Levey JM, Bonkovsky HL. Extrahepatic manifestations of infection with hepatitis C virus. Clin Liver Dis 2001;5:979–1008.

[10] Mehta SH, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. Ann Intern Med 2000;133:592–599.

[11] Wu X, Freeze HH. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative spice forms. Genomics 2002;80:553–557.

[12] Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;202:654–662.

[13] Godoy A, Ulloa V, Rodriguez F, Reinicke K, Yanez AJ, Garcia Mde L, et al. Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. J Cell Physiol 2006;207:614–627.

[14] Ban N, Yamada Y, Someya Y, Miyawaki K, Ihara Y, Hosokawa M, et al. Hepatocyte nuclear factor-1α recruits the transcriptional co-activator p300 on the GLUT2 gene promoter. Diabetes 2002;51:1409–1418.

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

[16] Hidajat R, Nagano-Fujii M, Deng L, Tanaka M, Takigawa Y, Kitazawa S, et al. Hepatitis C virus NS3 protein interacts with ELKS-δ and ELKS-α, members of a novel protein family involved in intracellular transport and secretory pathways. J Gen Virol 2005;86:2197–2208.

[17] Nomura-Takigawa Y, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K, et al. Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. J Gen Virol 2006;87:1935–1945.

[18] Inubushi S, Nagano-Fujii M, Kitayama K, Tanaka M, An C, Yokozaki H, et al. Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein–tyrosine kinase Syk. J Gen Virol 2008;89:1231–1242.

[19] Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. 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 2005;329:1350–1359.

[20] Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, et al. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its