this shall be referred to as the core protein in this study. Here, we demonstrate that the core protein contains a functional Bcl-2 homology 3 (BH3) domain that is essential for its proapoptotic property and ability to interact with human myeloid cell factor 1 (Mcl-1), a prosurvival member of the Bcl-2 family (31). Detailed molecular analysis and infection studies using the J6/JFH-1 infectious clone showed that the core protein is a bona fide BH3-only protein that contributes to the induction of apoptosis during HCV infection by mimicking Noxa and interfering with the prosurvival function of Mcl-1.

MATERIALS AND METHODS

Construction of plasmids. Expression plasmids for the wild-type core protein and mutants were generated by PCR using Titanium *Taq* DNA polymerase (Clontech Laboratories Inc., Palo Alto, CA). Two plasmids containing full-length HCV genomes were used as templates. The first one is a 1b strain cloned in Singapore (59), and the second is the JFH-1 clone, which is a 2a strain (68). All sequences were confirmed by sequencing performed by the core facilities at the Institute of Molecular and Cell Biology, Singapore. The pXJ40flag vector is used so that a flag epitope is fused to the N terminus of the core protein, and this allows the comparison of protein expression levels with an anti-flag antibody.

Transient transfections, CaspACE fluorometric assay, and Western blot analysis. Transient transfections of Huh7 cells were performed using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Approximately 16 h after transfection, the activation of caspase-3 was quantified by using a CaspACE fluorometric assay system from Promega Corporation (Madison, WI) as previously described (63).

Western blot analysis was performed as previously described (64). The primary antibodies (anti-myc monoclonal and anti-myc and anti-Mcl-1 polyclonal [Santa Cruz Biotechnology, Santa Cruz, CA], anti-Mcl-1 monoclonal [Calbiochem, La Jolla, CA], anti-actin monoclonal, anti-Hsp-60 monoclonal, anti-flag monoclonal and polyclonal [Sigma, St. Louis, MO], anti-poly[ADP-ribose] polymerase [PARP] polyclonal [Cell Signaling Technology Inc., Beverly, MA], anti-cytochrome *c* monoclonal [BD PharMingen, BD Biosciences, San Jose, CA], and anti-Noxa [Imgenex, San Diego, CA]) were purchased. Anti-core protein monoclonal antibody (clone 2H9; a kind gift from T. Wakita, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan) was used to detect the core protein of HCV (68).

Coimmunoprecipitation experiments. For the coimmunoprecipitation experiments, each 6-cm dish of cells was resuspended in 200 μl of immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholic acid, 0.005% sodium dodecyl sulfate [SDS], and 1 mM phenylmethylsulfonyl fluoride) and subjected to freeze-thawing six times. Anti-flag monoclonal antibody conjugated to Sepharose beads (Sigma) were added to 150 μl of the lysates, and the mixture was subjected to end-over-end mixing at 4°C for 6 h. Beads were washed four times with cold IP buffer, and then 15 μl of Laemmli's SDS buffer was added and the samples were boiled at 100°C for 5 min to release the immunocomplexes. Samples were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis.

Alternatively, rabbit anti-Mcl-1 polyclonal antibody was used to immunoprecipitate endogenous Mcl-1 protein. In this case, 7 μg of antibody (either anti-Mcl-1 or anti-hemagglutinin [HA] polyclonal antibody [Santa Cruz Biotechnology]) was added to the lysates obtained from two dishes of cells and allowed to mix for 1 h at room temperature. Protein A agarose beads (Roche, Indianapolis, IN) were added, and the mixture was subjected to end-over-end mixing at 4°C overnight. The coimmunoprecipitated proteins then were detected as described above.

Quantification of autoradiographs. An imaging densitometer (Bio-Rad, Hercules, CA) was used for the quantification of the intensities of specific bands on autoradiographs.

In vitro cytochrome *c* release assay. For the in vitro cytochrome *c* release assays, mitochondria were isolated from 293T cells as previously described (22). Briefly, 293T cells were suspended in isolation buffer (320 mM sucrose, 1 mM EDTA, 50 mM HEPES [pH 7.5]) and disrupted by 25 expulsions through a 27-gauge needle. The disrupted cells were spun at 1,000 × *g* for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 7,000 × *g* for 10 min, and the pellet was retained as the heavy membrane fraction containing the mitochondria. The mitochondrion-containing pellets then were resuspended in assay buffer (250 mM sucrose, 2 mM KH_2PO_4, 5 mM sodium succinate, 25 mM EGTA, and 10 mM HEPES [pH 7.5]) at 0.5 mg/ml. Equal amounts of mitochondria were treated with the indicated peptides for 30 min at room temperature, followed by centrifugation. Both the supernatant and pellet then were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis to determine the amount of cytochrome *c* released from the mitochondria. Hsp-60 was used as a loading control for the pellet.

Synthesis of peptides. A peptide that corresponds to residues 118 to 149 of the genotype 1b core protein (NLGKVIDTLTCGFADLMGYIPLVGAPLGG AAR) was synthesized and purified to 95% purity (Sigma Genosys, Japan). Peptides containing the BH3 domain of Bad (NLWAAQRYGRELRRMSDEF VDSFKK) or Noxa (VPADLKDECAQLRRIGDKVNLRQKL) also were synthesized and purified to 95% purity (Mimotopes, Clayton, Victoria, Australia).

Generation of recombinant HCV. The pFL-J6/JFH-1 plasmid encoding the entire viral genome of a chimeric strain of HCV genotype 2a, J6/JFH-1 (37), was kindly provided by C. M. Rice, Center for the Study of Hepatitis C, The Rockefeller University. To generate mutant virus possessing a core protein mutation, a nucleotide substitution was introduced into pFL-J6/JFH-1 by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All PCR-amplified DNA fragments were verified extensively using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). Each of the plasmids was linearized by XbaI digestion and in vitro transcribed by using T7 RiboMAX (Promega) to generate the full-length viral genomic RNA. The in vitro-transcribed RNA (10 μg) was transfected into Huh7.5 cells by means of electroporation (975 μF, 270 V) using a Gene Pulser (Bio-Rad). The cells then were cultured in complete medium, and the supernatant was propagated as a virus stock. Culture supernatants of uninfected cells served as a control (mock preparation). Virus infectivity was measured by indirect immunofluorescence as previously described (17) and expressed as cell-infecting units (CIU) per milliliter.

Proliferation, caspase-3, and DNA fragmentation assays. Huh7.5 cells were seeded in 96-well plates at a density of 1.0×10^4 cells per well and cultured overnight. The cells then were infected with recombinant HCV at a multiplicity of infection of 0.1 CIU/cell or with a mock preparation. At different time points postinfection (p.i.), cell viabilities were determined by WST-1 proliferation assays (Roche, Mannheim, Germany) as described previously (17, 48).

Caspase-3 and DNA fragmentation assays also were performed on the infected cells as previously described (17).

HCV RNA quantitation. To measure intracellular HCV RNA replication levels, total RNA was extracted from the cells using an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using a QuantiTect reverse transcription kit (Qiagen) with random primers and was subjected to quantitative real-time PCR analysis using SYBR premix Ex *Taq* (Takara Bio, Kyoto, Japan) in a MicroAmp 96-well reaction plate and an ABI PRISM 7000 (Applied Biosystems, Foster, CA). The primers used to amplify an NS5A region of the HCV genome were 5′-AGACGTATTGAGGTCCATGC-3′ (sense) and 5′-CCGCAGCGACGGTG CTGATAG-3′ (antisense). As an internal control, human glyceraldehyde-3-phosphate dehydrogenase expression levels were measured using primers 5′-G CCATCAATGACCCCTTCATT-3′ (sense) and 5′-TCTCGCTCCTGGAAGA TGG-3′ (antisense).

Statistical analysis. Either the two-tailed Student's *t* test or one-way analysis of variance (using SPSS version 16.0) was applied to evaluate the statistical significance of differences measured from the data sets. $P < 0.05$ was considered statistically significant.

RESULTS

A BH3-like domain is present in the core protein. The family of Bcl-2 proteins constitutes one of the biologically important gene products in the regulation of apoptosis (see recent reviews in references 1, 16, 67, and 75). The Bcl-2 proteins may be classified broadly into three classes: prosurvival members containing multiple Bcl-2 homology domains, proapoptotic members containing multiple Bcl-2 homology domains, and proapoptotic members containing the BH3 domain only. The examination of the amino acid sequence of the core protein revealed that there is a BH3-like domain near the C terminus. An alignment of this domain with BH3 domains of the Bcl-2 family of proteins is shown in Fig. 1A. The BH3-like domain of the core protein contains L (residue 119) and D (residue 124)

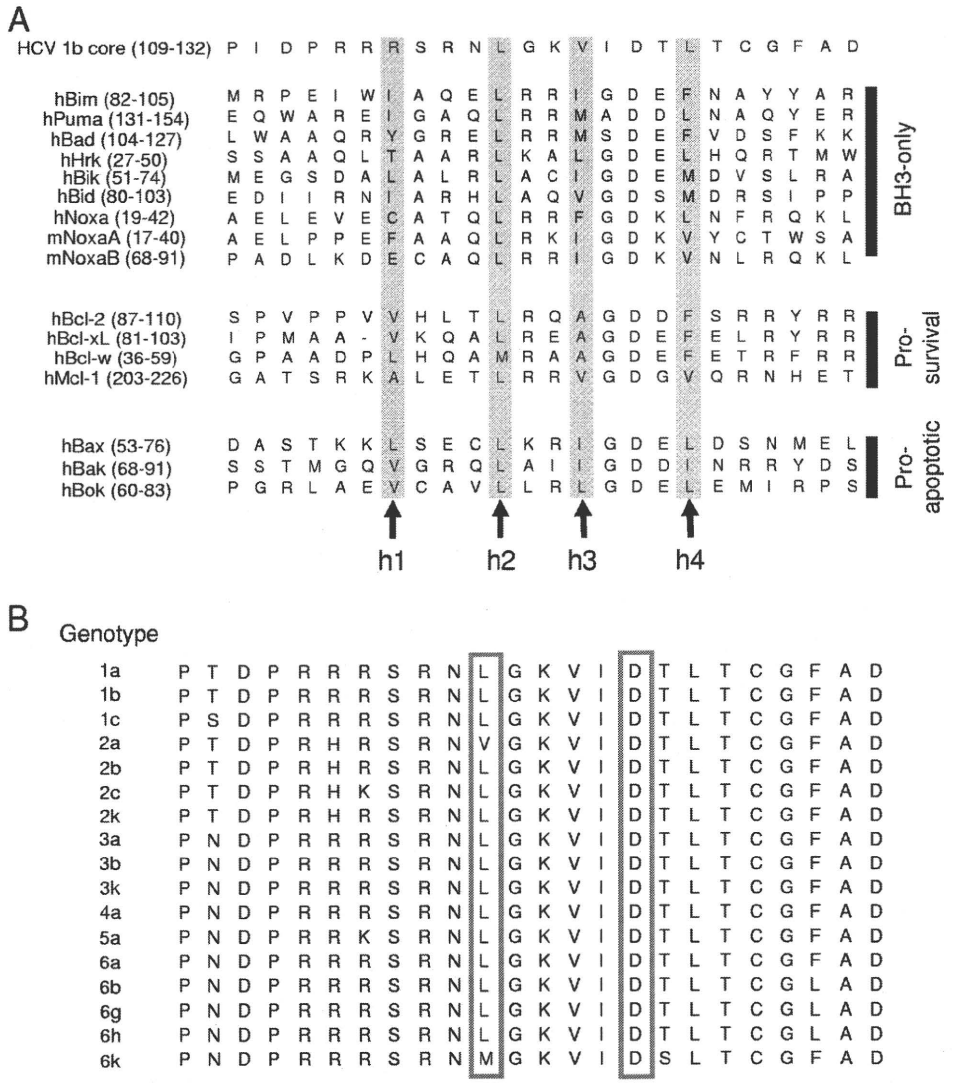

FIG. 1. Identification of a BH3-like domain in the core protein. (A) Alignment of the BH3 domain of the genotype 1b core protein with the BH3 domains of members of the Bcl-2 family. Numbers in parentheses represent the positions of amino acid residues of the respective proteins. The four hydrophobic amino acids that make critical contacts with residues in the BH3 recognition grooves present on the surfaces of the prosurvival Bcl-2 family proteins are indicated as h1 to h4. (B) Alignment of the core protein (residues 109 to 132) of different genotypes. The consensus sequences for these genotypes were obtained from http://hcv.lanl.gov/content/hcv-index. The highly conserved L and D residues, at positions 119 and 124 of the genotype 1b core protein, respectively, in BH3 domains are boxed.

separated by four residues, as in other known BH3 domains. This domain is highly conserved among the major HCV genotypes, with the exception of genotypes 2a and 6k, which have V and M residues at position 119, respectively (Fig. 1B).

The BH3 domain of the core protein is essential for the induction of apoptosis and its interaction with human Mcl-1. The overexpression of the core protein (with a flag epitope at the N terminus) in Huh7 cells, via the transient transfection of a cDNA expression plasmid containing the genotype 1b core protein gene, induced significant levels of apoptosis as determined by the activation of caspase-3, which is a hallmark of apoptosis (Fig. 2A). The deletion of the BH3 domain in the core protein (designated coreΔ115-128aa) abolished its proapoptotic property, indicating that this domain is essential for the induction of apoptosis. Consistently, the cleavage of endogenous PARP, a substrate of activated caspase-3, was clearly observed in Huh7 cells expressing the wild-type core protein but not in those expressing coreΔ115-128aa (Fig. 2B).

To understand how the core protein modulates the function of the Bcl-2 family of proteins, coimmunoprecipitation experiments were performed to determine if the core protein can interact with representative prosurvival members of the Bcl-2 family. As shown in the top panel of Fig. 3A, Mcl-1 was specifically coimmunoprecipitated by the core protein (lane 8) but not by an irrelevant protein, glutathione *S*-transferase (GST) (lane 7). The BH3 domain of the core protein is essential for its interaction with Mcl-1, as coreΔ115-128aa failed to coimmunoprecipitate Mcl-1 (lane 9). These results indicate that the core protein induces apoptosis by interfering directly with the prosurvival function of Mcl-1. In contrast, no significant inter-

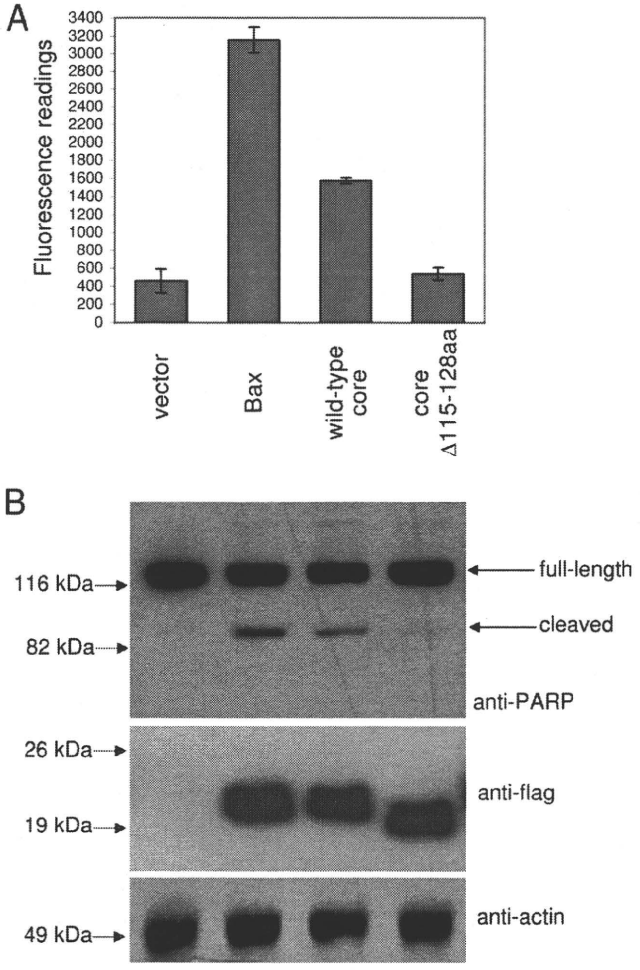

FIG. 2. Induction of apoptosis by the overexpression of the core protein in Huh7 cells. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3, which is a hallmark of apoptosis, in Huh7 cells that were transfected with vector only, a classical apoptosis inducer (Bax), the wild-type core protein, and a core protein mutant lacking the putative BH3 domain (coreΔ115-128aa). 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, which is a substrate of activated caspase-3, from 116 to 83 kDa (top). Similarly, the expression levels of the different proteins were determined using anti-flag antibody (middle). The amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

action was observed between the core protein and two other prosurvival proteins, Bcl-X_L and Bcl-w (lanes 1 to 6).

The interaction between the core protein and endogenous Mcl-1 was determined by overexpressing the core protein in Huh7 cells. As shown in Fig. 3B, the core protein was coimmunoprecipitated with endogenous Mcl-1 (lane 3). On the other hand, the core protein was not coimmunoprecipitated when an irrelevant antibody (anti-HA) (lane 4) was used for IP. Consistently with the results in Fig. 3A, only a small amount of coreΔ115-128aa was coimmunoprecipitated with endogenous Mcl-1 (lane 5). To estimate the degree of reduction in the binding of coreΔ115-128aa to endogenous Mcl-1, an imaging densitometer was used to quantify the intensities of specific bands on the autoradiographs obtained in three independent coimmunoprecipitation experiments (see Fig. S1 in the supplemental material). The ratios of signals for the expression of flag-tagged core protein, endogenous Mcl-1, and actin (internal control) all are close to 1 (0.94 to 1.14), indicating that the expression levels of these proteins in the two sets of cells (either transfected with cDNA construct for expressing flag-core or flag-coreΔ115-128aa) were similar. From the three independent experiments, the average amount of coreΔ115-128aa coimmunoprecipitated specifically by the Mcl-1 antibody is 14.2% (± 10.7%) of the amount of wild-type core protein coimmunoprecipitated. This implies that the deletion of the BH3 domain does not completely abolish the interaction between the core protein and endogenous Mcl-1 but reduces the interaction greatly.

Overexpression of Mcl-1 or Bcl-X_L prevents core protein-induced apoptosis. To examine the protective effects of the prosurvival Bcl-2 proteins in Huh7 cells, transfections were performed with plasmids expressing myc-tagged Bcl-2, Bcl-X_L, Bcl-w, or Mcl-1 (Fig. 4A and B). Consistent with studies of other cell lines (12, 66), the transient high-level expression of Bcl-2 also caused apoptosis in Huh7 cells (lane 1). Interestingly, the overexpression of Bcl-w also induced a significant level of apoptosis in Huh7 cells (lane 3), and this phenomenon has not been reported previously. Cells overexpressing Bcl-X_L, but not Mcl-1, also had a slightly higher level of apoptosis than that of the vector control cells (lanes 2 and 4).

Huh7 cells were cotransfected with plasmids for expressing myc-Mcl-1 and flag-core or myc-Bcl-X_L and flag-core. As shown in Fig. 4C and D, the level of apoptosis was significantly reduced in cells expressing both Mcl-1 and the core protein (lane 3) compared to those expressing the core protein only (lane 2). When the same experiment was repeated using Bcl-X_L, the level of apoptosis was reduced to a lesser extent (lane 5). However, this may be due to the low level of apoptosis induced by the overexpression of Bcl-X_L (lane 6). The level of the core protein expressed in the presence of Bcl-X_L also was decreased greatly, but the smaller amount of the core protein expressed still induced a high level of apoptosis (lane 5). However, when a broad caspase inhibitor (z-VAD-fmk) was used, the core protein level in cells coexpressing Bcl-X_L increased (see Fig. S2 in the supplemental material), indicating that the transfection efficiencies were similar in the different samples. To resolve this uncertainty, the experiment was repeated with a smaller amount of Bcl-X_L plasmid (0.5 μg). Under this condition, the overexpression of Bcl-X_L did not induce apoptosis (lane 8), and the level of apoptosis also was reduced in cells expressing both Bcl-X_L and the core protein (lane 7) compared to those expressing the core protein only (lane 2). Thus, the results show that the overexpression of either Mcl-1 or Bcl-X_L protects against core protein-induced apoptosis.

Bad enhances the ability of the core protein to release cytochrome *c* from isolated mitochondria. The ability of a core protein peptide, which contains residues 118 to 149 of the genotype 1b core protein, to release cytochrome *c* from the mitochondria was tested using 293T cells instead of Huh7 cells, as the method for the isolation of mitochondria from 293T cells is well established. The core protein induced apoptosis in 293T cells in the same manner as that in Huh7 cells (see Fig.

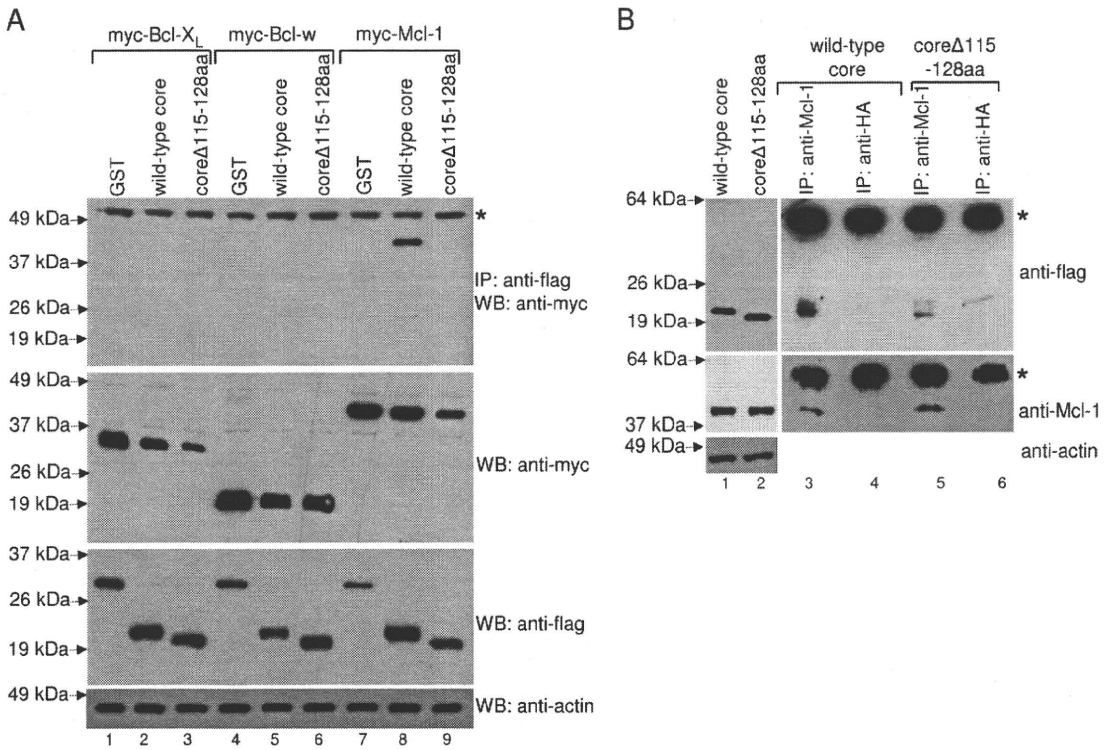

FIG. 3. Interaction of the core protein with prosurvival members of the Bcl-2 family determined by coimmunoprecipitation experiments. (A) Huh7 cells were transfected with cDNA constructs for expressing flag-GST (negative control), flag-core, or flag-coreΔ115-128aa, and myc-tagged prosurvival members of the Bcl-2 family (myc-Bcl-X_L [lanes 1 to 3], myc-Bcl-w [lanes 4 to 6], and myc-Mcl-1 [lanes 7 to 9]). The cells were harvested at ~16 h posttransfection, lysed, and subjected to IP with anti-flag monoclonal antibody conjugated to Sepharose beads. 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 protein marked with an asterisk represents the heavy chain of the antibody used for IP (top), and the amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom). (B) Huh7 cells were transfected with cDNA constructs for expressing flag-core or flag-coreΔ115-128aa. IP then was performed using anti-Mcl-1 or anti-HA rabbit polyclonal antibodies and protein A agarose beads. The amounts of flag-tagged core protein in the lysates before IP (lanes 1 and 2) or coimmunoprecipitated (lanes 3 to 6) were determined by Western blot analysis with an anti-flag monoclonal antibody (top). Similarly, the amounts of endogenous Mcl-1 in these samples were detected using an anti-Mcl-1 monoclonal antibody (middle). The protein marked with an asterisk represents the heavy chain of the antibody used for IP (top), and the amounts of total cell lysates loaded were verified by measuring the levels of endogenous actin (bottom).

S3 in the supplemental material). As shown in Fig. 5A, both the Bad and core protein peptides were inefficient in inducing the release of cytochrome *c*, as only a small amount of cytochrome *c* was detected in the supernatant from the treated mitochondria when 200 μM of either peptide was used. However, when Bad and core protein peptides were used in combination, the release of cytochrome *c* was observed at the much lower concentration of 50 μM (consisting of 25 μM Bad peptide and 25 μM core protein peptide). Furthermore, the release of cytochrome *c* increased in a dose-dependent manner. The amount of cytochrome *c* left in the treated mitochondria (i.e., pellet) decreased correspondingly, while the amount of control protein, Hsp-60, was not affected.

The same experiment was repeated using the Noxa peptide (Fig. 5B). Consistently with a previous study (11), the Noxa peptide alone was inefficient in inducing the release of cytochrome *c*, but when it was combined with the Bad peptide, the release was significantly enhanced. The peptide(s) dosage required was similar to the amount required for the core protein and Bad, indicating that the complementation between the core protein and Bad is similar to the complementation between Noxa and Bad. In addition, complementation between the core protein and Bad also was observed when they were coexpressed in Huh7 cells (see Fig. S4 in the supplemental material).

The three hydrophobic residues in the BH3 domain of the core protein are important for apoptosis induction. Site-directed mutagenesis and structural studies of the interactions between the prosurvival Bcl-2 proteins and BH3 domains have revealed the mechanism by which BH3 domains are bound to the hydrophobic grooves present on the surfaces of the prosurvival Bcl-2 proteins (see reviews in references 50 and 69). In particular, the BH3 domain usually contains four hydrophobic residues (h1, h2, h3, and h4) (Fig. 1A) that make contacts critical for the stability of the complex. Interestingly, the core protein contains hydrophobic residues at the h2, h3, and h4 positions (Fig. 1A). An alanine substitution experiment was performed to determine if these residues are essential for the proapoptotic property of the core protein (Fig. 6A and B). The results showed that replacement of either L119, V122, or L126

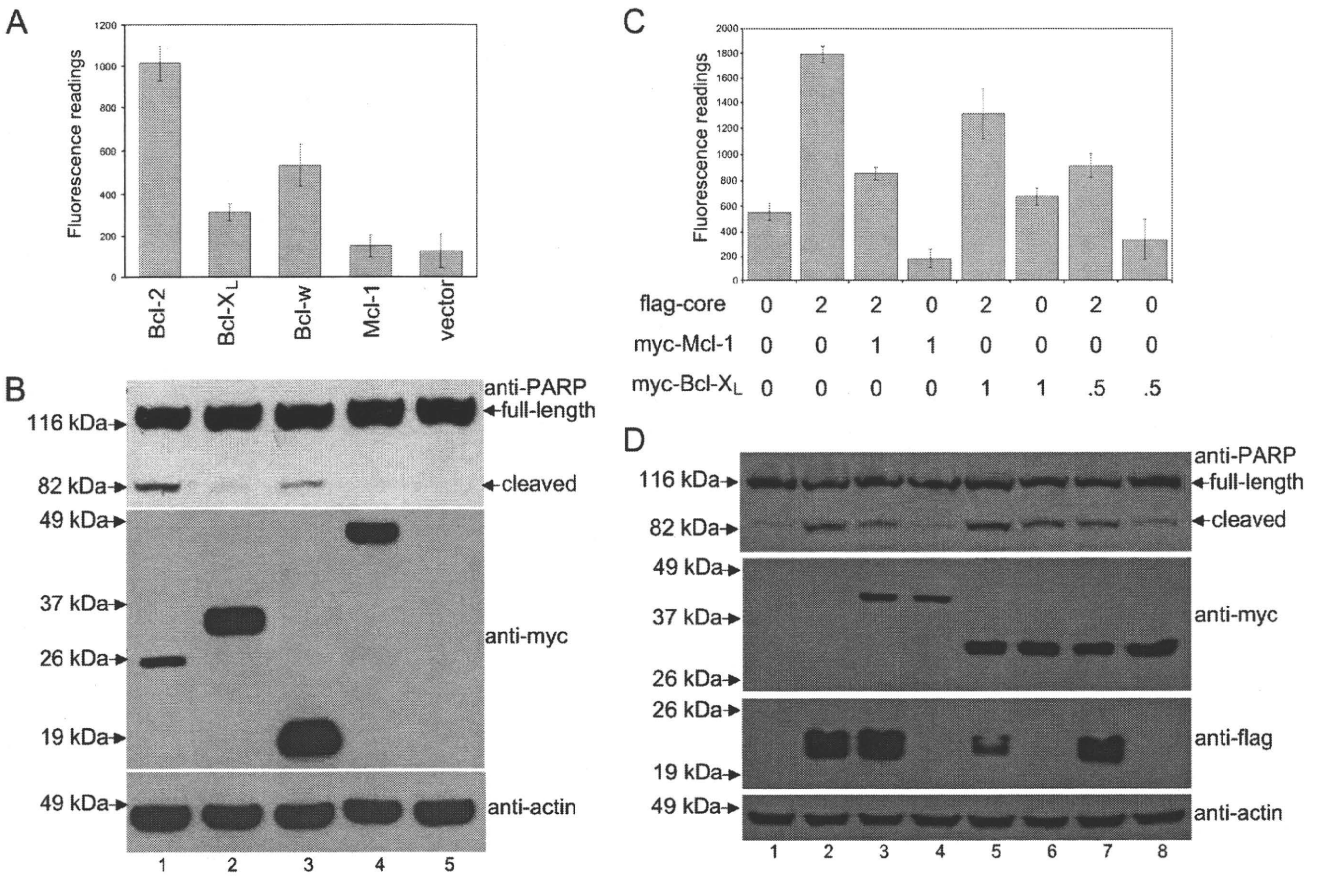

FIG. 4. Effects of Mcl-1 and Bcl-X_L overexpression on the proapoptotic property of the core protein. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were transfected with Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, or 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).

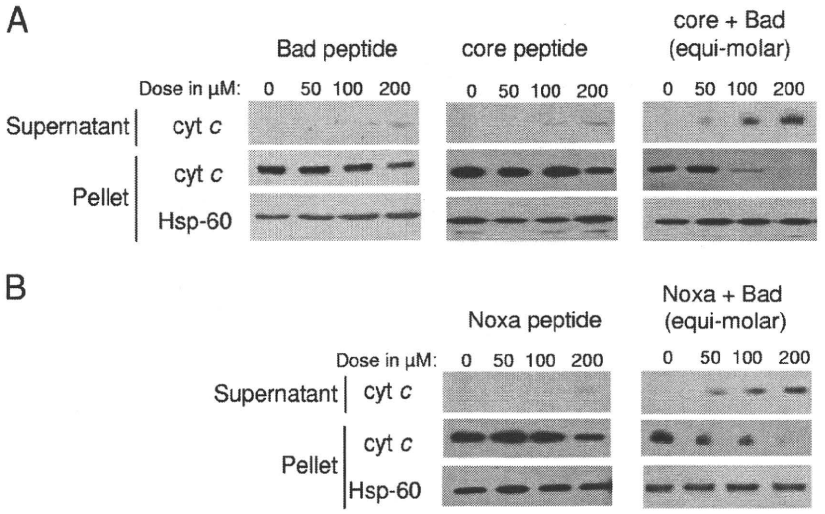

FIG. 5. Release of cytochrome *c* from isolated mitochondria by a combination of the core-BH3 and Bad-BH3 peptides or Noxa-BH3 and Bad-BH3 peptides. (A) Mitochondria isolated from 293T cells were incubated with either Bad peptide, core protein peptide, or a combination of the two peptides in equimolar 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

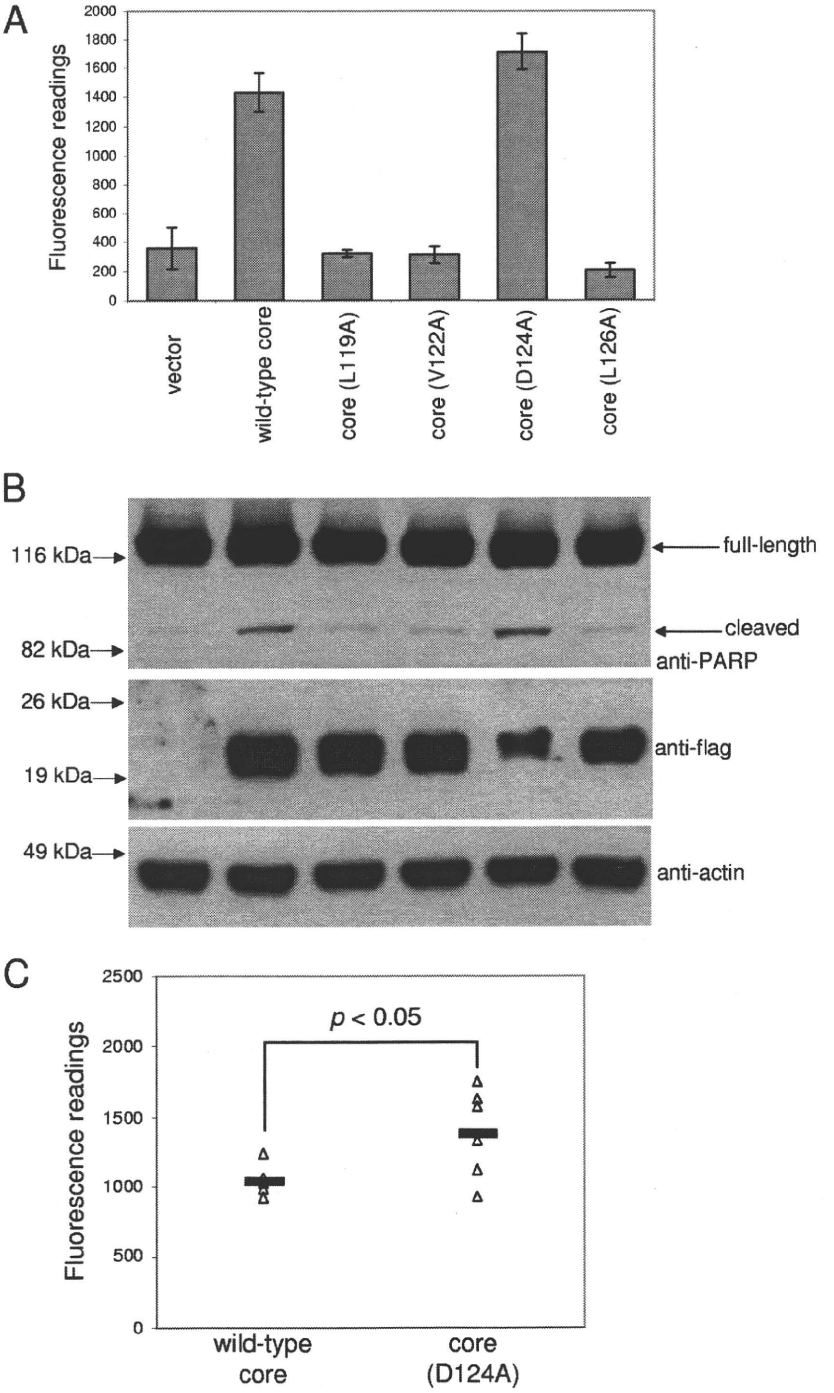

FIG. 6. Effects of alanine substitutions on the proapoptotic property of the core protein. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, wild-type core, or alanine substitution 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-

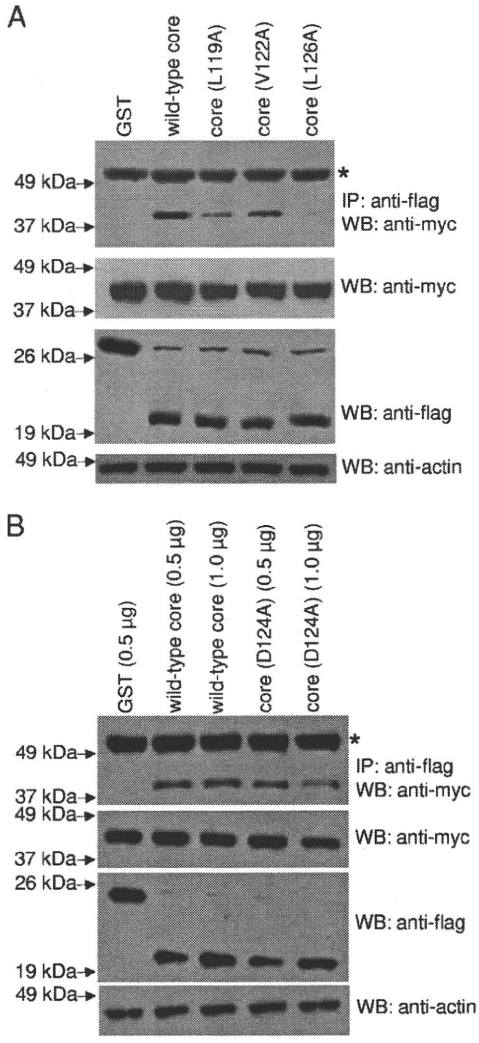

FIG. 7. Effects of alanine substitutions on the binding of the core protein to Mcl-1. (A) Huh7 cells were transfected with cDNA constructs (1.0 μg) for expressing flag-GST (negative control), flag-tagged wild-type core protein, or single-alanine-substitution mutants (L119A, V122A, and L126A). All cells 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.

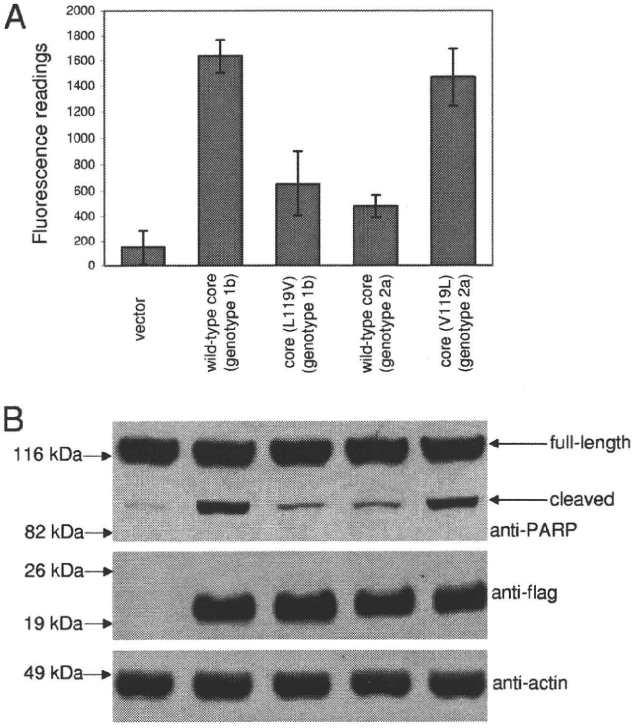

FIG. 8. Comparison of the proapoptotic properties of the core proteins of genotypes 1b and 2a. (A) A CaspACE fluorometric assay system from Promega Corporation (Madison, WI) was used to measure the activation of caspase-3 in Huh7 cells that were transfected with vector only, wild-type core of genotype 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-

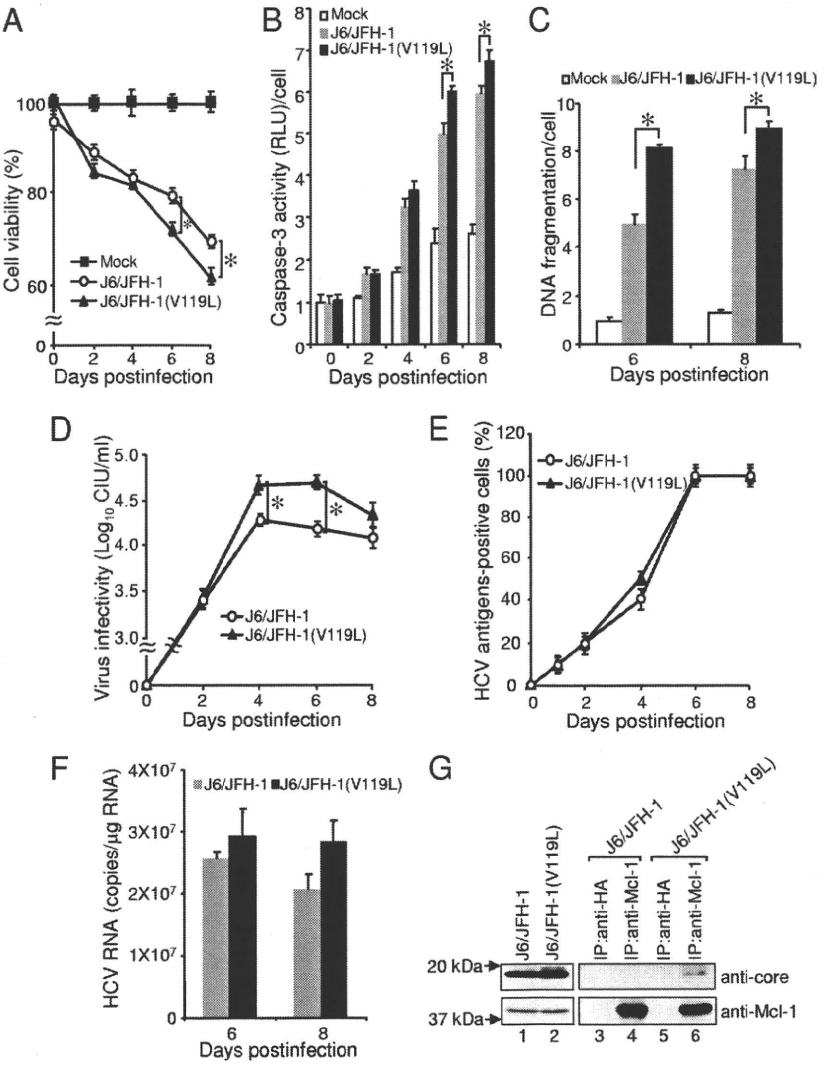

FIG. 9. Comparison of parental J6/JFH-1 and mutant J6/JFH-1(V119L) recombinant viruses. Huh7.5 cells were infected with recombinant HCV at a multiplicity of infection of 0.1 CIU/cell or with a mock preparation, and various assays were performed at different days after infection. (A) Cell viabilities 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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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.

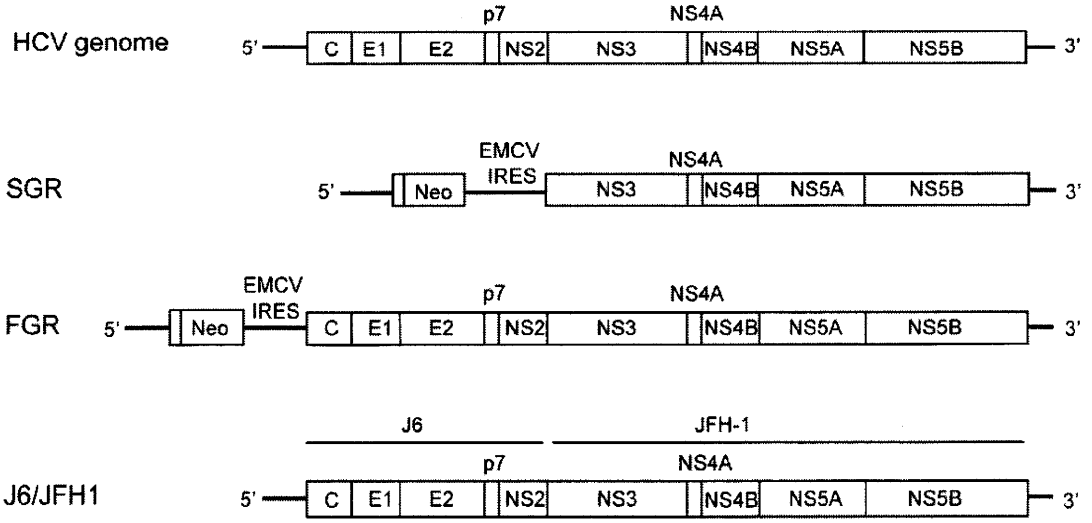

Fig. 1. The HCV genome and HCV RNA replicons. Schematic diagram of the HCV genome, SGR, FGR and the chimeric HCV J6/JFH1 genome are shown. EMCV IRES, encephalomyocarditis virus internal ribosome entry site; Neo, neomycin-resistance gene.

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 *Photimus 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	

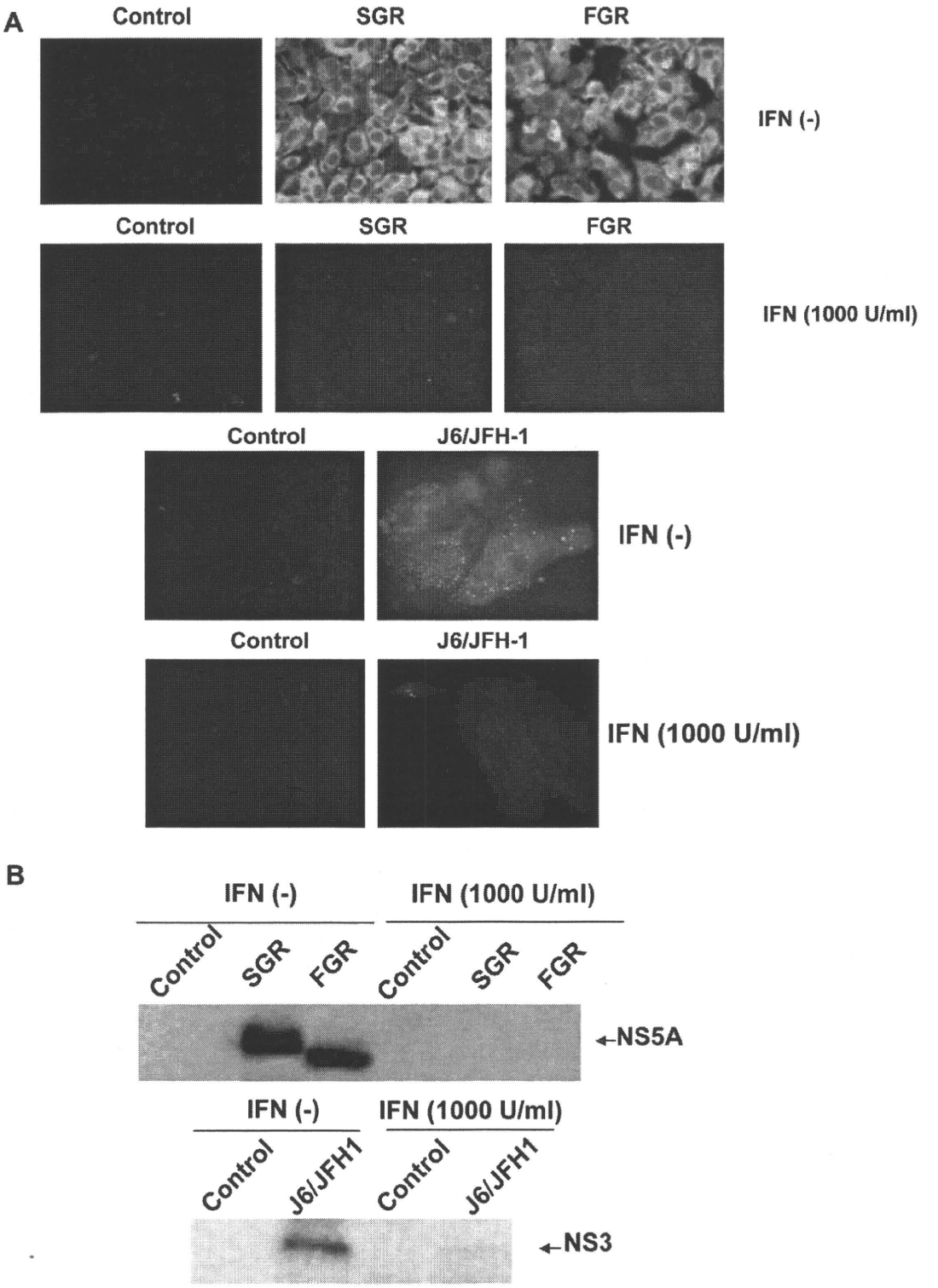

Fig. 2. Expression of HCV proteins in SGR, FGR, HCV-infected cells and the respective cells treated with IFN. (A) Cells were immunostained with anti-NS5A antibody (for SGR, FGR and the control cells) or anti-core antibody (for HCV-infected cells and the control). In parallel, cells were treated with 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).

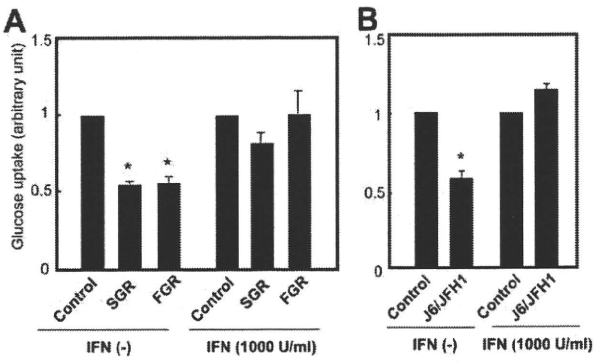

Fig. 3. Selective suppression of cellular glucose uptake by HCV replication. (A) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in SGR, FGR and HCV-negative control. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis. 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

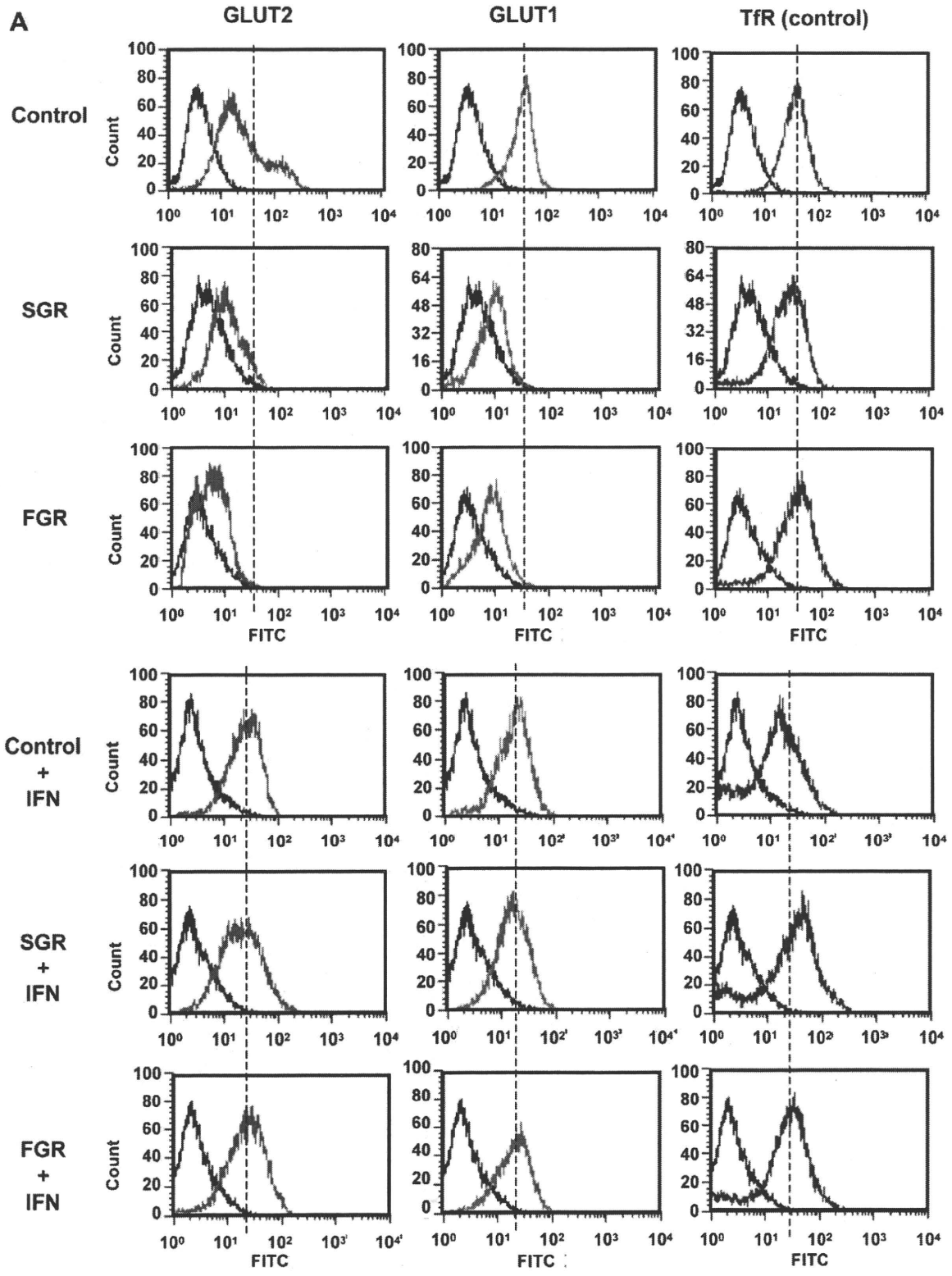

Fig. 4. Down-regulation of cell surface expressions of GLUT2 and GLUT1 by HCV replication. (A) SGR, FGR, the HCV-negative control cells were stained with specific antibodies, followed by FITC-conjugated second antibody (GLUT2, red line; GLUT1, green line) or stained with FITC-conjugated antibody alone (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.

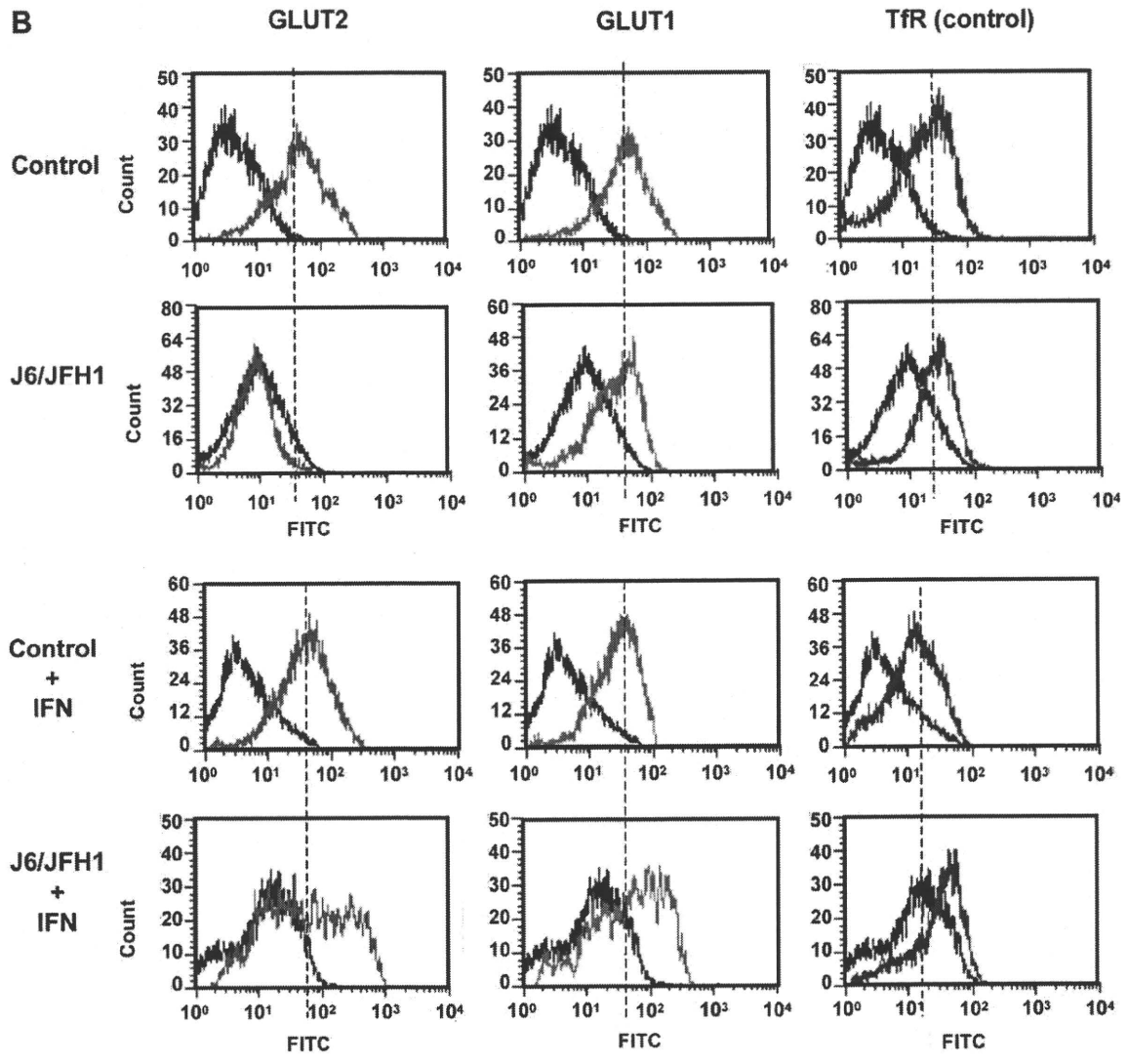

Fig. 4 (*continued*)

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