ELSEVIER

Journal of Hepatology 51 (2009) 853–864

Journal of Hepatology

www.elsevier.com/locate/jhep

Nonstructural 5A protein activates β-catenin signaling cascades: Implication of hepatitis C virus-induced liver pathogenesis☆

Chul-Yong Park[1], Soo-Ho Choi[1], Sang-Min Kang[1], Ju-Il Kang[2], Byung-Yoon Ahn[2], Hoguen Kim[3], Guhung Jung[4], Kang-Yell Choi[5], Soon B. Hwang[1,*]

[1]*National Research Laboratory of Hepatitis C Virus and Ilsong Institute of Life Science, Hallym University, 1605-4 Gwanyang-dong, Dongan-gu, Anyang 431-060, Republic of Korea*
[2]*School of Science and Biotechnology, Korea University, Seoul, Republic of Korea*
[3]*Department of Pathology, College of Medicine, Yonsei University, Seoul, Republic of Korea*
[4]*School of Biological Sciences, Seoul National University, Seoul, Republic of Korea*
[5]*Department of Biotechnology, Yonsei University, Seoul, Republic of Korea*

***Background/Aims*: The nonstructural 5A (NS5A) protein of hepatitis C virus (HCV) has been implicated in HCV-induced liver pathogenesis. Wnt/β-catenin signaling has also been involved in tumorigenesis. To elucidate the molecular mechanism of HCV pathogenesis, we examined the potential effects of HCV NS5A protein on Wnt/β-catenin signal transduction cascades.**

***Methods*: The effects of NS5A protein on β-catenin signaling cascades in hepatic cells were investigated by luciferase reporter gene assay, confocal microscopy, immunoprecipitation assay, and immunoblot analysis.**

***Results*: β-Catenin-mediated transcriptional activity is elevated by NS5A protein, in the context of HCV replication, and by infection of cell culture-produced HCV. NS5A protein directly interacts with endogenous β-catenin and colocalizes with β-catenin in the cytoplasm. NS5A protein inactivates glycogen synthase kinase 3β and increases subsequent accumulation of β-catenin in HepG2 cells. β-Catenin was also accumulated in HCV patients' liver tissues. In addition, the accumulation of β-catenin in HCV replicon cells requires both activation of phosphatidylinositol 3-kinase and inactivation of GSK3β.**

***Conclusions*: NS5A activates β-catenin signaling cascades through increasing the stability of β-catenin. This modulation is accomplished by the protein interplay between viral and cellular signaling transducer. These data suggest that NS5A protein may directly be involved in Wnt/β-catenin-mediated liver pathogenesis.**

***Keywords*: Hepatitis C virus; Liver pathogenesis; NS5A protein; β-Catenin signaling; Tumorigenesis**

Received 10 February 2009; received in revised form 8 June 2009; accepted 22 June 2009; available online 12 August 2009
Associate Editor: F. Zoulim

☆ The authors declared that they do not have anything to disclose regarding funding from industry or conflict of interest with respect to this manuscript.

* Corresponding author. Tel.: +82 31 3801732; fax: +82 31 3845395.
E-mail address: sbhwang@hallym.ac.kr (S.B. Hwang).

Abbreviations: HCV, hepatitis C virus; NS5A, nonstructural 5A; HCC, hepatocellular carcinoma; GST, glutathione S-transferase; HA, hemaglutinin; IFN, interferon; PBS, phosphate-buffered saline; GFP, green fluorescence protein; TRITC, tetramethylrhodamine isothiocyanate; EGF, epidermal growth factor; PI3K, Phosphatidylinositol 3-kinase; GSK3β, glycogen synthase kinase 3β; LEF/TCF, lymphoid enhancing factor/T-cell factor; Grb2, growth factor receptor-bound protein 2.

1. Introduction

Hepatitis C virus (HCV) is the causative agent of non-A, non-B hepatitis. HCV infection often leads to chronic hepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma (HCC) [1]. However, the molecular events that lead to HCC development during HCV infection are poorly defined. HCV is an enveloped, positive-sense RNA virus belonging to the *Flaviviridae* family. Its genome encodes a single polyprotein precursor of more than 3000 amino acids, which is cleaved by host and viral proteases at the endoplasmic reticulum, yielding structural (core E1

0168-8278/$36.00
doi:10.1016/j.jhep.2009.06.026

and E2) and nonstructural (p7, NS2 to NS5B) proteins. The nonstructural 5A (NS5A) protein is generated by the NS3/4A serine protease. NS5A is a phosphoprotein consisting of 447 amino acid residues. NS5A exists in two forms of polypeptide, p56 and p58, which are phosphorylated at serine residues by cellular kinase [2]. Phosphorylation is involved in the HCV life cycle [3]. NS5A protein is localized in the cytoplasm and forms a part of the HCV RNA replication complex [4]. NS5A is a multifunctional protein involved in cellular signal modulations. NS5A modulates interferon (IFN) signaling through protein kinase R (PKR) interaction and also interferes with host cell signaling pathways, including Grb2 and p85 subunits of phosphatidylinositol 3-kinase (PI3K) [5–7]. In addition, NS5A protein has been found to stimulate anchorage-independent growth of murine fibroblast cell lines [8] and induced chromosome instability by mitotic cell cycle dysregulation of HepG2 cell lines [9].

The Wnt/β-catenin signaling pathway was initially discovered by genetic analysis in the wing development of *Drosophila* [10] and has now been implicated in many human cancers. The Wnt family of proteins consists of 350–380 amino acids that serve as the ligands for Frizzled receptors [11]. There are 19 known Wnt ligands in humans [12]. These proteins are highly conserved throughout evolution and play major roles in embryonic patterning, cell polarity, and cell fate determination [12]. β-Catenin was first identified on the basis of its association with cadherin adhesion molecules and is now widely recognized as a key molecule of the Wnt signaling cascade [13]. In the presence of Wnt-1, GSK3β activity is suppressed, which in turn leads to β-catenin stabilization. Abnormalities in the regulation of Wnt/β-catenin signaling have been implicated in various human cancers, including colon cancer, HCC, leukemia, and melanoma [14,15]. It has previously been shown that mutations of β-catenin, specifically stabilizing mutations in exon 3 [16], were detected in approximately 30% of primary HCCs [17,18]. Furthermore, the Wnt/β-catenin signaling pathway has been identified as a common target for perturbation by viruses. For example, hepatitis B virus X protein achieves β-catenin stabilization by suppressing GSK3β activity in a Src-kinase-dependent manner [19]. The Vpu protein of HIV-1 binds to βTrCP and blocks the ubiquitinylation and proteasomal degradation of β-catenin [20]. In addition, HCV NS5A protein is involved in PI3K-mediated β-catenin stabilization [21]. In the present study, we demonstrated that NS5A-activated β-catenin signaling cascades through the stabilization of β-catenin and that this activity may play an important role in HCV pathogenesis.

2. Materials and methods

2.1. Plasmids

Plasmids expressing NS5A (genotype 1b), Myc-tagged NS5A, GST-NS5A, and GFP-NS5A were described elsewhere [22,23]. Either Myc-tagged NS3 or NS4B of HCV (genotype 1b) expression plasmid was generated by PCR and inserted into the EcoRI site in pEF6A/His-Myc vector (Invitrogen, Carlsbad, CA, USA). NS5A mutants were constructed using either the pFlag-CMV-2 or pEF6A/His-Myc vector. Both wild-type and mutants of β-catenin were subcloned into the BamHI site of the pEF6B/His-Myc vector. pTOPFLASH, pFOPFLASH, and TCF-4 expression plasmids were kindly provided by Drs. B. Vogelstein and K. Kinzler (Johns Hopkins University, Baltimore, Maryland, USA). Flag-tagged β-catenin was obtained from Dr. Eric R. Fearon (University of Michigan, Ann Arbor, USA), and HA-human GSK3β was provided by Dr. J. Woodgett (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ont., Canada).

2.2. Cell culture, transfection, and HCV infection

Huh7, HepG2, and Cos7 cells were grown as described previously [24]. Stable cells expressing NS5A were grown as reported previously [23]. HCV subgenomic replicon cells were described elsewhere [23]. RNA prepared from the infectious cDNA clone (JFH-1) was transfected into Huh7 cells, and cell culture-produced HCV was generated as described previously [25].

2.3. Luciferase reporter gene assays

Luciferase and β-galactosidase assays were performed as described previously [24] using 1 μg reporter plasmid (pTOPFLASH or pFOPFLASH) and 0.1 μg of pCH110 reference plasmid (Amersham Biosciences, GE Healthcare UK Ltd., Buckinghamshire, UK) containing the *Escherichia coli lac*Z gene under the control of the Simian virus 40 promoter.

2.4. Immunoblot analysis

Cell lysates were prepared and immunoblotted as described previously [23]. Quantification of the band intensity was determined using a calibrated GS-800 densitometer (Bio-Rad, Hercules, CA, USA).

2.5. Glutathione S-transferase pull-down assay and coimmunoprecipitation

Glutathione S-transferase (GST)-NS5A fusion protein was expressed and purified as described previously [23]. Both in vitro and *in vivo* binding assays between NS5A and β-catenin, and coimmunoprecipitation assay were performed as described elsewhere [24].

2.6. Confocal microscopy

Cos7 cells grown on cover glass were cotransfected with the GFP-NS5A and Flag-catenin expression plasmids, fixed in 4% paraformaldehyde and 0.1% Triton X-100, and analyzed using the LSM 510 laser confocal microscopy system (Carl Zeiss, Inc., Thornwood, New York, USA), as reported previously [22].

2.7. Patient tissues

Human liver tissue specimens were obtained from the Liver Cancer Specimen Bank at Yonsei University in Seoul, South Korea. All patients participating in this study gave informed consent before surgery, and the use of human tissue for this research was authorized by the Institutional Review Board of the College of Medicine at Yonsei University.

2.8. Statistical analysis

The data are presented as mean ± SD. The Student *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant. Error bars represent SDs of three independent experiments.

3. Results

3.1. HCV NS5A protein activates TCF-4 dependent transcriptional activity

The Wnt/β-catenin signaling cascade controls the cellular transcriptional properties of DNA-binding proteins of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family. In the presence of Wnt, β-catenin associates with TCF transcription factors. The TCF/β-catenin complexes bind to DNA and activate Wnt target genes. Because NS5A has been shown to have pleiotropic functions in many cellular signaling events, we first assessed the effects of NS5A protein on Wnt/β-catenin signaling by measuring TCF-4 dependent transcriptional activity. NS5A stable cells were transfected with reporter plasmids containing either wild-type (pTOPFLASH) or mutant (pFOPFLASH) TCF-4 binding sites [26]. The pTOPFLASH or pFOPFLASH reporter gene contains either multimerized wild-type or mutant TCF-4, with the binding site positioned at 5′ of the luciferase reporter gene, respectively. At 24 h after transfection, the luciferase assay was performed. As shown in Fig. 1A, endogenous reporter activity was maintained at a basal level, and this activity was 3-fold activated by NS5A protein alone. We found that TCF-4-dependent transcriptional activity was also activated by transiently expressing NS5A protein in Huh7 cells (data not shown). Wnt/β-catenin signaling was not activated by the Wnt ligand itself in Huh7 cells (data not shown), as reported previously [19]. However, Wnt/β-catenin signaling was activated in cells cotransfected with TCF-4 and Flag-catenin expression plasmid, and this TCF-4 dependent transcriptional activity was approximately 11-fold activated by NS5A protein (Fig. 1A). To investigate the possible impact of NS5A protein on TCF-4-dependent transcription, we determined Wnt/β-catenin signal activation in the absence of ectopic β-catenin transfection. For this purpose, stable cells expressing either NS5A or vector were cotransfected with pTOPFLASH reporter and TCF-4 plasmids in the absence or presence of β-catenin. We found that endogenous reporter activity was approximately 3-fold activated by NS5A protein and that TCF-4-dependent transcriptional activity was also approximately 3-fold increased by TCF-4 in the absence of β-catenin in NS5A stable cells (Supplementary Fig. 1, lane 2 vs. lane 5). However, TCF-4-dependent transcriptional activity was approximately 8-fold activated in cells cotransfected with TCF-4 and β-catenin (Supplementary Fig. 1, lane 3 vs. lane 6), indicating that Wnt/β-catenin signal activation was mediated through β-catenin. Huh7 cells transfected with pFOPFLASH reporter plasmid were not activated in transcriptional activity regardless of NS5A protein (Fig. 1A). We further investigated whether the stimulatory effect of NS5A on Wnt/β-catenin signaling might occur in the context of viral RNA replication. Both IFN-cured and HCV subgenomic replicon cells were cotransfected with TCF-4 and Flag-catenin expression plasmid, and luciferase reporter gene assay was performed. Fig. 1B showed that TCF-4-dependent transcriptional activity in HCV subgenomic replicon cells was increased by 2.4-fold compared with IFN-cured cells. We then asked whether Wnt/β-catenin signaling might be activated in HCV-infected cells. Huh7 cells were either mock-infected or infected with cell culture-produced HCV [25]. At 3 days after infection, cells were transfected with pTOPFLASH reporter plasmid. Luciferase reporter activity was then determined at 24 h after transfection. Fig. 1C showed that TCF-4-dependent transcriptional activity in HCV-infected cells was increased by 2.8-fold as compared with mock-infected Huh7 cells. This is the first report that TCF-4-dependent transcriptional activity is activated by HCV infection.

3.2. β-Catenin is accumulated in NS5A stable cells and HCV replicon cells

Wnt/β-catenin signaling activation leads to stabilization of cytosolic β-catenin. To investigate whether the β-catenin level can be accumulated by HCV NS5A protein, we analyzed the β-catenin level in NS5A stable cells. HepG2 cells expressed two forms of β-catenin: a wild-type and a mutant form lacking amino acids 25–140 [17,27]. The mutant form of β-catenin cannot be phosphorylated and thus accumulated in the nucleus, where it was constitutively active. Two forms of β-catenin can be detected because this anti-β-catenin antibody binds to the C terminus of β-catenin. Fig. 2A showed that wild-type β-catenin was accumulated in NS5A stable cells but not in control vector stable cells. On the other hand, the protein level of mutant β-catenin was unchanged in both control and NS5A stable cells. We found that β-catenin levels were also increased in HepG2 cells transiently expressing NS5A protein and in other isolates of NS5A stable cells (data not shown), confirming that this phenomenon was not caused by clonal selection of NS5A stable cells. To further examine whether the β-catenin level was also increased in the context of HCV RNA replication, we analyzed the β-catenin level in both IFN-cured and HCV subgenomic replicon cells. As expected, β-catenin was accumulated in HCV subgenomic replicon cells but not in IFN-cured cells (see Fig. 2B, top panel). Furthermore, the nonphosphorylated β-catenin level was also increased in the

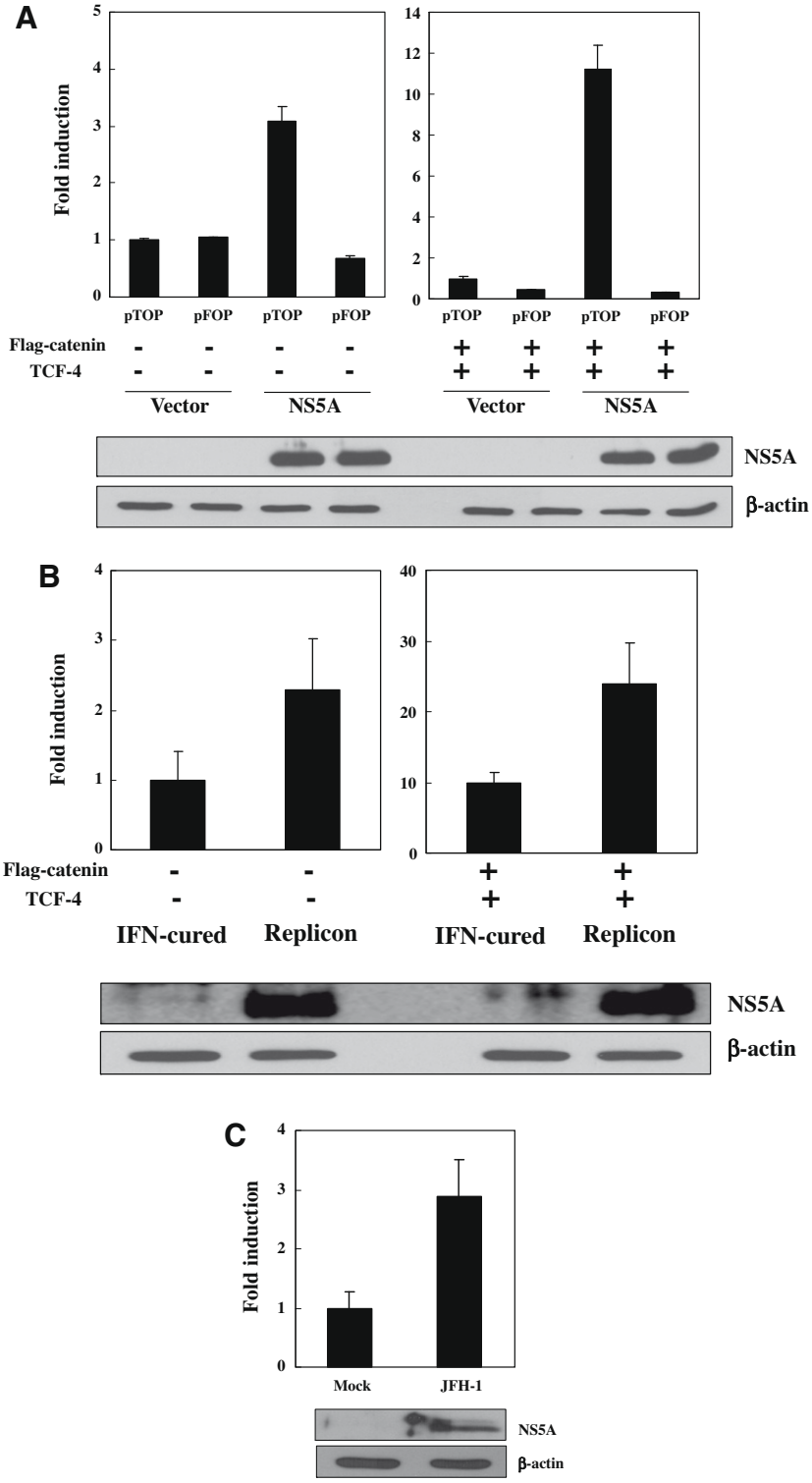

Fig. 1. HCV NS5A proteins activates TCF-4-dependent transcriptional activity. (A) Huh7 cells stably expressing either vector or NS5A were transfected with either pTOPFLASH (pTOP) or pFOPFLASH (pFOP) reporter plasmids together with the indicated expression plasmids. The total DNA concentration in each transfection mixture was kept constant by adjusting with an empty vector. At 24 h after transfection, cells were harvested and luciferase activities were determined. Equal amounts of cell lysates were subjected to immunoblotting with anti-NS5A polyclonal antibody and anti-actin monoclonal antibody (lower two panels). (B) Both IFN-cured and HCV subgenomic replicon cells were transfected with pTOP reporter plasmid together with the indicated expression plasmids. Equal amounts of cell lysates were subjected to immunoblotting with anti-NS5A polyclonal antibody and anti-actin monoclonal antibody (lower two panels). (C) HCV infection activates TCF-4-dependent transcriptional activity. Huh7 cells were either mock-infected or infected with cell culture-produced HCV. At 3 days after infection, cells were transfected with pTOP reporter plasmid. Cells were harvested at 24 h after transfection and luciferase activities were determined. Equal amounts of cell lysates were subjected to immunoblotting with anti-NS5A polyclonal antibody and anti-actin monoclonal antibody (lower two panels).

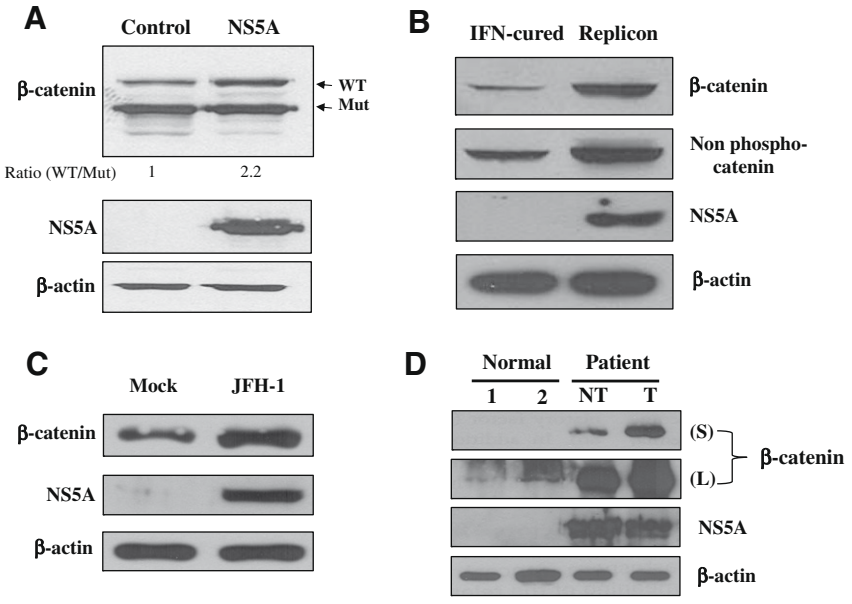

Fig. 2. β-Catenin is accumulated in HCV NS5A stable cells, HCV subgenomic replicon cells, and HCV-infected cells. (A) Both vector stable and NS5A stable cell lysates were immunoblotted with anti-β-catenin monoclonal antibody (top panel) and anti-β-actin monoclonal antibody (bottom panel). Protein expression of NS5A was confirmed by anti-NS5A antibody (middle panel). Quantification of the band intensity was determined using a calibrated GS-800 densitometer. (B) Total cell lysates harvested from IFN-cured and subgenomic replicon were immunoblotted with either anti-β-catenin antibody (1st panel) or nonphosphorylated β-catenin antibody (2nd panel). Protein expression of HCV replicon is confirmed by immunoblotting with anti-NS5A antibody (3rd panel). (C) Huh7 cells were either mock-infected or infected with cell culture-produced HCV. At 3 days after infection, total cell lysates were immunoblotted with either anti-β-catenin antibody (1st panel) or anti-NS5A antibody (2nd panel). (D) Tissue samples isolated from normal human liver and either nontumor or tumor regions of an HCV patient were immunoblotted with anti-β-catenin antibody. Protein expression of HCV in liver tissue was confirmed by immunoblotting with anti-NS5A antibody. Protein expression of β-actin was used as a loading control. NT, nontumor region; T, tumor region; (S), short exposure; (L), long exposure.

HCV subgenomic replicon cells (Fig. 2B, second panel). We then examined whether the β-catenin level was also increased by HCV infection. We analyzed the β-catenin level in both mock-infected and HCV-infected cells. Indeed, the β-catenin level in HCV-infected cells (JFH-1) was also increased as compared with mock-infected Huh7 cells (Fig. 2C). We then asked if the β-catenin level could be altered in HCV patients. We therefore compared the β-catenin levels in human livers of both normal and HCV patients. As demonstrated in Fig. 2D, the β-catenin level was maintained at the basal level in normal human livers, whereas it was accumulated in the liver of HCV patients. It was noteworthy that the β-catenin level was higher in the tumor region than in the nontumor region in many HCV patient samples (Fig. 2D and Supplementary Fig. 2).

It has been previously reported that active mutations of β-catenin were found in HCC associated with HCV infection [17,28]. It is well known that β-catenin is phosphorylated at codons 33, 37, 41, and 45 by GSK3β [29] and CKI [30]. To investigate whether any β-catenin genes used in our studies were mutated, we isolated total RNAs from Huh7, IFN-cured, HCV subgenomic replicon cells, and HCV patients' liver tissues. We analyzed cDNA sequences of β-catenin and found that all cDNA sequences were identical to wild-type sequences (data not shown) and hence that β-catenin accumulations in both NS5A stable cells and HCV subgenomic replicon cells were not due to genetic mutations. In addition, we determined the transcription level of β-catenin gene in HCV subgenomic replicon cells. Using total cellular RNA isolated from IFN-cured and HCV subgenomic replicon cells, mRNA levels were determined by quantitative PCR. There were no differences in the mRNA levels of β-catenin in both IFN-cured and HCV subgenomic replicon cells (Supplementary Fig. 3A and B). This result indicated that the accumulation of β-catenin in HCV subgenomic replicon cells occurred at a post-transcriptional level.

3.3. β-Catenin levels are increased in both cytosolic and nuclear fractions in HCV subgenomic replicon cells

The activation of Wnt/β-catenin signaling involves increasing the post-translational stability of β-catenin and subsequent accumulation of cytoplasmic and nuclear levels of β-catenin. To investigate the subcellular distribution of β-catenin, both nuclear and cytoplasmic fractions were prepared as reported previously [23], and each fraction was analyzed by immunoblotting. As shown in Fig. 3A, protein levels of both total and nonphosphorylated catenin were increased in

cytoplasmic and nuclear fractions in HCV subgenomic replicon cells as compared with IFN-cured cells. In addition, immunofluorescent staining data showed that nuclear translocated β-catenin proteins were observed only in HCV subgenomic replicon cells but not in IFN-cured cells (Fig. 3B). These results indicated that TCF-4-dependent transcriptional activation observed in NS5A expressing cells and in HCV subgenomic replicon cells were due to nuclear translocated β-catenin.

3.4. GSK3β is inactivated in NS5A stable cells and in HCV subgenomic replicon cells

In Wnt/β-catenin signaling cascades, β-catenin levels are regulated by GSK3β protein. Since β-catenin was accumulated in NS5A stable cells and in HCV subgenomic replicon cells, we examined whether GSK3β was inactivated in these cells by immunoblotting with phospho-GSK3β antibody. The phosphorylated GSK3β is inactive and hence unable to phosphorylate β-catenin, resulting in accumulation of β-catenin in cells. Indeed, the level of GSK3β phosphorylation at Ser^9 residue was increased in NS5A stable cells (Fig. 4A, middle panel). We confirmed that the phosphorylated GSK3β level was also increased in HCV subgenomic replicon cells (Fig. 4B, middle panel). These data indicate that the accumulation of β-catenin was attributed to the inactivated GSK3β in NS5A expressing cells and in HCV replicon cells.

3.5. PI3K activity is required for β-catenin accumulation in HCV subgenomic replicon cells

It has previously been reported that HCV NS5A protein was involved in PI3K-Akt-mediated β-catenin stabilization in HepG2 cells [21]. To investigate whether β-catenin accumulation in HCV subgenomic replicon cells was mediated through the activation of PI3K, we treated both IFN-cured and replicon cells with PI3K inhibitor, LY294002. As shown in Fig. 5, the β-catenin level in IFN-cured cells was unchanged by the treatment of PI3K inhibitor. However, the β-catenin level in cells harboring HCV subgenomic replicon was significantly reduced by LY294002 (Fig. 5A, lane 4), indicating that PI3K activation was required for the accumulation of β-catenin in HCV subgenomic replicon cells. We further

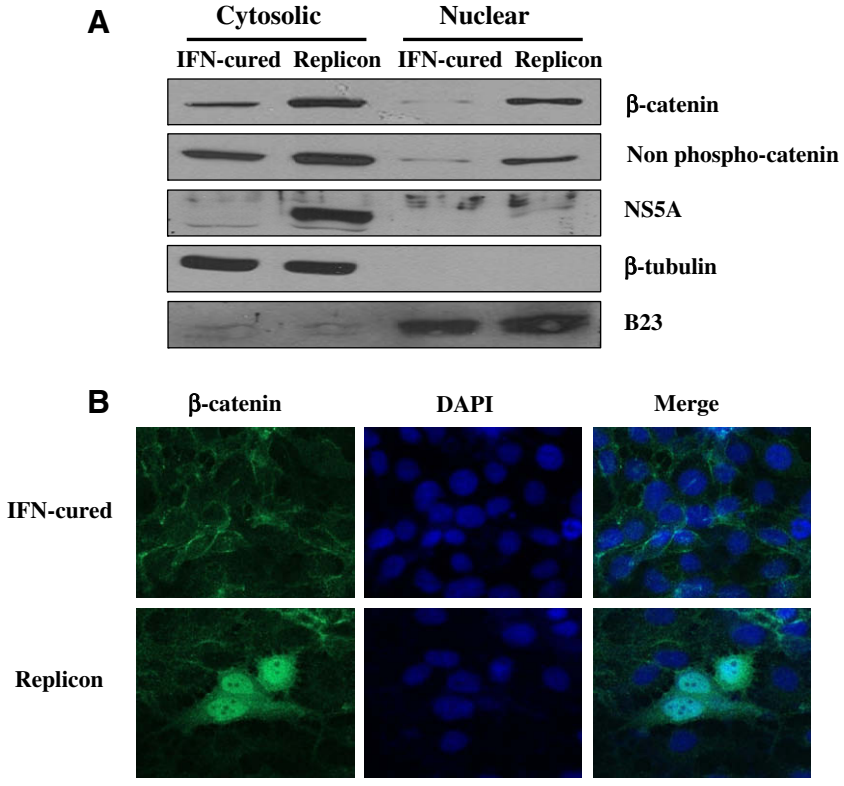

Fig. 3. β-Catenin levels are increased in both cytosolic and nuclear fractions in the HCV subgenomic replicon cells. (A) Both cytosolic and nuclear fractions prepared from IFN-cured and subgenomic replicon cells were immunoblotted with anti-β-catenin or nonphosphorylated β-catenin antibody (1st and 2nd panels). Protein expression of HCV replicon was confirmed by immunoblotting with anti-NS5A antibody (3rd panel). Both cytosolic and nuclear fractions were verified by immunoblot analysis using either anti-β-tubulin or B23 antibodies (4th and 5th panels). (B) Both IFN-cured and subgenomic replicon cells grown on cover glass were fixed and incubated with anti-β-catenin monoclonal antibody for 2 h. After being washed with PBS, cells were further incubated with FITC-conjugated goat anti-mouse IgG for 1 h. Samples were analyzed for immunofluorescence staining using the LSM 510 laser confocal microscopy system. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to label nuclei.

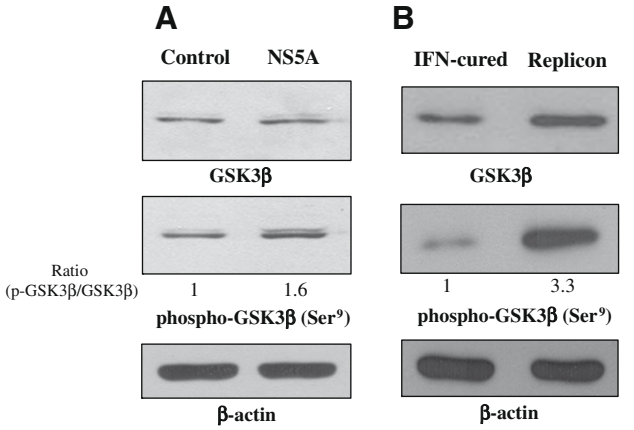

Fig. 4. GSK3β is inactivated in NS5A stable cells and in HCV subgenomic replicon cells. (A) Both vector stable and NS5A stable cell lysates were immunoblotted with anti-GSK3β (top panel) and anti-phospho-GSK3β antibody (middle panel). Protein expression of β-actin was used as a loading control (bottom panel). (B) Total cell lysates harvested from IFN-cured and subgenomic replicon were immunoblotted with either anti-GSK3β antibody (top panel) or phospho-GSK3β antibody (middle panel). β-Actin was used as a loading control (bottom panel). Quantification of the band intensity was determined using a calibrated GS-800 densitometer.

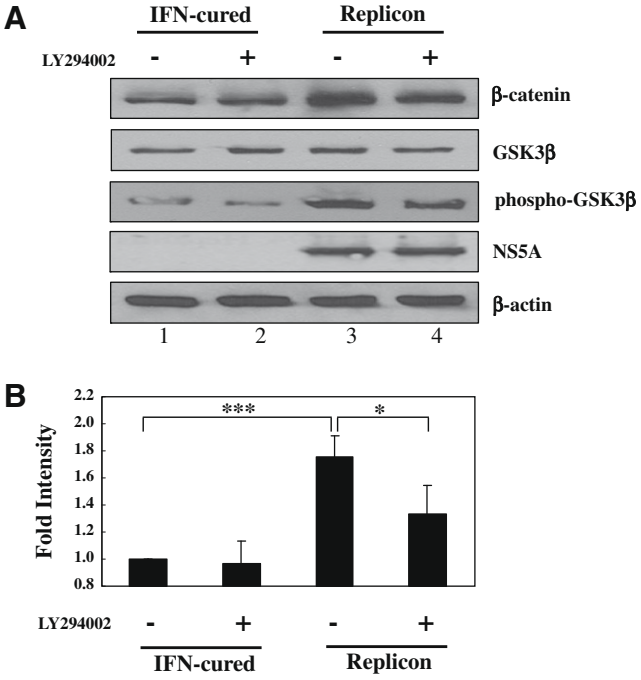

Fig. 5. PI3K activity is required for β-catenin accumulation in HCV subgenomic replicon cells. (A) Both IFN-cured and HCV subgenomic replicon cells were either left untreated (DMSO only) or treated with 10 μM of LY294002 (dissolved in DMSO) for 6 h. Total cell lysates were immunoblotted with anti-β-catenin antibody (1st panel). The same cell lysate was immunoblotted with anti-GSK3β antibody (2nd panel) and anti-phospho-GSK3β antibody (3rd panel). Protein expression of HCV replicon was confirmed by immunoblotting with anti-NS5A antibody (4th panel). Protein expression of β-actin was used as a loading control for the same amount of cell lysates (5th panel). (B) Data from triplicate experiments shown in (a) were quantified, and each bar represents the average intensity of the β-catenin level. Quantification of the band intensity was determined as described above. $^{***}P < 0.001$, replicon cells vs. IFN-cured cells; $^{*}P < 0.05$, LY294002-treated HCV subgenomic replicon cells vs. untreated replicon cells.

confirmed this result by repeating these experiments three times in HCV replicon cells, as demonstrated in Fig. 5B. We further demonstrated that the phosphorylated GSK3β level was also decreased in HCV subgenomic replicon cells (Fig. 5A, lane 4).

3.6. HCV NS5A protein interacts with β-catenin both in vitro and in vivo

Because NS5A protein stabilizes the β-catenin level in hepatic cells, we speculated that NS5A might activate Wnt/β-catenin signaling through interaction with β-catenin, which is a key molecule in the Wnt/β-catenin signaling pathway. We first examined the interaction between NS5A and β-catenin by in vitro GST pull-down assay using GST and GST-NS5A fusion protein expressed in *E. coli*. Cell extracts containing Flag-tagged β-catenin were incubated with GST beads for 2 h at 4 °C. As shown in Fig. 6A, GST-NS5A selectively bound to β-catenin, whereas GST failed to interact with β-catenin. To further confirm in vitro interaction between NS5A and β-catenin, we performed a coimmunoprecipitation assay. Myc-tagged NS5A was coexpressed with Flag-tagged β-catenin in Cos7 cells. We took advantage of the high efficiency of the protein expression level in Cos7 cells paired with a recombinant vaccinia virus (vTF7-3) system, as reported previously [23]. Cell lysates were immunoprecipitated with anti-Flag monoclonal antibody, and the coprecipitated protein was detected by immunoblot analysis using an anti-Myc monoclonal antibody. Indeed, NS5A specifically interacted with β-catenin *in vivo* (Fig. 6B). We confirmed this result by a reciprocal experiment using an anti-Myc antibody for coimmunoprecipitation and an anti-Flag antibody to detect coprecipitated protein (Supplementary Fig. 4A). Next, we investigated whether NS5A interacted with endogenous β-catenin protein. Both IFN-cured and HCV subgenomic replicon cell lysates were immunoprecipitated with anti-NS5A antibody, and bound proteins were analyzed by immunoblotting with an anti-β-catenin monoclonal antibody. Fig. 6C shows that NS5A interacts with endogenous β-catenin protein. These data suggest that NS5A protein may colocalize with exogenously expressed β-catenin. To determine this possibility, Cos7 cells were cotransfected with GFP-tagged NS5A and Flag-tagged β-catenin expression plasmids, and we examined the subcellular localization by confocal microscopy. As shown in Supplementary Fig. 4B, NS5A was localized in the cytoplasm, and β-catenin

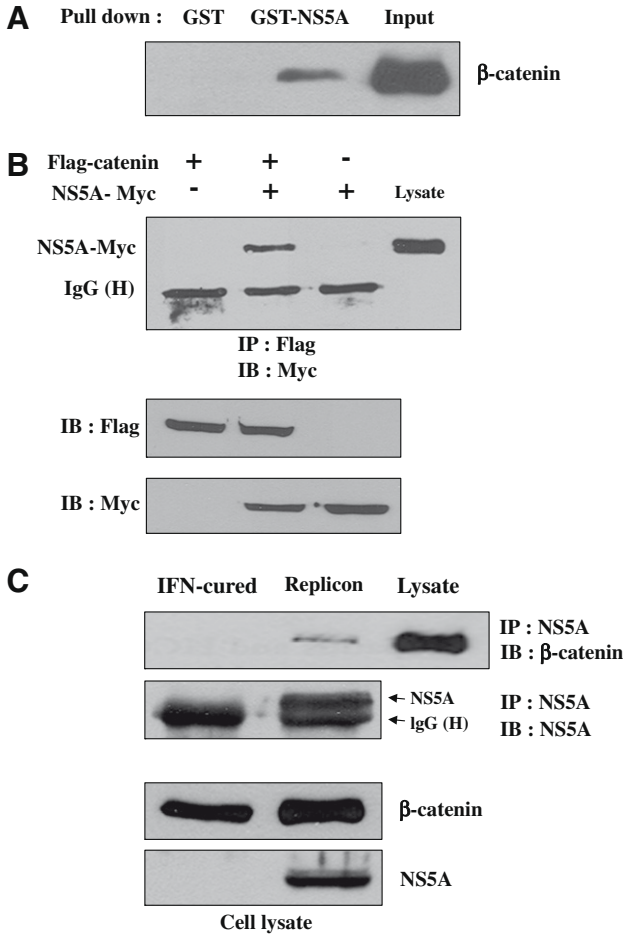

Fig. 6. HCV NS5A protein interacts with β-catenin both in vitro and *in vivo*. (A) In vitro GST pull-down assay. 293T cell lysates expressing Flag-catenin were incubated with either GST or GST-5A fusion protein purified from *E. coli*. Bound proteins were precipitated by glutathione-sepharose beads and detected by immunoblotting with anti-Flag monoclonal antibody. (B) *In vivo* coimmunoprecipitation assay. Cos7 cells were transfected with Flag-tagged β-catenin and Myc-tagged NS5A protein expression plasmids paired with a recombinant vaccinia virus vTF7-3. At 12 h after transfection, cell lysates were immunoprecipitated with anti-Flag monoclonal antibody, and bound proteins were detected by immunoblot analysis using anti-Myc monoclonal antibody (top panel). Protein expressions of Flag-tagged β-catenin and Myc-tagged NS5A were verified by immunoblotting with either anti-Flag monoclonal antibody (middle panel) or anti-Myc monoclonal antibody (bottom panel). (C) NS5A protein interacts with endogenous β-catenin in HCV replicon cells. Both IFN-cured and HCV subgenomic replicon cell lysates were immunoprecipitated with anti-NS5A antibody, and bound proteins were detected by immunoblotting with anti-β-catenin monoclonal antibody. Control, HCV replicon cell lysate was immunoblotted with β-catenin antibody.

was localized in both the nucleus and cytoplasm. In the merged image, two proteins were colocalized in the cytoplasm. We have further confirmed the colocalization of NS5A and β-catenin in Huh7 cells (data not shown). Taken together, these data indicate that NS5A specifically interacts with β-catenin both in vitro and *in vivo*.

3.7. NS5A interacts with β-catenin through the N-terminal region of NS5A and the ARM region 1–6 of β-catenin

To determine the region in NS5A that is responsible for β-catenin binding, the interaction of β-catenin with various deletion mutants of NS5A (Fig. 7A) was determined by a transfection-based coprecipitation assay in Cos7 cells infected with the recombinant vaccinia virus vTF7-3. As shown in Fig. 7B, β-catenin interacted with an N-terminal region of NS5A (amino acids 1–147). Next, we determined the region in β-catenin that is responsible for NS5A binding. We constructed various deletion mutants of β-catenin (Fig. 7C) and the binding domain was determined as described above. Fig. 7D showed that NS5A interacted with either N-terminal or C-terminal deletion mutants of β-catenin (M1 and M4, respectively). However, a mutant lacking the N-terminus plus ARM 1–6 of β-catenin (M2) no longer interacted with NS5A, suggesting that NS5A interacted with β-catenin through ARM 1–6 (amino acids 153–390) of β-catenin.

To investigate whether the N-terminal region of NS5A protein mediates the activation of TCF-4-dependent transcriptional activity and the accumulation of β-catenin, Huh7 cells transfected with either wild-type or mutant forms of NS5A were analyzed for luciferase reporter activity and β-catenin level. Indeed, the N-terminal region of NS5A protein was involved in TCF-4-dependent transcriptional activation (Fig. 8A) and an accumulation of β-catenin in Huh7 cells (Fig. 8B). However, neither TCF-4 dependent transcriptional activity nor β-catenin accumulation was affected by the middle region of NS5A protein. These data further support that β-catenin is accumulated through interaction with the N-terminus of NS5A protein.

3.8. HCV NS5A protein inhibits protein interaction between GSK3β and β-catenin

Since NS5A interacted with β-catenin both in vitro and *in vivo*, we examined the possibility that NS5A might affect GSK3β and β-catenin interaction. For this purpose, Flag-tagged β-catenin and HA-tagged GSK3β were cotransfected with either NS5A or other control plasmids in Cos7 cells infected with the recombinant vaccinia virus vTF7-3. At 12 h after transfection, cell lysates were immunoprecipitated with anti-HA antibody, and then coimmunoprecipitated catenin was analyzed by immunoblot analysis using anti-Flag monoclonal antibody. As reported previously, GSK3β formed a complex with β-catenin, and this complex was significantly inhibited by NS5A protein (Supplementary Fig. 5A, lane 5), but not by vector or NS3 protein. Interestingly, NS4B slightly inhibited GSK3β/β-catenin complex formation. We further confirmed that N-terminal region of NS5A protein mediated the inhibition of

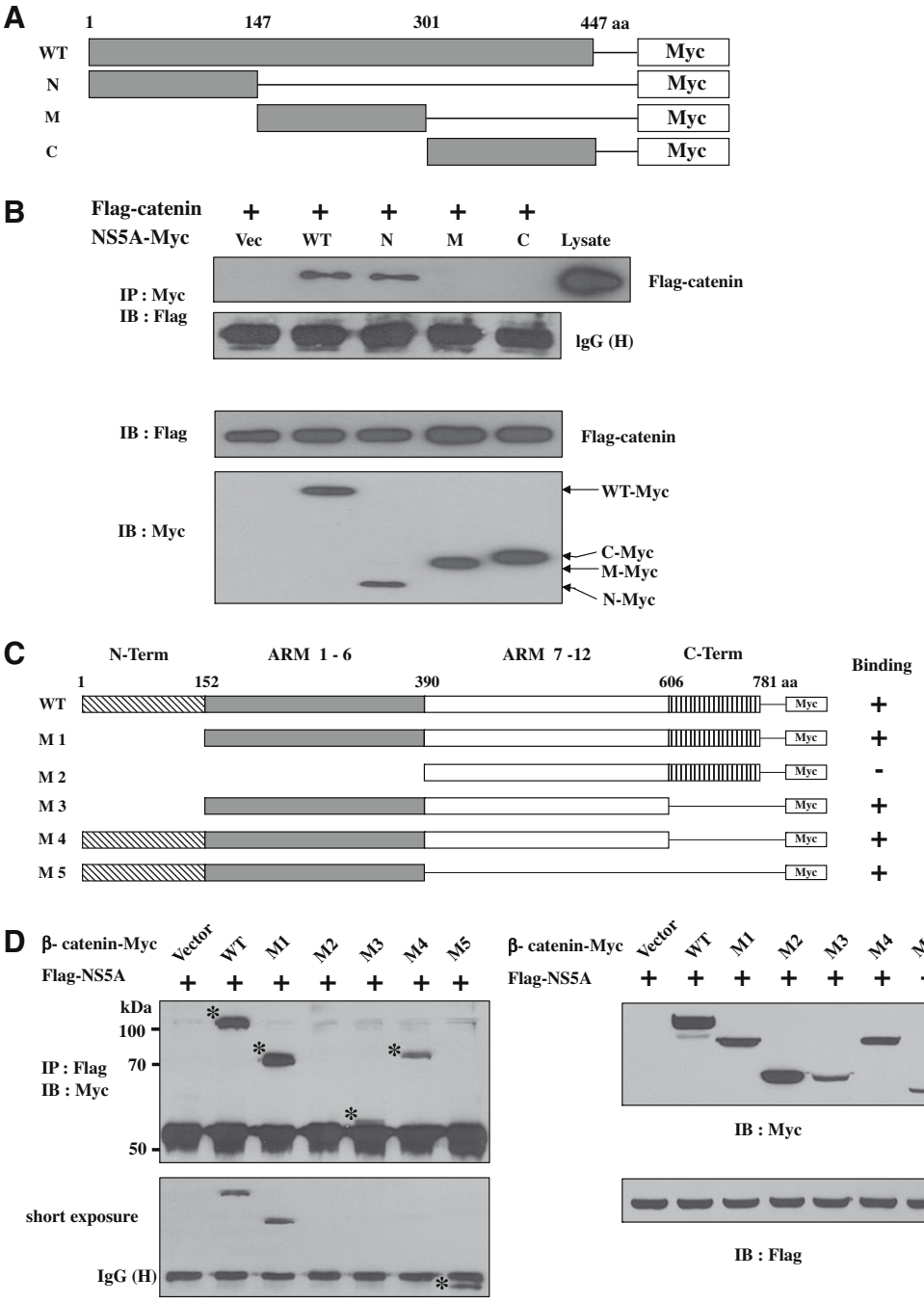

Fig. 7. HCV NS5A protein interacts with β-catenin through the amino terminal region of NS5A and ARM region 1–6 of β-catenin. (A) Schematic diagram shows both wild-type and mutant forms of Myc-tagged NS5A. aa, amino acids. (B) NS5A interacts with β-catenin through the N-terminal region of NS5A. Cos7 cells were cotransfected with Flag-tagged β-catenin and mutant forms of Myc-tagged NS5A protein expression plasmids paired with the recombinant vaccinia virus vTF7-3. At 12 h after transfection, cell lysates were immunoprecipitated with anti-Myc monoclonal antibody, and bound proteins were immunoblotted with anti-Flag monoclonal antibody (1st panel). IgG shows the loading control for Myc antibody (2nd panel). Protein expressions of Flag-tagged β-catenin (3rd panel) and Myc-tagged NS5A (4th panel) were confirmed using the same cell lysates by immunoblotting with anti-Flag and anti-Myc monoclonal antibody. IP, immunoprecipitation; IB, immunoblot. (C) Schematic diagram shows both wild-type and mutant forms of Myc-tagged β-catenin. (D) β-Catenin interacts with NS5A through ARM 1–6 region of β-catenin. 293T cells were cotransfected with Flag-tagged NS5A and mutant forms of Myc-tagged β-catenin expression plasmids. At 24 h after transfection, cell lysates were immunoprecipitated with anti-Flag monoclonal antibody, and bound proteins were immunoblotted with anti-Myc monoclonal antibody (left two panels). Protein expressions of Myc-tagged β-catenin and Flag-tagged NS5A were verified by immunoblotting with anti-Myc and anti-Flag monoclonal antibody (right two panels) using the same cell lysates.

GSK3β/β-catenin complex formation (Supplementary Fig. 5B). Because the protein expression levels of GSK3β and β-catenin were not affected by NS5A, these results might explain the underlying mechanism of

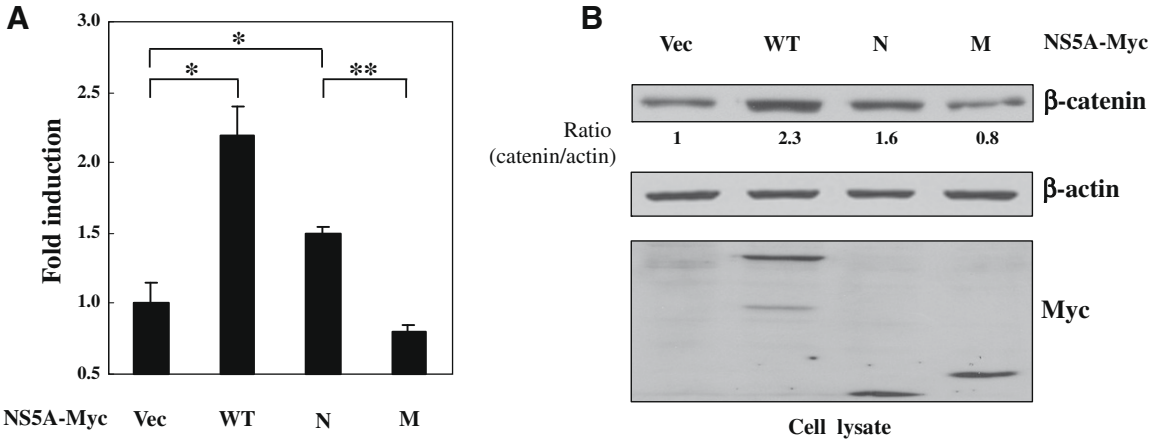

Fig. 8. Amino terminal region of NS5A protein mediates TCF-4-dependent transcriptional activation and β-catenin accumulation in hepatoma cells. (A) Huh7 cells were transfected with pTOPFLASH reporter plasmid together with the indicated expression plasmids. At 24 h after transfection, cells were harvested and luciferase activities were determined. $^{*}P < 0.05$, vector transfected cells vs. wild-type NS5A or vector transfected cells vs. mutant expressing N-terminal region of NS5A; $^{**}P < 0.01$, N-terminal region of NS5A vs. middle (M) region of NS5A. (B) Huh7 cells were transfected with either wild-type or mutant forms of NS5A. Total cell lysates were immunoblotted with anti-β-catenin antibody (top panel). Expressions of both wild-type and mutants of NS5A were verified using the same cell lysates by immunoblotting with anti-Myc monoclonal antibody (bottom panel). Quantification of the band intensity was determined by using a calibrated GS-800 densitometer.

β-catenin-dependent signaling activation by NS5A protein.

4. Discussion

HCV is the major cause of non-A, non-B hepatitis, which frequently leads to liver cirrhosis and HCC. To date, the molecular events during HCV infection that lead to HCC development have been poorly defined. NS5A is a pleiotropic protein involved in viral RNA replication and modulation of the physiology of the host cells. NS5A interacts with cellular signaling transducers, transcriptional activation machinery, and cell cycle regulatory kinases. NS5A has also been associated with cellular transformation in vitro [8,31]. Although the oncogenicity of NS5A is controversial [32], NIH3T3 cells transfected with NS5A resulted in a transformed phenotype defined by increased proliferation and colony formation in soft-agar medium [8,31]. To investigate the molecular mechanism of HCV-induced pathogenesis, we investigated the potential involvement of HCV NS5A protein in the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is initiated by the binding of Wnt ligand to a receptor encoded by the Frizzled gene. Activation of the receptor phosphorylates Disheveled protein, which then prevents glycogen synthase kinase 3β (GSK3β) from phosphorylating β-catenin. Unphosphorylated β-catenin escapes from the E3 ubiquitin pathway, resulting in accumulation and translocation into the nucleus. In the nucleus, β-catenin forms a transcription complex with TCF/LEF and leads to the activation of target genes. Aberrant activations of Wnt target genes are considered to be the basis for tumorigenesis. Since NS5A protein is implicated in HCV pathogenesis, we asked whether NS5A could modulate β-catenin signaling cascades. We demonstrated that TCF-4-dependent transcriptional activity was increased in the presence of NS5A, in the context of HCV replication and in HCV infection. NS5A protein increased the stability of β-catenin through protein interplay in hepatoma cell lines, and thus β-catenin was accumulated in NS5A stable cells and in the HCV replicon cells. β-Catenin accumulation occurred in both cytosolic and nuclear fractions in HCV subgenomic replicon cells. Quantitative PCR data indicated that the accumulation of β-catenin occurred at post-transcriptional level. Since mutations in β-catenin were associated with β-catenin stabilization in HCC [16–18], we have sequenced all β-catenin genes used in our studies and confirmed that there was no mutation in β-catenin genes.

Activating mutations of Wnt components lead to nuclear localization of β-catenin and are involved in tumor formation and development [33]. Nevertheless, Wnt-independent signaling is also involved in regulation of β-catenin transactivation and tumorigenesis [34]. β-Catenin-TCF/LEF-1 signaling can be activated by growth factors, such as epidermal growth factor (EGF), hepatocyte growth factor, insulin-like growth factor I, insulin-like growth factor II, and insulin [35–38]. In response to insulin stimulation, phosphatidylinositol 3-kinase-activated Akt phosphorylates GSK3β at Ser^{9}, which leads to inactivation of GSK3β and augmentation of β-catenin-TCF/LEF-1 transcriptional activity [39]. Interestingly, viral proteins also increase the stability of β-catenin through various mechanisms. Hepatitis B virus X protein stabilizes β-catenin by suppressing GSK3β activity through Src kinase [19]

and Erk [40]. Epstein–Barr virus latent membrane protein 2A activates PI3K and Akt, resulting in GSK3β inactivation and β-catenin stabilization [41]. Tomita et al. reported that the Tax protein of human T-cell leukemia virus type 1 activates β-catenin through the Akt signaling pathway [42]. Likewise, we demonstrated that the phospho-GSKβ (Ser^9) level was increased in both NS5A stable cells and HCV subgenomic replicon cells, indicating that HCV-induced β-catenin accumulation occurred through the inactivation of GSKβ. It has previously been shown that NS5A activated PI3K and the downstream effector serine/threonin kinase Akt/protein kinase B [7], and that NS5A was involved in PI3K-Akt-mediated β-catenin stabilization [21]. We also found that β-catenin accumulation in HCV subgenomic replicon cells required the activation of PI3K protein. We further demonstrated that the accumulation of β-catenin was mediated through protein interplay between HCV NS5A and β-catenin. We consider that these two mechanisms are independent of each other in terms of β-catenin accumulation.

NS5A directly interacted with β-catenin through the amino terminal region of NS5A and the ARM region 1–6 of β-catenin. Protein interaction between NS5A and β-catenin was confirmed by GST pull-down assay, coimmunoprecipitation assay, and confocal microscopy. Moreover, NS5A protein interacted with endogenous β-catenin in HCV replicon cells. We also found that the amino terminal region of NS5A protein activated TCF-4 dependent transcriptional activity and increased the protein level of β-catenin. We further showed that NS5A increased the β-catenin stability by disrupting GSK3β from complex formation with its β-catenin substrate. It has previously been reported that T-antigen encoded by the human neurotropic polyomavirus JCV interacted with β-catenin and elevated the level of β-catenin in cells due to prolonged stability of the protein [43]. In JCV, the interaction of β-catenin with T-antigen facilitated the nuclear import of β-catenin. However, how HCV NS5A increased the nuclear β-catenin level is unclear because NS5A localizes in the cytoplasm, although the nuclear localization signal exists at its C-terminus. It is possible that NS5A may either form a structural barrier between β-catenin and GSK3β or inhibit APC/Axin/GSK3β complex formation such that GSK3β can no longer phosphorylate β-catenin and perform subsequent ubiquitinylation. In fact, we demonstrated that the HCV NS5A protein inhibited protein interaction between GSK3β and β-catenin. We therefore speculate that the accumulation of β-catenin in cytoplasm by NS5A protein may facilitate the nuclear translocation of β-catenin, and hence that more target genes will be aberrantly activated in HCV patients. Taken together, our results indicate that NS5A protein increases the stability of β-catenin through protein interplay between NS5A and β-catenin, and hence that Wnt/β-catenin signaling is activated. We propose that NS5A protein involved in aberrant Wnt/β signaling cascade may promote HCV pathogenesis.

Acknowledgments

We thank Dr. Takaji Wakita (National Institute of Infectious Disease, Tokyo) for providing us with an HCV JFH-1 cDNA clone. This study was supported by a grant from the National R&D program for Cancer Control, Ministry of Health & Welfare (0620100), Korea, National Research Laboratory (ROA-2007-000-20051-0) and Biotechnology Development (2008-04100) from the Ministry of Education, Science and Technology, Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.06.026.

References

[1] Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–6549.
[2] Ide Y, Tanimoto A, Sasaguri Y, Padmanabhan R. Hepatitis C virus NS5A protein is phosphorylated in vitro by a stably bound protein kinase from HeLa cells and by cAMP-dependent protein kinase A-alpha catalytic subunit. Gene 1997;201:151–158.
[3] Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc Natl Acad Sci USA 2004;101:13038–13043.
[4] Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, et al. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J Virol 2003;77:5487–5492.
[5] Tan SL, Nakao H, He Y, Vijaysri S, Neddermann P, Jacobs BL, et al. NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling. Proc Natl Acad Sci USA 1999;96:5533–5538.
[6] He Y, Nakao H, Tan SL, Polyak SJ, Neddermann P, Vijaysri S, et al. Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and P85 phosphatidylinositol 3-kinase. J Virol 2002;76:9207–9217.
[7] Street A, Macdonald A, Crowder K, Harris M. The Hepatitis C virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. J Biol Chem 2004;279:12232–12241.
[8] Gimenez-Barcons M, Wang C, Chen M, Sanchez-Tapias JM, Saiz JC, Gale Jr M. The oncogenic potential of hepatitis C virus NS5A sequence variants is associated with PKR regulation. J Interferon Cytokine Res 2005;25:152–164.
[9] Baek KH, Park HY, Kang SM, Kim SJ, Jeong SJ, Hong EK, et al. Overexpression of hepatitis C virus NS5A protein induces chromosome instability via mitotic cell cycle dysregulation. J Mol Biol 2006;359:22–34.

[10] Miller JR, Moon RT. Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes Dev 1996;10:2527–2539.
[11] Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006;127:469–480.
[12] Branda M, Wands JR. Signal transduction cascades and hepatitis B and C related hepatocellular carcinoma. Hepatology 2006;43: 891–902.
[13] Nusse R. Wnt signaling in disease and in development. Cell Res 2005;15:28–32.
[14] Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. Science 2005;307:1904–1909.
[15] Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature 2005;434:843–850.
[16] Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 1998;58:1130–1134.
[17] de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, et al. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci USA 1998;95:8847–8851.
[18] Wei Y, Fabre M, Branchereau S, Gauthier F, Perilongo G, Buendia MA. Activation of beta-catenin in epithelial and mesenchymal hepatoblastomas. Oncogene 2000;19:498–504.
[19] Cha MY, Kim CM, Park YM, Ryu WS. Hepatitis B virus X protein is essential for the activation of Wnt/beta-catenin signaling in hepatoma cells. Hepatology 2004;39:1683–1693.
[20] Besnard-Guerin C, Belaidouni N, Lassot I, Segeral E, Jobart A, Marchal C, et al. HIV-1 Vpu sequesters beta-transducin repeat-containing protein (betaTrCP) in the cytoplasm provokes the accumulation of beta-catenin and other SCFbetaTrCP substrates. J Biol Chem 2004;279:788–795.
[21] Street A, Macdonald A, McCormick C, Harris M. Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular beta-catenin and stimulation of beta-catenin-responsive transcription. J Virol 2005;79:5006–5016.
[22] Park KJ, Choi SH, Lee SY, Hwang SB, Lai MM. Nonstructural 5A protein of hepatitis C virus modulates tumor necrosis factor alpha-stimulated nuclear factor kappa B activation. J Biol Chem 2002;277:13122–13128.
[23] Choi SH, Hwang SB. Modulation of the transforming growth factor-beta signal transduction pathway by hepatitis C virus nonstructural 5A protein. J Biol Chem 2006;281:7468–7478.
[24] Choi SH, Jeong SH, Hwang SB. Large hepatitis delta antigen modulates transforming growth factor-beta signaling cascades: implication of hepatitis delta virus-induced liver fibrosis. Gastroenterology 2007;132:343–357.
[25] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.
[26] Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, et al. Constitutive transcriptional activation by a beta-catenin–Tcf complex in APC−/− colon carcinoma. Science 1997;275:1784–1787.
[27] Carruba G, Cervello M, Miceli MD, Farruggio R, Notrarbartolo M, Virruso L, et al. Truncated form of beta-catenin and reduced expression of wild-type catenins feature HepG2 human liver cancer cells. Ann NY Acad Sci 1999;886:212–216.
[28] Huang H, Fujii H, Sankila A, Mahler-Araujo BM, Matsuda M, Cathomas G, et al. Beta-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C virus infection. Am J Pathol 1999;155:1795–1801.
[29] Yost C, Torres M, Miller JR, Huang E, Kimelman D, Moon RT. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev 1996;10:1443–1454.
[30] Price MA. CKI, there's more than one: casein kinase I family members in Wnt and hedgehog signaling. Genes Dev 2006;20:399–410.
[31] Gale Jr M, Kwieciszewski B, Dossett M, Nakao H, Katze MG. Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. J Virol 1999;73:6506–6516.
[32] Majumder M, Steele R, Ghosh AK, Zhou XY, Thornburg L, Ray R, et al. Expression of hepatitis C virus non-structural 5A protein in the liver of transgenic mice. FEBS Lett 2003;555: 528–532.
[33] Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta 2003;1653:1–24.
[34] Lu Z, Hunter T. Wnt-independent beta-catenin transactivation in tumor development. Cell Cycle 2004;3:571–573.
[35] Muller T, Bain G, Wang X, Papkoff J. Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Exp Cell Res 2002;280:119–133.
[36] Lu Z, Ghosh S, Wang Z, Hunter T. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell 2003;4:499–515.
[37] Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoel MJ, Bertrand F, Cherqui G, Perret C, et al. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation. Oncogene 2001; 20:252–259.
[38] Morali OG, Delmas V, Moore R, Jeanney C, Thiery JP, Larue L. IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition. Oncogene 2001;20:4942–4950.
[39] Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–789.
[40] Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, et al. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. Mol Cell 2005;19: 159–170.
[41] Morrison JA, Klingelhutz AJ, Raab-Traub N. Epstein–Barr virus latent membrane protein 2A activates beta-catenin signaling in epithelial cells. J Virol 2003;77:12276–12284.
[42] Tomita M, Kikuchi A, Akiyama T, Tanaka Y, Mori N. Human T-cell leukemia virus type 1 tax dysregulates beta-catenin signaling. J Virol 2006;80:10497–10505.
[43] Gan DD, Khalili K. Interaction between JCV large T-antigen and beta-catenin. Oncogene 2004;23:483–490.