

MODULATION OF TGF- β SIGNAL TRANSDUCTION PATHWAY BY HEPATITIS C VIRUS NONSTRUCTURAL 5A PROTEIN*

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Running Title: HCV NS5A regulates TGF- β signaling cascades

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Transforming growth factor- β (TGF- β) is implicated in the pathogenesis of liver disease. TGF- β is involved both in liver regeneration and in the fibrotic and cirrhotic transformation with hepatitis viral infection. Hepatitis C virus (HCV) infection often leads to cirrhosis and hepatocellular carcinoma. HCV nonstructural 5A (NS5A) protein is a multifunctional protein that modulates cytokine-mediated signal transduction pathways. To elucidate the molecular mechanism of HCV pathogenesis, we examined the effect of NS5A protein on TGF- β stimulated signaling cascades. We showed that the NS5A protein inhibited TGF- β -mediated signaling pathway in hepatoma cell lines as determined by reporter gene assay. To further investigate the role of NS5A, we examined the protein-protein interaction between NS5A and TGF- β signaling transducers. Both *in vitro* and *in vivo* binding data showed that NS5A protein directly interacted with type I TGF- β receptor I (T β R-I) in hepatoma cell lines. This interaction was mapped to the amino acids 148-238 of NS5A. We also found that NS5A protein was colocalized with T β R-I in

the cytoplasm of Huh7 cells and inhibited TGF- β -mediated nuclear translocation of Smad2. Furthermore, we demonstrated that the NS5A protein abrogated the phosphorylation of Smad2 and the heterodimerization of Smad3 and Smad4. To further explore the relevance to the viral infection, we examined the effect of HCV subgenomic replicon on TGF- β signaling pathway. Indeed, we showed that HCV subgenomic replicon also inhibited TGF- β -induced signaling cascades. These results indicate that HCV NS5A modulates TGF- β signaling through the interaction with T β R-I and NS5A may be an important risk factor in HCV-associated liver pathogenesis.

INTRODUCTION

Hepatitis C virus (HCV)¹ is a causative agent of non-A, non-B hepatitis (1, 2), which often leads to liver cirrhosis and hepatocellular carcinoma (3, 4). HCV is a single-stranded, positive-sense RNA virus belonging to the *Flaviviridae* family (5). The viral genome is approximately 9.6 kb in length and encodes a single polyprotein precursor of a little more than 3,000 amino acid residues.

This polyprotein is processed by a combination of host and viral proteases into 10 individual mature proteins (6). The three N-terminal structural proteins (core, E1, and E2) are required for nucleocapsid formation and assembly of virus particles. The remainders are the nonstructural proteins with various enzymatic activities including serine protease, RNA helicase, and RNA-dependent RNA polymerase. The nonstructural 5A (NS5A) is a multifunctional phosphoprotein consisting of 447 amino acid residues. NS5A protein exists in two different sizes of polypeptide, p56 and p58, which is phosphorylated mainly at serine residues by cellular kinase (7). NS5A protein is implicated in antiviral resistance to interferon (IFN). NS5A from HCV genotype 1b interacts with the IFN-inducible double-stranded RNA-activated protein kinase (PKR) through IFN-sensitivity-determining region (ISDR) in the middle domain of the protein and functions as a repressor of PKR (8). NS5A protein has a nuclear localization signal (NLS) at C-terminus that can direct a heterologous protein to the nucleus, although native NS5A is localized in the cytoplasm of HCV-infected patients and mammalian cells (9). NS5A protein can perturb the cellular signaling pathway by selectively targeting the growth factor receptor-bound protein 2 (Grb2) adapter protein (10). Moreover, NS5A protects cells from TNF- α and p53-mediated apoptosis, and promotes tumor growth and a number of cell cycle regulatory genes (11-15).

Transforming growth factor- β (TGF-

β) is a member of multifunctional cytokines that are involved in regulation of cell proliferation, differentiation, apoptosis, and matrix production (16, 17). TGF- β -dependent signaling pathway involves two transmembrane serine/threonine kinases, namely type I TGF- β receptor and type II TGF- β receptor (T β R-I and T β R-II) (18, 19). Upon ligand binding, T β R-II phosphorylates T β R-I at glycine-serine rich domain (GS domain). The phosphorylated T β R-I initiates intracellular signal transduction pathway through the activation of receptor-specific Smads (R-Smads) and common-partner Smad (Co-Smad). An R- and Co-Smad complex translocates to the nucleus and regulates the transcription of target genes either positively or negatively (20, 21). TGF- β has a major regulatory role in hepatic fibrosis and cirrhosis (22). TGF- β is also a potent growth inhibitor in many cell types including carcinoma cells, endothelial cells, hepatocytes, and lymphocytes (23-27).

In the present study, we examined the effects of HCV NS5A protein on TGF- β dependent signaling pathway. We demonstrate that NS5A physically associates with T β R-I and thereby inhibits TGF- β -mediated signaling pathway in hepatoma cells and in HCV subgenomic replicon cells. These data suggest that the association of NS5A with T β R-I can negatively regulate TGF- β -stimulated transcriptional activities of target genes, which may be involved in HCV pathogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Constructions-cDNA encoding NS5A of HCV (genotype 1b) was amplified by polymerase chain reaction (PCR) using the Korean isolate of HCV (28) as a template and subcloned into the pcDNA3 (Invitrogen, Carlsbad, CA) and pGEX-4T-1 (Amersham Bioscience, Uppsala, Sweden) vectors. HA-T β R-I (T204D), Flag-Smad2, Flag-Smad3, and Flag-Smad4 expression vectors were provided from Dr. Masa Kawabata (The Cancer Institute, Tokyo, Japan). Smad4-Myc and NS5A-Myc expression plasmids were subcloned into the pEF6/His-Myc vector (Invitrogen). NS5A deletion mutants were generated by PCR and subcloned into the pcDNA3 expression vector.

Cell Culture and Transfection Experiment-All cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin-streptomycin/mL. For transfection, $\sim 5 \times 10^5$ cells plated on 60-mm dishes were transfected with plasmid DNA using either Lipofectamine (Invitrogen) or the calcium phosphate method as described previously (29). To make stable cell lines of NS5A, Huh7 cells were transfected with pcDNA3-NS5A expression plasmid. Single clones were selected in the presence of 500 μ g/mL of G418 in culture medium. Four to five weeks after transfection, positive clones were selected by Western blot analysis using anti-NS5A polyclonal antibody. Huh7 cells transfected with empty vector were selected as described above and used as a control. Huh7 cells containing HCV subgenomic replicons

were kindly provided by Dr. C. Seeger (Fox Chase Cancer Center, Philadelphia), and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin-streptomycin/mL, 0.1 mM nonessential amino acids (Gibco, Invitrogen Corp., Grand Island, NY), and 500 μ g/mL of G418 (Qbiogen, Carlsbad, CA). To establish the interferon (IFN)-cured cells, HCV replicon cells were treated with 100 U/ml of IFN- α (Sigma-Aldrich, St. Louis, MO) for two weeks. Elimination of HCV replicon RNA was confirmed by RT-PCR, Western blotting, and by the loss of resistance to G418.

Luciferase Reporter Gene Assay-Either HepG2 cells or Huh7 cells were transfected by the calcium phosphate method with 2 μ g of expression plasmid, 0.1 μ g of SBE4-Luc consisting of four Smad binding elements in tandem (30), 0.1 μ g of 3TP-Lux reporter plasmid consisting of TGF- β -inducible elements in the promoter of human plasminogen activator inhibitor-type I gene (31), p21-Luc reporter plasmid containing p21 promoter region (gift from Dr. Jae Yong Lee, Hallym University, Chuncheon, Korea), and 0.1 μ g of pCH110 (Amersham Biosciences) reference plasmid, respectively. Huh7 cells harboring HCV subgenomic replicons were transfected with 0.1 μ g of SBE4-Luc, 0.1 μ g of 3TP-Lux reporter plasmid, and 0.1 μ g of pCH110 reference plasmid, respectively. Total DNA amount in each transfection was kept constant by adjusting with an empty vector. At 24 h after transfection, cells were stimulated

with human TGF- β (5 ng/mL; R&D system, Minneapolis, MN) for 24 h. Luciferase and β -galactosidase assays were performed as described previously (29).

MTT assay-Huh7 cells stably expressing empty vector and NS5A were plated at a density of 2×10^5 cells in 6-well plates. At 24 h after plating, cells were treated with 5 ng/ml human TGF- β for 48 h and stained with 1 mg of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) for 3 h. The percentage of cell death was calculated based on the OD₅₇₀ according to the following formula: percentage of cell death = (OD₅₇₀ of control-OD₅₇₀ of sample)/OD₅₇₀ of control x 100. Each bar represents the means from triplicate experiments.

GST Pull-down Assay and Coimmunoprecipitation-GST-NS5A fusion protein was expressed in *Escherichia coli* BL21 (DE) (Novagen, Madison, WI) and purified with glutathione-Sepharose 4B beads (Amersham Bioscience) according to the manufacturer's instructions. Huh7 cells were transfected with HA-T β R-I (T204D) expression plasmid. At 36 h after transfection, cells were lysed in 400 μ L of cell lysis buffer [20 mmol/L Tris-HCl, (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerolphosphate, 1 mmol/L Na₃VO₄, 1 μ g/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride]. The cell lysate was centrifuged at 15,000 rpm for 10 min, and protein concentration was

determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

For *in vitro* binding assay, HA-T β R-I (T204D) was incubated with 2 μ g of either GST or GST-NS5A fusion protein for 2 h at 4°C in cell lysis buffer. Samples were washed four times in cell lysis buffer, and the bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by Western blot analysis using anti-HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

For coimmunoprecipitation assay, Cos7 cells were infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (32). Two h after infection, cells were transfected with 5 μ g of corresponding plasmids. Following incubation at 37°C for 12 h, cells were harvested and lysed in buffer B [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EGTA, 1% Triton X-100, 1.5 mmol/L MgCl₂, 10 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 1 mmol/L Na₃VO₄, and 1 mmol/L phenylmethylsulfonyl fluoride]. The cell lysates were triturated by 10 passes through a 26-gauge needle on ice and centrifuged at 15,000 rpm for 10 min. The supernatant was incubated at 4°C for 2 h with either anti-HA monoclonal antibody (Santa Cruz Biotechnology) or anti-NS5A rabbit polyclonal antibody. The samples were further incubated with 30 μ L of protein-A beads (Zymed Laboratories, San Francisco, CA) for 1 h. Beads were washed five times in cell lysis buffer, and the bound proteins were separated

by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by Western blot analysis using either anti-NS5A rabbit polyclonal antibody or anti-HA monoclonal antibody.

For *in vivo* binding assay between NS5A and endogenous T β R-I, Huh7 cells were transfected with NS5A-Myc plasmid. At 36 h after transfection, Huh7 cells were either untreated or treated with human TGF- β (5 ng/mL) for 1 h. Cellular supernatant was prepared as described above and incubated with anti-T β R-I polyclonal antibody (Santa Cruz Biotechnology) for 2 h at 4°C. The samples were further incubated with 30 μ L of protein-A beads for 1 h. These bound proteins were then separated by SDS-PAGE, and detected by Western blot analysis using anti-Myc monoclonal antibody (Santa Cruz Biotechnology).

Confocal Microscopy-Either Huh7 cells or Cos7 cells grown on chamber slides (Nunc Inc., Naperville, IL) were cotransfected with either NS5A-Myc and HA-T β R-I (T204D), or Flag-Smad2 and GFP-NS5A expression plasmids. At 36 h after transfection, slides were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde and 0.1% Triton X-100 for 20 min at 37°C. Cells were incubated in 5% bovine serum albumin (BSA) for 20 min at 37°C and then incubated with either anti-HA monoclonal antibody, anti-Myc monoclonal antibody, or anti-Flag monoclonal antibody for 2 h at 37°C. After being washed in PBS three times, cells were further incubated with

tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (American Qualex, Santa Clemente, CA) for 1 h at 37°C. After two washes in PBT (0.1% Triton X-100 in PBS) and three washes in PBS, cells were analyzed using LSM 510 laser confocal microscopy system (Carl Zeiss, Inc., Thornwood, NY).

Coimmunoprecipitation of Smad3/Smad4 Complex-Approximately, 5 \times 10⁵ cells plated on 60-mm dishes were transfected with Flag-Smad3, Smad4-Myc, and HA-T β R-I (T204D) expression plasmids using Lipofectamine (Invitrogen). At 36 h after transfection, cells were lysed in buffer B and subjected to immunoprecipitation with anti-Flag monoclonal antibody. The bound Smad4 protein was detected by Western blot analysis using anti-Myc monoclonal antibody.

Cytoplasmic and Nuclear Fractionation-Huh7 cells plated on 60-mm dishes were transfected with the corresponding plasmids. At 36 h after transfection, cells were washed twice in PBS, resuspended in buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride] for 2 min on ice. Cells were harvested and vortexed for 5 seconds and centrifuged at 10,000 rpm for 2 min. The supernatant was saved as a cytoplasmic fraction. The pellet was washed twice in buffer C [20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 420 mmol/L KCl, 25% glycerol, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.5 mmol/L

phenylmethylsulfonyl fluoride] and resuspended in buffer C. The samples were vortexed for 10 seconds, incubated with agitation for 30 min, and then centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was collected as a nuclear extract. Protein concentration was determined by the Bradford method using a Bio-Rad protein assay kit. Equal amounts of protein were subjected to Western blot analysis using either anti-Flag monoclonal antibody (Sigma-Aldrich), anti- β -Actin monoclonal antibody (Sigma-Aldrich), anti- α -B23 polyclonal antibody (Santa Cruz Biotechnology), and anti-NS5A rabbit polyclonal antibody.

Western Blot Analysis-HepG2 cells and Huh7 cells were transfected with the corresponding expression plasmids. At 36 h after transfection, cells were treated with human TGF- β (5 ng/mL) for the indicated times and incubated in lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EGTA, 1% Triton X-100, 1.5 mmol/L MgCl₂, 10 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 1 mmol/L Na₃VO₄, and 1 mmol/L phenylmethylsulfonyl fluoride]. Equal amounts of proteins were subjected to electrophoresis in 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 5% nonfat dry milk for 1 h and then incubated overnight at 4°C with anti-phospho Smad2 polyclonal antibody (Cell Signaling Technology, Beverly, MA), anti-Flag monoclonal antibody (Sigma-Aldrich), anti-NS5A polyclonal antibody,

respectively, in TTBS [20 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, 0.05% Tween-20] overnight at 4°C. Following two washes in TTBS, the membrane was incubated with either HRP-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) in TTBS for 90 min at room temperature. Proteins were detected by an ECL kit (Amersham Bioscience).

Electrophoretic Mobility Shift Assay (EMSA)-Huh7 cells stably expressing NS5A, HCV subgenomic replicon, and empty vector were plated at a density of $\sim 3 \times 10^5$ in 6-well dishes. Following incubation at 37°C overnight, cells were treated with human TGF- β (5 ng/mL) for 24 h and then nuclear fractions were prepared as described previously (33). Briefly, cells were harvested by scraping in PBS and incubated in 200 μ L of buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.1 % Nonidet P-40, 0.5 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride] for 3 min on ice. Crude nuclei were collected by centrifugation at 10,000 rpm for 2 min at 4°C. The pellets were rinsed once in buffer A and resuspended in 100 μ L of buffer C [20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 420 mmol/L KCl, 25% glycerol, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride]. The sample was further incubated with agitation in the cold room for 30 min and centrifuged at 15,000 rpm for 10 min at 4°C. Protein concentration in nuclear extract was

determined by the Bradford method (Bio-Rad). Nuclear extract (20 μ g) was incubated with the oligonucleotide probe (34) labeled with 32 P (1×10^5 cpm) in 15 μ L of binding buffer [20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L dithiothreitol, 250 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), and 0.25 mg/ml poly (dI-dC)] at room temperature for 20 min. The Smad-DNA complexes were separated by electrophoresis on a 5% native polyacrylamide gel using 0.25 \times Tris borate EDTA buffer and detected by autoradiography. For competition assay, unlabeled oligonucleotide was incubated with nuclear extract in binding buffer for 20 min before the addition of radiolabeled oligonucleotide.

RESULTS

HCV NS5A protein inhibits TGF- β -induced transcriptional activation and cell death-TGF- β has a pivotal role in pathogenic hepatic fibrosis and cirrhosis in chronic liver disease (22). Since we found that TGF- β was differentially expressed in hepatocellular carcinoma of NS5A transgenic mice (data not shown) and NS5A regulates several cellular signaling events, we investigated the role of NS5A in the TGF- β -dependent transcriptional activation by using a luciferase reporter gene assay. Human hepatoma cell lines were transiently cotransfected with NS5A expression plasmid and either TGF- β responsive 3TP-luc reporter plasmid (31) or a SBE4-luc reporter plasmid (30) containing four Smad binding elements. At 24 h after transfection, cells were either left untreated or

treated with human TGF- β (5 ng/mL) for 24 h and luciferase assay was performed. As shown in Fig.1A, TGF- β induced 40- to 60-fold transactivation of the reporters in Huh7 cells. Endogenous luciferase activity was maintained at a basal level, and this activity was not affected by NS5A. Overexpression of NS5A protein inhibited TGF- β -induced transcriptional activation by \sim 40 to 60% as compared to the vector control in both reporter plasmids. It is noteworthy that HepG2 cells are less responsive to reporter plasmids than Huh7 cells. We found that overexpression of NS5A protein inhibited TGF- β -induced transcriptional activation in a dose dependent manner (Fig. 1B). However, overexpression of GFP protein showed no effect on TGF- β -induced transcriptional activation (data not shown). These results suggest that NS5A protein inhibits TGF- β -induced signal transduction pathway on transcriptional level in hepatoma cell lines. In most cell types, TGF- β is a potent activator of p21 and induces cell cycle arrest at G1 phase (17, 35). p21 is a cyclin-dependent kinase inhibitor and correlates with cell proliferation, differentiation, apoptosis, and transformation (36, 37). Recently, Majumder *et al.* have shown that HCV NS5A protein represses p21 expression in p53 dependent manner (11). To investigate the direct effect of NS5A protein on p21 transcriptional activity, we performed luciferase reporter gene assay using p21-Luc plasmid. Huh7 cells were cotransfected with NS5A expression plasmid, T β R-I (T204D) expression plasmid, and p21-Luc reporter

plasmid. At 36 h after transfection, TGF- β -induced p21 promoter activity was analyzed. As shown in Fig. 1C, overexpression of NS5A protein inhibited TGF- β -induced p21 promoter activity in a dose-dependent manner. It is noteworthy that the expression level of exogenous T β R-I (T204D) was not affected by the NS5A protein in Fig. 1B and 1C. Next, we investigated the effect of NS5A protein on TGF- β -induced cell death. Huh7 cells stably expressing either empty vector or NS5A were treated with human TGF- β for 48 h and then MTT assay was performed. Upon TGF- β treatment, only 40% cells remained viable in cells stably transfected with empty vector (Fig. 1D). However, the majority of Huh7 cells stably expressing NS5A were viable, indicating that these cells were significantly less sensitive to TGF- β -induced cell death than vector control cells (Fig. 1D). These results suggest that HCV NS5A protein inhibits TGF- β -induced cell death through downregulation of p21 activity.

NS5A protein specifically interacts with type I TGF- β receptor (T β R-I) in vitro and in vivo-To further investigate how NS5A protein inhibits TGF- β -mediated signaling pathway, we examined whether NS5A protein interacted with TGF- β signaling transducers. Since many viral proteins regulate TGF- β signaling through Smad proteins, we first examined the interaction between NS5A and Smad proteins by coimmunoprecipitation assay. NS5A expression plasmid was cotransfected into Huh7 cells with Flag-tagged Smad2, Smad3, and Smad4, individually. Cell

lysates were immunoprecipitated with Flag antibody and the bound proteins were detected by NS5A antibody. Reciprocally, cell lysates were immunoprecipitated with NS5A antibody and the bound proteins were detected by Flag antibody. In either case, NS5A protein failed to interact with Smad proteins (data not shown). We then examined the interaction between NS5A and T β R-I *in vitro*. We used a GST pull-down assay using *E. coli*-expressed GST and GST-NS5A fusion protein. Cell extracts containing HA-T β R-I (T204D) were incubated with either GST or GST-NS5A beads for 2 h at 4°C. As shown in Fig. 2A, GST-NS5A selectively bound to T β R-I, whereas GST protein failed to interact with T β R-I. To further confirm *in vitro* interaction between NS5A and T β R-I, we performed a coimmunoprecipitation assay. HA-T β R-I (T204D) was coexpressed with NS5A in Cos7 cells paired with a recombinant vaccinia virus (vTF7-3) system. At 12 h after transfection, cell lysates were immunoprecipitation with either anti-HA mouse monoclonal antibody or control mouse IgG and coprecipitated proteins were detected by Western blot analysis using anti-NS5A rabbit polyclonal antibody. Indeed, NS5A interacts with T β R-I *in vivo* (Fig. 2B). We confirmed this result by reciprocal experiment using NS5A antibody for immunoprecipitation in Fig. 2C. Next, we investigated whether NS5A could interact with endogenous T β R-I. Huh7 cells were transfected with increasing amount of NS5A-Myc expression plasmid. At 36 h after transfection, cells were either left untreated or

treated with human TGF- β (5 ng/mL) for 1 h. Cell lysates were subjected to immunoprecipitation with rabbit anti-T β R-I polyclonal antibody and the immunoprecipitated proteins were analyzed by Western blot analysis using anti-Myc monoclonal antibody. In Fig. 2D, NS5A protein interacts with endogenous T β R-I regardless of TGF- β treatment. We also confirmed that NS5A interacted with T β R-I in Huh7 cells stably expressing NS5A protein (data not shown). Both *in vitro* and *in vivo* protein-protein interaction data suggest that NS5A protein may colocalize with the exogenously expressed T β R-I (T204D). To determine this possibility, Huh7 cells were cotransfected with NS5A-Myc and HA-T β R-I (T204D) expression plasmids and were examined the subcellular localization by confocal microscopy. It has previously been reported that NS5A protein is localized to the ER and Golgi apparatus in Huh7 cells (38). Fig. 2E shows that NS5A is localized in the cytoplasm and dual staining shows colocalization of NS5A and T β R-I (T204D) in the cytoplasm as yellow fluorescence. We have further confirmed that NS5A and T β R-I (T204D) were colocalized in non-hepatic Cos7 cells (data not shown). Taken together, these results indicate that NS5A protein specifically interacts with T β R-I both *in vitro* and *in vivo*.

The middle region of NS5A mediates the NS5A-T β R-I (T204D) interaction-To determine the region in NS5A that is responsible for T β R-I binding, the interaction of T β R-I with various deletion mutants of

NS5A (Fig. 3A) was determined by a transfection-based coprecipitation assay in Cos7 cells infected with the recombinant vaccinia virus (vTF7-3). As shown in Fig. 3B, HA-T β R-I (T204D) interacts with an N-terminal deletion mutant of NS5A (148-447 aa) and a C-terminal deletion mutant of NS5A (1-301 aa), respectively. However, NS5A (238-447 aa) mutant no longer interacts with HA-T β R-I (T204D), suggesting that the middle region (148-237 aa) of NS5A is required for NS5A-T β R-I (T204D) interaction.

NS5A blocks TGF- β -mediated nuclear translocation and phosphorylation of Smad2-TGF- β activation stimulates phosphorylation and nuclear translocation of Smad proteins. In the control Huh7 cells, Smad2 protein is mostly localized in the cytoplasm as shown in Fig. 4A. To understand the molecular mechanism of inhibitory effect of NS5A on TGF- β -mediated transactivation, we examined whether NS5A could repress T β R-I (T204D)-mediated nuclear translocation of Smad2 protein. Huh7 cells were cotransfected with HA-T β R-I (T204D) and Flag-Smad2 in the absence or presence of NS5A. Cells were fractionated into distinct cytoplasmic and nuclear fraction, and nuclear translocation of the Smad2 protein was determined. Indeed, overexpression of HA-T β R-I (T204D) induced nuclear translocation of Smad2 protein (Fig. 4B, lane 2), whereas NS5A protein stayed in the cytoplasm. Importantly, HA-T β R-I (T204D)-induced nuclear translocation of Smad2 protein was significantly inhibited by NS5A protein (Fig.

4B, lane 4). To further confirm this *in vivo*, we examined the effect of NS5A protein on TGF- β -induced nuclear translocation of Smad2 by confocal microscopy. As previously reported (39), Flag-Smad2 was dispersedly localized in the cytoplasm and in the nucleus of Huh7 cells. Upon TGF- β treatment, Flag-Smad2 was accumulated in the nucleus (Fig. 4C, top panel). However, TGF- β -induced nuclear translocation of Smad2 was inhibited by NS5A protein (Fig. 4C, middle and bottom panels). These results indicate that TGF- β -dependent nuclear translocations of Smad proteins are inhibited by NS5A protein in hepatoma cell line.

Since NS5A interacts with T β R-I and T β R-I plays an important role in TGF- β -dependent signaling pathway through phosphorylation of R-Smad proteins, we examined the role of NS5A protein in Smad2 phosphorylation. To investigate whether NS5A could inhibit TGF- β -mediated Smad2 phosphorylation, Huh7 cells stably transfected with either vector control or NS5A were treated with TGF- β for the indicated times. Fig. 4D shows that NS5A dramatically inhibits TGF- β -mediated phosphorylation of endogenous Smad2 in a time-dependent manner. These data indicate that NS5A inhibits TGF- β -mediated phosphorylation of Smad2 protein through interaction with T β R-I.

NS5A inhibits T β R-I-induced heterodimerization of Smad3 and Smad4-The ligand-activated T β R-I phosphorylates R-Smads at C-terminal SXS motif (20, 40), which induces a conformational change in R-

Smads, thereby facilitating heterodimerization with Co-Smad (21). To determine the effect of NS5A on T β R-I-induced heterodimerization of Smad3 and Smad4, Huh7 cells were cotransfected with constitutively activated HA-T β R-I (T204D), Flag-Smad3, and Smad4-Myc in the absence or presence of NS5A. At 36 h after transfection, cell lysates were immunoprecipitated with anti-Flag monoclonal antibody, and then bound complex was analyzed by Western blot analysis using anti-Myc monoclonal antibody. As expected, constitutively activated T β R-I induced the heterodimer formation between Smad3 and Smad4, whereas coexpression of NS5A dramatically inhibited the complex formation of Smad3 and Smad4 (Fig. 5, lane 3). Since the protein expression level of Smad3 and Smad4 were not affected by NS5A, this result may explain how NS5A modulates TGF- β -dependent signaling pathway.

NS5A protein inhibits TGF- β -induced Smad-DNA binding activity-To examine how the inhibitory effect of NS5A protein on Smads activities resulted in TGF- β -induced Smad-DNA complex, EMSA was performed. At 24 h after plating, Huh7 cells stably expressing NS5A or empty vector were treated with TGF- β for 24 h and then nuclear extracts were prepared. EMSA was performed using TGF- β -responsive element in the plasminogen activator inhibitor-1 promoter (-586 ~ -551). Upon TGF- β treatment, retarded bands of Smad-DNA complexes were increased in stable vector control cells (Fig. 6, lane 2), whereas these complexes were

markedly decreased in stable cells expressing NS5A protein (Fig. 6, lane 5). It is noteworthy that TGF- β -induced Smad-DNA complexes form distinctive triple bands, as previously reported (34). Also, the disappearance of TGF- β -response complexes using an unlabeled probe further suggest the specificity of Smad-DNA binding (Fig. 6, lanes 3 and 6).

HCV subgenomic replicon suppresses TGF- β -induced signal transduction pathway-We further asked whether the inhibitory effect of NS5A on TGF- β -induced Smad-DNA complexes might occur in the context of viral infection or even viral RNA replication. Currently, we do not have an *in vitro* system that supports efficient production of infectious HCV viral particles. For this purpose, we investigated TGF- β -induced signaling cascades in a HCV subgenomic replicon system in which the HCV subgenomic RNA autonomously replicates in Huh7 cells (41). Both IFN-cured and HCV subgenomic replicon cells were transfected with SBE4-Luc reporter plasmids. At 24 h after transfection, cells were left either untreated or treated with human TGF- β for 24 h. Luciferase reporter gene assay was then performed. As shown in Fig. 7A, TGF- β -induced promoter activity in HCV subgenomic replicon cells is suppressed by ~ 60% as compared to the IFN-cured Huh7 cells. We then examined whether NS5A protein could interact with T β R-I in the context of viral RNA replication. For this purpose, both IFN-cured and HCV subgenomic replicon cell lysates were immunoprecipitated with anti-

T β R-I antibody and then bound proteins were immunoblotted with anti-NS5A antibody. As shown in Fig. 7B, NS5A protein in HCV subgenomic replicon cells specifically interacted with T β R-I. We further asked whether TGF- β -mediated endogenous Smad2 phosphorylation was affected by HCV subgenomic replicon. Both IFN-cured and HCV subgenomic replicon cells were treated with human TGF- β for the indicated times and Smad2 phosphorylation was determined by anti-phospho Smad2 polyclonal antibody. TGF- β treatment immediately stimulated endogenous Smad2 phosphorylation in IFN-cured cells (Fig. 7C, lane 2). However, the phosphorylation level of endogenous Smad2 was inhibited approximately 40% in HCV subgenomic replicon cells (Fig. 7C, lanes 5 and 6). We also examined Smad-DNA binding activity in IFN-cured and HCV subgenomic replicon cells by EMSA. Indeed, TGF- β -stimulated Smad-DNA complexes were also decreased in HCV subgenomic replicon cells (data not shown). These data strongly support that NS5A inhibits TGF- β -mediated signaling cascades through interaction with T β R-I.

DISCUSSION

TGF- β is one of the multifunctional cytokines that is implicated in diverse cellular phenomena including cell growth control, cell adhesion and motility, alteration of cellular phenotype, production and degradation of extracellular matrix protein, and apoptosis of hepatic cell lines. TGF- β is known to activate a tumor suppressor pathway by a reversible

arrest of cell proliferation (24, 42-44). However, tumor cells commonly acquire the ability to resist the growth inhibitory effect of TGF- β .

This study demonstrates the novel regulatory role of the HCV NS5A protein in TGF- β -dependent signaling pathway of transiently transfected human hepatoma cells and cells harboring HCV subgenomic replicons. Several viruses encode gene products that modulate TGF- β signaling pathway through interaction with cellular proteins. For example, X protein of hepatitis B virus activates TGF- β signaling through direct interaction with Smad4 (45). Adenovirus E1A protein inhibits TGF- β signaling through binding to Smad proteins (46), and human papilloma virus E7 protein inhibits TGF- β signaling pathway by blocking DNA binding activity of the Smad complexes (34). Likewise, the hepatitis C virus core protein inhibits p21 promoter activity by inhibiting a TGF- β signaling pathway (47).

NS5A is a multifunctional protein and regulates various cellular signaling events. In the present study, we investigated the role of NS5A in the TGF- β -dependent signaling cascades. NS5A inhibited TGF- β -stimulated transcriptional activities and this inhibition was mediated through the interaction with T β R-I. We have shown that NS5A directly interacted with T β R-I through the middle region (amino acids 148-237) of NS5A. NS5A-T β R-I interaction was confirmed by coimmunoprecipitation assay and confocal microscopy. Since NS5A inhibits TNF- α -

induced NF- κ B activation by interaction with TRAF2 through the middle domain (amino acids 148-301) of NS5A (48), this region plays a key role in HCV to modulate many cytokine-dependent cellular signaling events. Although NS5A protein did not affect the autophosphorylation of T β R-I (data not shown), phosphorylation level of Smad2 was decreased by NS5A and hence complex formation between R- and Co-Smads was subsequently disrupted by NS5A protein.

We previously demonstrated that NS5A and NS5B (RNA-dependent RNA polymerase) interacted with 33 kDa human vesicle-associated membrane protein-associated protein (hVAP-33) (49). Since NS5A may be a part of the RNA replication complex, we asked whether NS5B could also modulate TGF- β -dependent signaling pathway. Using luciferase reporter gene assay, we found that NS5B protein had no effect on TGF- β signaling pathway (data not shown).

TGF- β induces hepatic fibrogenesis and modulates proliferation and differentiation of epithelial cells (50-52). TGF- β inhibits cell cycle progression during G1 phase through enhanced expression of cyclin-dependent kinase (CDK) inhibitors such as p21^{WAF1/CIP1} (53). Inhibition of cell proliferation by TGF- β is an important step in tumor development. Therefore, escape from TGF- β -induced anti-proliferation response plays a central role in many cancer cells. HCV NS5A protein not only regulates cell cycle regulatory genes at transcriptional level (10, 11, 54) but also inhibits apoptosis (13, 14, 55, 56), and leads to the production of profibrogenic mediator (57).

TGF- β -dependent signaling pathway involves liver fibrogenesis, chronic hepatitis (58, 59), and hepatocellular carcinoma (60). In this study, we have shown that NS5A is a negative regulator of TGF- β -dependent signaling intermediates through interaction with T β R-I. We demonstrate that NS5A protein inhibits phosphorylation of Smad2, abrogates interaction between Smad3 and Smad4, prevents nuclear translocation of Smad proteins, inhibits the formation of Smad-DNA complexes, and downregulates p21 expression in hepatoma cell lines. These results and our previous findings (33, 48) suggest that NS5A may play a key role in regulating cytokine signaling pathways. Indeed, we demonstrated that transgenic mice harboring HCV NS5A gene, but not HCV core gene, developed hepatocellular carcinoma (61). In conclusion, HCV regulates TGF- β -induced signal transduction pathway and NS5A protein may play an important role in the pathogenesis of HCV.

REFERENCES

1. Houghton, M., Weiner, A., Han, J., Kuo, G., and Choo, Q. L. (1991) *Hepatology* **14**, 381-388
2. Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.-L., Houghton, M., and Kuo, G. (1990) *Proc Natl Acad Sci U S A* **87**, 6547-6549
3. Di-Bisceglie, A. M., Simpson, L. H., Lotze, M. T., and Hoofnagle, J. H. (1994) *J. Clin. Gastroenterol.* **19**, 222-226
4. Aach, R. D., Stevens, C. E., Hollinger, F. B., Mosley, J. W., Peterson, D. A., Taylor, P. E., Johnson, R. G., Barbosa, L. H., and Nemo, G. J. (1991) *N. Engl. J. Med.* **325**, 1325-1329
5. Houghton, M. (1996) in *Fields Virology* (Fields, B. N., Knipe, D. M., Howley, P. M., Chanock, R. M., Melnick, J. L., Monath, T. P., Roizman, B., and Straus S. E., eds) 3rd ed., pp. 1035-1058, Lippincott-Raven, Philadelphia
6. Reed, K. E., and Rice, C. M. (2000) *Curr. Top. Microbiol. Immunol.* **242**, 55-84
7. Ide, Y., Tanimoto, A., Sasaguri, Y., and Padmanabhan, R. (1997) *Gene* **201**, 151-8
8. Gale, M. Jr, Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R., and Katz, M. G. (1997) *Virology* **230**, 217-227
9. Ide, Y., Zhang, L., Chen, M., Inchauspe, G., Bahl, C., Sasaguri, Y., and Padmanabhan, R. (1996) *Gene* **182**, 203-211
10. Tan, S. L., Nakao, H., He, Y., Vijaysri, S., Neddermann, P., Jacobs, B. L., Mayer, B. J., and Katze, M. G., (1999) *Proc. Natl. Acad. Sci. USA* **96**, 5533-5538
11. Ghosh, A. K., Steele, R., Meyer, K., Ray, R., and Ray, R. B. (1999) *J. Gen. Virol.* **80**, 1179-1183
12. Majumder, M., Ghosh, A. K., Steele, R., Ray, R., and Ray, R. B. (2001) *J. Virol.* **75**, 1401-1407
13. Ghosh, A. K., Majumder, M., Steele, R., Meyer, K., Ray, R., and Ray, R. B. (2000) *Virus. Res.* **67**, 173-178
14. Majumder, M., Ghosh, A. K., Steele, R., Zhou, X. Y., Phillips, N. J., Ray, R., and Ray, R. B. (2002) *Virology* **294**, 94-105
15. Lan, K. H., Sheu, M. L., Hwang, S. J., Yen, S. H., Chen, S. Y., Wu, J. C., Wang, Y. J., Kato, N., Omata, M., Chang, F. Y., and Lee, S. D. (2002) *Oncogene* **21**, 4801-4811
16. Massagué, J. (1998) *Annu. Rev. Biochem.* **67**, 753-791
17. Derynck, R., Akhurst, R. J., and Balmain, A. (2001) *Nat. Genet.* **29**, 117-129
18. Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massagué J. (1994) *Biochim. Biophys. Acta.* **1222**, 71-80
19. Derynck, R. (1994) *Trends. Biochem. Sci.* **19**, 548-553
20. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996) *Cell* **87**, 1215-1224

21. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) *EMBO J.* **16**, 5353-5362
22. Castilla, A., Prieto, J., and Fausto, N. (1991) *N. Engl. J. Med.* **324**, 933-940
23. Coffey, R. J. Jr, Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B.E, and Moses, H. L. (1988) *Mol. Cell. Biol.* **8**, 3088-3093
24. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M, and Massague, J. (1990) *Cell* **62**, 175-185
25. Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990) *Cell* **63**, 245-247
26. Newman, M. J. (1993) *Cancer Metastasis Rev.* **12**, 239-254
27. Ewen, M. E. (1994) *Cancer Metastasis Rev.* **13**, 45-66
28. Cho, Y. G., Yoon, J. W., Jang, K.L., Kim, C. M., Sung, Y.C. (1993) *Mol. Cells* **3**, 195-202
29. Kim, B. C., Lee, M. N., Kim, J. Y., Lee, S. S., Chang, J. D., Kim, S. S., Lee, S. Y., and Kim, J. H. (1999) *J. Biol. Chem.* **274**, 24372-24377
30. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) *Mol Cell* **1**, 611-617
31. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) *EMBO J.* **17**, 3091-3100
32. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) *Proc. Natl. Acad. Sci. U S A* **83**, 8122-8126
33. Park, K. J., Choi, S. H., Choi, D. H., Park, J. M., Yie, S. W., Lee, S. Y., and Hwang, S. B. (2003) *J. Biol. Chem.* **278**, 30711-30718
34. Lee, D. K., Kim, B. C., Kim, I. Y., Cho, E. A., Satterwhite, D. J., and Kim, S. J. (2002) *J. Biol. Chem.* **277**, 38557-3864
35. Massagué, J., Blain, S. W., and Lo, R. S. (2000) *Cell* **103**, 295-309
36. Harper, J. W., and Elledge, S. J. (1996) *Curr. Opin. Genet. Dev.* **6**, 56-64
37. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., et al. (1993) *Cell* **75**, 817-825
38. Shi, S. T., Polyak, S. J., Tu, H., Taylor, D. R., Gretch, D. R., and Lai, M. M. C. (2002) *Virology* **292**, 198-210
39. Matsuzaki, K., Date, M., Furukawa, F., Tahashi, Y., Matsushita, M., Sugano, Y., Yamashiki, N., Nakagawa, T., Seki, T., Nishizawa, M., Fujisawa, J., and Inoue, K. (2000) *Hepatology* **32**, 218-227
40. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) *Proc. Natl. Acad. Sci. U S A* **94**, 10669-10674
41. Guo, J. T., Bichko, V. V., and Seeger, C. (2001) *J. Virol.* **75**, 8516-8523
42. Kim, S. J., Im, Y. H., Markowitz, S. D., and Bang, Y. J. (2000) *Cytokine Growth Factor Rev.* **11**,

43. Howe, P. H., Draetta, G., and Leof, E. B. (1991) *Mol. Cell. Biol.* **11**, 1185-1194
44. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M., and Moses, H. L. (1990) *Cell* **61**, 777-785
45. Lee, D. K., Park, S. H., Yi, Y., Choi, S. G., Lee, C., Park, W. T., Cho, H., et al. (2001) *Genes Dev.* **15**, 455-466
46. Nishihara, A., Hanai, J., Imamura, T., Miyazono, K., and Kawabata, M. (1999) *J. Biol. Chem.* **274**, 28716-28723
47. Lee, M. N., Jung, E. Y., Kwun, H. J., Jun, H. K., Yu, D. Y., Choi, Y. H., and Jang, K. L. (2002) *J. Gen. Virol.* **83**, 2145-2151
48. Park, K. J., Choi, S. H., Lee, S. Y., Hwang, S. B., and Lai, M. M. C. (2002) *J. Biol. Chem.* **277**, 13122-13128
49. Tu, H., Gao, L., Shi, S. T., Taylor, D. R., Yang, T., Mircheff, A. K., Wen, Y., Gorbalenya, A. E., Hwang, S. B., and Lai, M. M. *Virology* **263**, 30-41
50. Saltis, J. (1996) *Mol. Cell. Endocrinol.* **116**, 227-232
51. Lyons, R. M., and Moses, H. L. (1990) *Eur. J. Biochem.* **187**, 467-473
52. Jetten, A. M., Shirley, J. E., and Stoner, G. (1986) *Exp. Cell. Res.* **167**, 539-549
53. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., Wang, X. F. (1995) *Proc. Natl. Acad. Sci. U S A* **92**, 5545-5549
54. Qadri, I., Iwahashi, M., and Simon, F. (2002) *Biochim. Biophys. Acta.* **1592**, 193-204
55. Gale, M. Jr, Kwieciszewski, B., Dossett, M., Nakao, H., and Katze, M. G. (1999) *J. Virol.* **73**, 6506-6516
56. He, Y., Nakao, H., Tan, S. L., Polyak, S. J., Neddermann, P., Vijaysri, S., Jacobs, B. L., and Katz, M. G. (2002) *J. Virol.* **76**, 9207-9217
57. Schuppan, D., Krebs, A., Bauer, M., Hahn, E. G. (2003) *Cell Death Differ.* 10 Suppl. **1**, S59-67
58. Ray, S., Broor, S. L., Vaishnav, Y., Sarkar, C., Girish, R., Dar, L., Seth, P., and Broor, S. (2003) *J. Gastroenterol. Hepatol.* **18**, 393-403
59. Shimizu, I. (2001) *Curr. Drug Targets Infect. Disord.* **1**, 227-240
60. Ueno, T., Hashimoto, O., Kimura, R., Torimura, T., Kawaguchi, T., Nakamura, T., Sakata, R., Koga, H., and Sata, M. (2001) *Int. J. Oncol.* **18**, 49-55
61. Wang, A. G., Choi, S. H., Moon, H. B., Yu, D. Y., and Hwang, S. B. (2004) 11th International Symposium on HCV and related viruses. 242

FOOTNOTES

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¹The abbreviations used are: HCV, hepatitis C virus; TGF- β , transforming growth factor- β ; NS5A, nonstructural 5A; T β R-I, TGF- β receptor I; R-Smads, receptor-specific Smads; Co-Smad, common-partner Smad; EMSA, electrophoretic mobility shift assay.

FIGURE LEGENDS

Fig. 1. HCV NS5A protein suppresses TGF- β -mediated transcriptional activation. *A*, HCV NS5A protein suppresses TGF- β -stimulated transcriptional activation. Both Huh7 (upper panels) and HepG2 (bottom panels) cells were transfected with either SBE4-luc (left panels) or 3TP-luc (right panels) reporter plasmids together with empty vector or NS5A expression plasmid. One day after transfection, cells were left either untreated or treated with human TGF- β (5 ng/mL) for 24 h. Luciferase activities were then measured and values were normalized based on β -gal activities. The data shown represent triplicate experiments. *B*, NS5A suppresses TGF- β -mediated transcriptional activation in a dose-dependent manner. Huh7 cells were cotransfected with the indicated plasmids together with selected amounts (0.5, 1, and 2 μ g) of HCV NS5A. At 36 h after transfection, cells were harvested and 3TP-reporter activity was determined. Equal amounts of cell lysates were subjected to immunoblotting with anti-HA monoclonal antibody and anti-NS5A polyclonal antibody (bottom two panels). *C*, NS5A inhibits TGF- β -mediated p21 promoter activation. Huh7 cells were cotransfected with the indicated plasmids together with selected amounts (1, 2, and 5 μ g) of HCV NS5A plasmid. Total DNA amount was kept constant at 7.2 μ g by adjusting with empty vector. At 36 h after transfection, cells were harvested and luciferase activity was measured. Equal amounts of cell lysates were subjected to immunoblotting with anti-HA monoclonal antibody and anti-NS5A polyclonal antibody (bottom two panels). *D*, NS5A protein inhibits TGF- β -induced cell death. Huh7 cells stably expressing empty vector and NS5A were treated with human TGF- β for 48 h and MTT assay was performed as described in EXPERIMENTAL PROCEDURES.

Fig. 2. NS5A protein interacts with Type I TGF- β receptor both *in vitro* and *in vivo*. *A*, Huh7 cells were transfected with HA-T β R-I (T204D) expression plasmid. At 36 h after transfection, cell lysates were incubated with either GST or GST-NS5A fusion protein purified from *E. coli*. Bound proteins were precipitated by glutathione beads and detected by Western blot analysis using anti-HA monoclonal antibody (top panel). T β R-I expression in Huh7 cells was verified by HA antibody (middle panel). Both GST and GST-GST-NS5A fusion protein used for the binding analysis are shown by staining with Coomassie brilliant blue (bottom panel). *B*, Using recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3), both NS5A and HA-T β R-I (T204D) proteins were coexpressed in Cos7 cells. At 12 h after transfection, cell lysates were immunoprecipitated with either anti-HA monoclonal antibody or normal mouse IgG. Bound proteins were detected by Western blot analysis using rabbit anti-NS5A polyclonal antibody (top panel). Both HA-T β R-I

(T204D) and NS5A proteins were verified using the same cell lysates by Western blotting with either anti-HA monoclonal antibody (middle panel) or anti-NS5A polyclonal antibody (bottom panel). *C*, Cell lysates used in *B* were immunoprecipitated with either anti-NS5A polyclonal antibody or normal rabbit IgG. Bound proteins were analyzed by Western blot analysis using anti-HA monoclonal antibody (top panel). Both NS5A and HA-T β R-I (T204D) proteins were verified using the same cell lysates by Western blotting with either anti-NS5A polyclonal antibody (middle panel) or anti-HA monoclonal antibody (bottom panel). *D*, Huh7 cells were transfected with NS5A-Myc expression plasmid in a dose dependent manner (0, 2, and 5 μ g). At 36 h after transfection, cells were left either untreated or treated with human TGF- β (5 ng/mL) for 1 h. Cell lysates were immunoprecipitated with anti-T β R-I polyclonal antibody. The immunoprecipitates were analyzed by Western blot analysis using anti-Myc monoclonal antibody. The membrane was reprobed with either anti-T β R-I polyclonal antibody (middle panel) or anti-Myc monoclonal antibody (bottom panel). An asterisk indicates the heavy chain of IgG. *E*, Colocalization of NS5A and Type I TGF- β receptor. Both NS5A-Myc and HA-T β R-I (T204D) plasmids were cotransfected into Huh7 cells. At 36 h after transfection, cells were fixed in 4% paraformaldehyde, and immunofluorescence staining was performed using an anti-HA polyclonal antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG to detect T β R-I (green) and using anti-Myc monoclonal antibody and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG to detect NS5A (red). Dual staining showed colocalization of NS5A and HA-T β R-I (T204D) as yellow fluorescence in the merge. Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) to label nuclei.

Fig. 3. NS5A interacts with HA-T β R-I (T204D) through the amino acids 148-237 of NS5A. *A*, Schematic diagram shows both wild type and mutant forms of NS5A. *B*, Cos7 cells were cotransfected with HA-T β R-I (T204D) and NS5A mutant plasmids paired with recombinant vaccinia virus (vTF7-3). At 12 h after transfection, cell lysates were immunoprecipitated with anti-HA monoclonal antibody, and bound proteins were detected by Western blotting with anti-NS5A polyclonal antibody (top panel). Both NS5A and T β R-I (T204D) proteins were verified using the same cell lysates by Western blotting with either anti-NS5A polyclonal antibody (middle panel) or anti-HA monoclonal antibody (bottom panel).

Fig. 4. NS5A protein inhibits TGF- β -induced nuclear translocation of exogenous Smad2 protein and phosphorylation of endogenous Smad2. *A*, Huh7 cells were transfected with Flag-Smad2 in the absence of ligand. At 36 h after transfection, cells were fractionated into cytoplasmic and nuclear fractions and Smad2 protein was detected by Western blot analysis using anti-Flag monoclonal antibody. β -Actin and B23 were detected for the indication of cytoplasmic (C) and nuclear fraction (N), respectively. *B*, Huh7 cells were cotransfected with constitutively active form

of HA-T β R-I (T204D) and Flag-Smad2 expression plasmid in the absence or presence of NS5A plasmid. At 36 h after transfection, cells were fractionated into cytoplasmic (C) and nuclear (N) fractions. Smad2 protein was detected by Western blot analysis using anti-Flag monoclonal antibody (top panel). NS5A protein was verified by Western blotting with anti-NS5A polyclonal antibody in the same cell lysate (bottom panel). *C*, NS5A protein inhibits TGF- β -induced Smad2 nuclear translocation. Both Flag-Smad2 and GFP-NS5A plasmids were cotransfected into Huh7 cells. At 36 h after transfection, cells were treated with human TGF- β for 1 h and immunofluorescence staining was performed using an anti-Flag monoclonal antibody and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG to detect Smad2 (red). Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) to label nuclei. *D*, Huh7 cells stably expressing NS5A and empty vector were stimulated with human TGF- β (5 ng/mL) for the indicated times. The phosphorylation level of endogenous Smad2 was detected by phospho-Smad2 polyclonal antibody (top panels). Protein expressions of both total endogenous Smad2 and NS5A were verified using the same cell lysates by Western blotting with either anti-Smad2 polyclonal antibody (middle panels) or anti-NS5A polyclonal antibody (bottom panels).

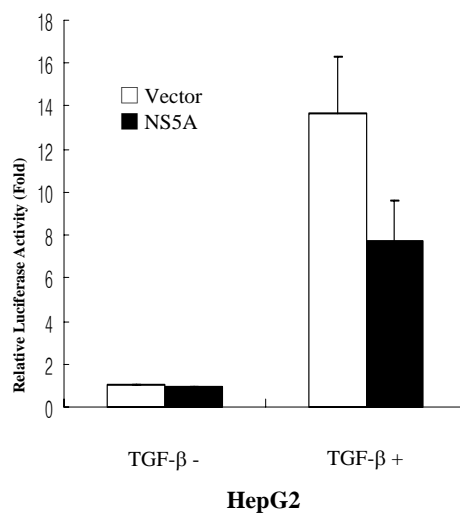
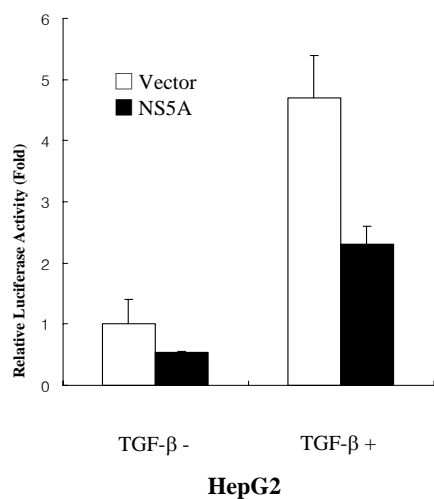
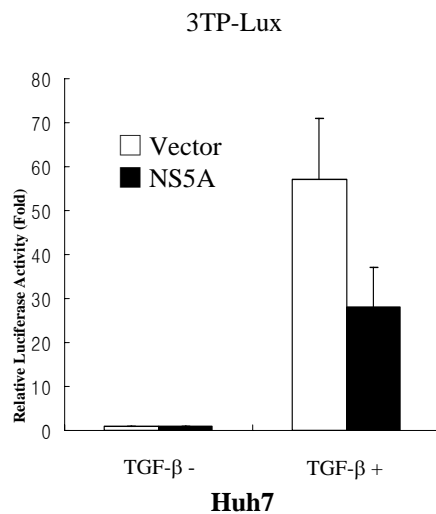
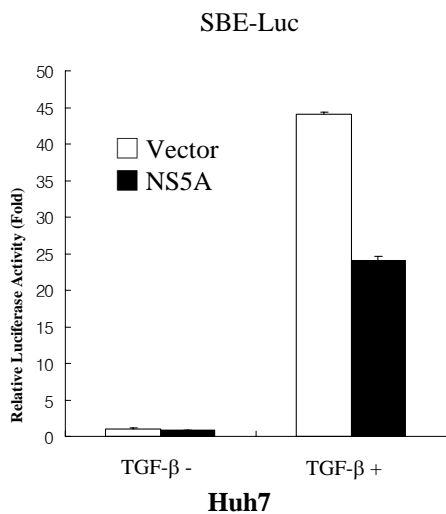
Fig. 5. NS5A protein abrogates T β R-I-induced heterodimerization of Smad3 and Smad4. Huh7 cells were cotransfected with constitutively active HA-T β R-I (T204D), Flag-Smad3, and Smad4-Myc in the absence or presence of NS5A. Cell lysates were immunoprecipitated with anti-Flag monoclonal antibody and then complex formation between Smad3 and Smad4 was detected by Western blot analysis using anti-Myc monoclonal antibody (top panel). Protein expressions of Smad3, Smad4, and NS5A were verified by Western blotting with anti-Flag, anti-Myc, and anti-NS5A antibodies (bottom three panels).

Fig. 6. NS5A inhibits TGF- β -induced Smad-DNA complex formation. *A*, Huh7 cells stably expressing either NS5A or empty vector were stimulated with human TGF- β (5 ng/mL) for 24 h, and nuclear extracts were prepared and assessed for the formation of activated Smad and DNA complex using 32 P-labeled TGF- β response sequence as a probe. *B*, Nuclear extracts used in EMSA (*A*) were verified by staining with Coomassie brilliant blue (CBB).

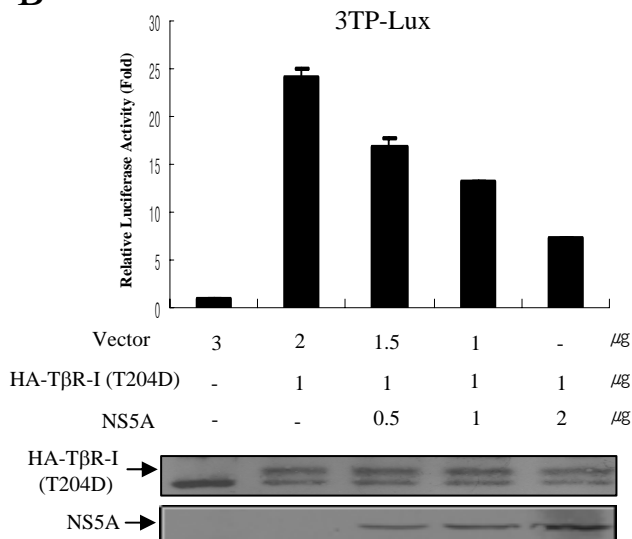
Fig. 7. HCV subgenomic replicon inhibits the TGF- β -induced signal transduction pathway. *A*, Both IFN-cured and HCV subgenomic replicon cells were transfected with SBE4-luc reporter plasmid. At 24 h after transfection, cells were left either untreated or treated with human TGF- β (5 ng/mL) for 24 h. The luciferase activities were then measured and values were normalized based on β -gal activities. Total cellular extracts were subjected by SDS-PAGE and immunoblotted with anti-NS5A polyclonal antibody and anti- β -Actin monoclonal antibody (bottom two panels). The data

shown represent three independent experiments. *B*, Both IFN-cured and HCV subgenomic replicon cell lysates were immunoprecipitated with anti-T β R-I polyclonal antibody. Bound proteins were detected by immunoblotting with rabbit anti-NS5A polyclonal antibody (top panel). Protein expressions of both endogenous T β R-I and NS5A were verified using the same cell lysates by immunoblotting with either anti-T β R-I polyclonal antibody (middle panel) or anti-NS5A polyclonal antibody (bottom panel). IB, immunoblot. *C*, Both IFN-cured and HCV subgenomic replicon cells were stimulated with human TGF- β (5 ng/mL) for the indicated times and phosphorylation level of endogenous Smad2 was detected by anti-phospho-Smad2 polyclonal antibody (top panel). Protein expressions of both endogenous Smad2 and NS5A were verified by immunoblotting with either anti-Smad2 polyclonal antibody (middle panel) or anti-NS5A polyclonal antibody (bottom panel) using the same cell lysates.

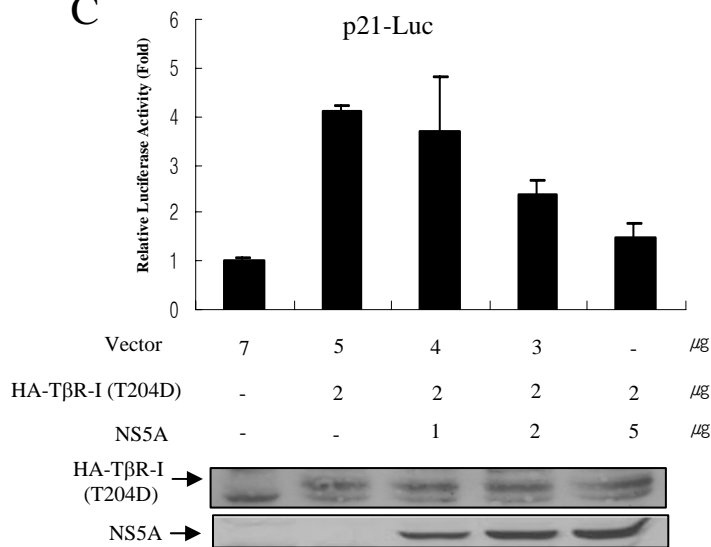
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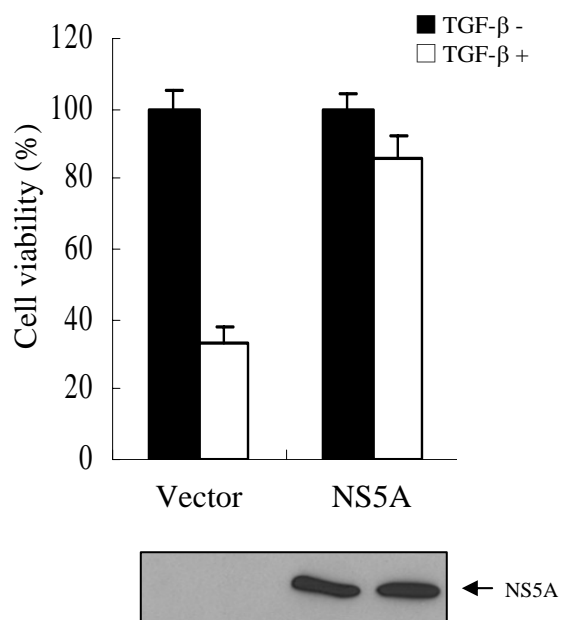
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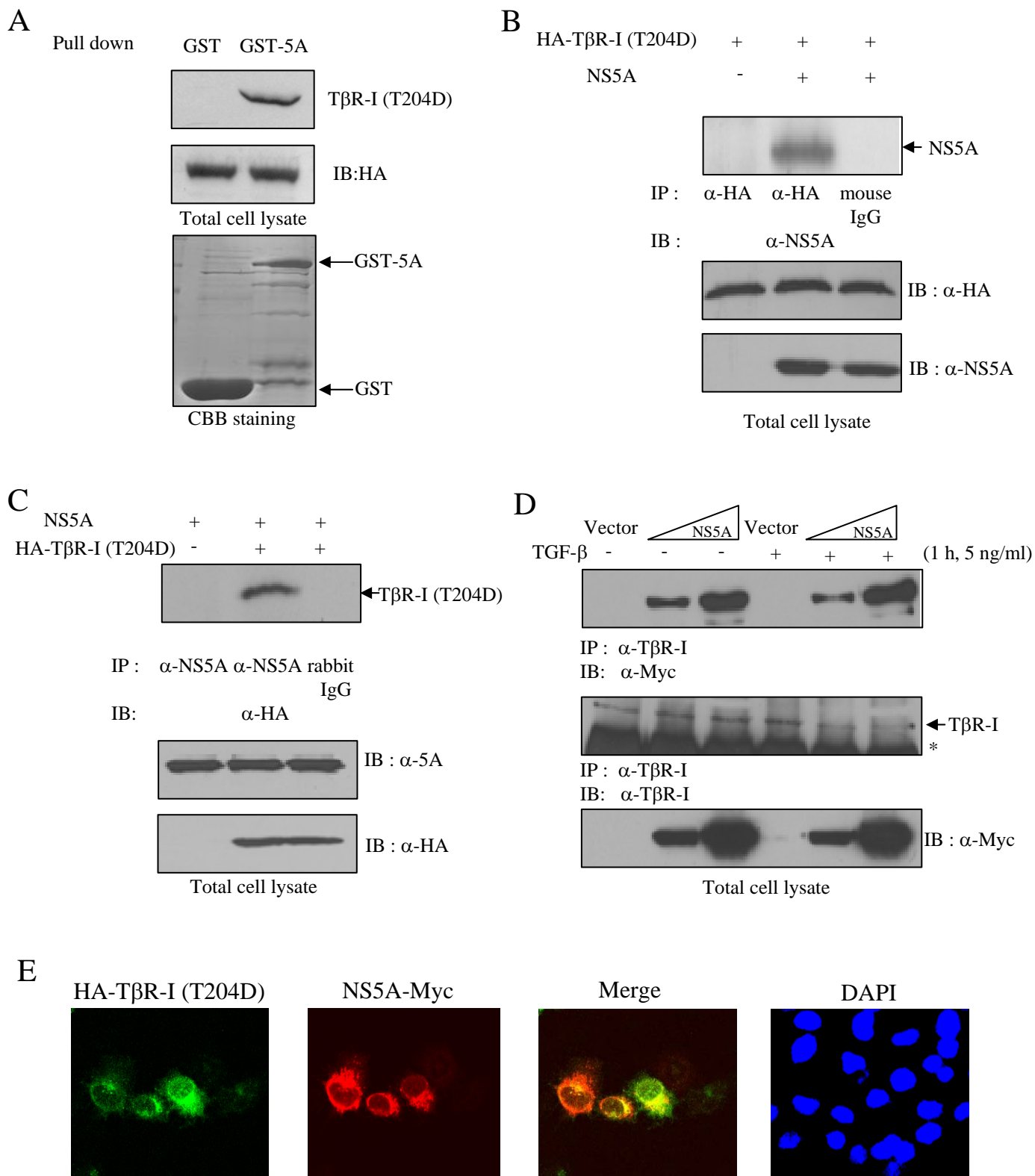


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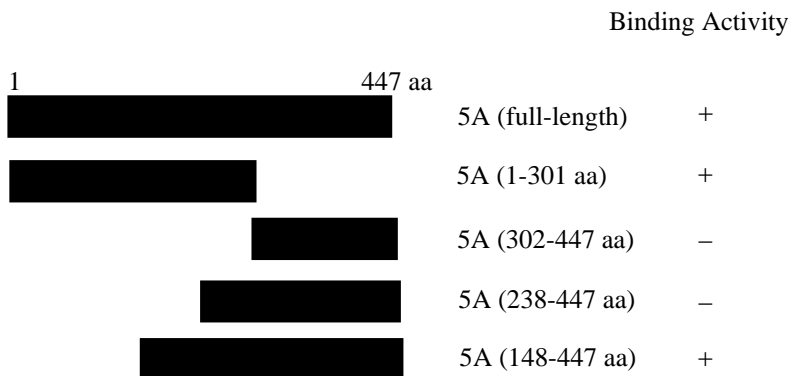


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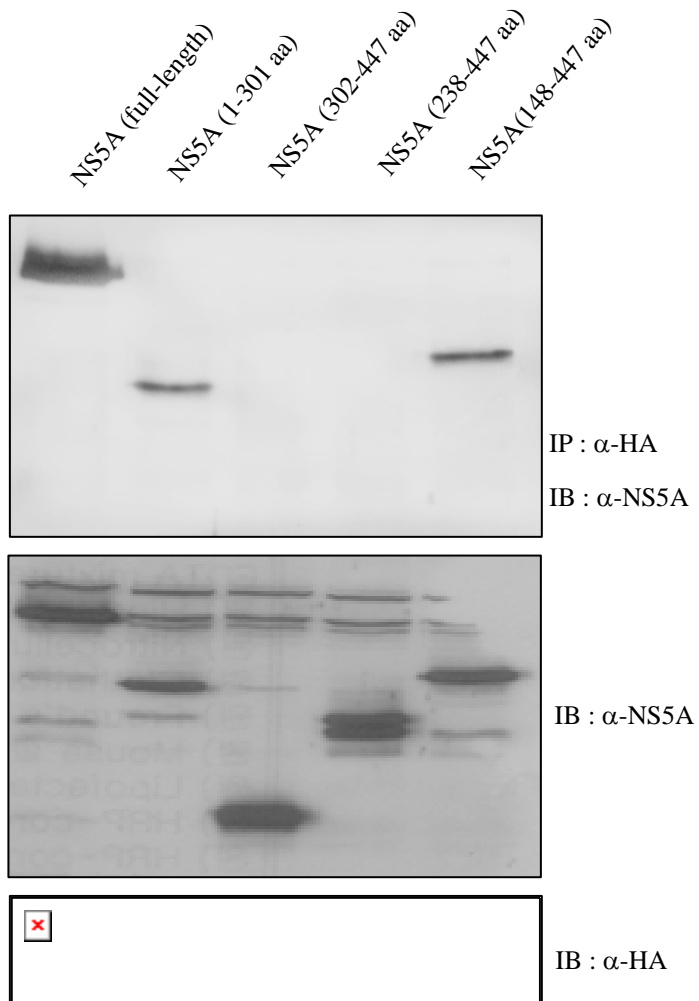




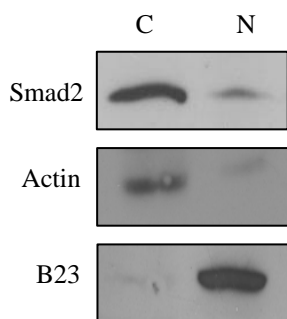
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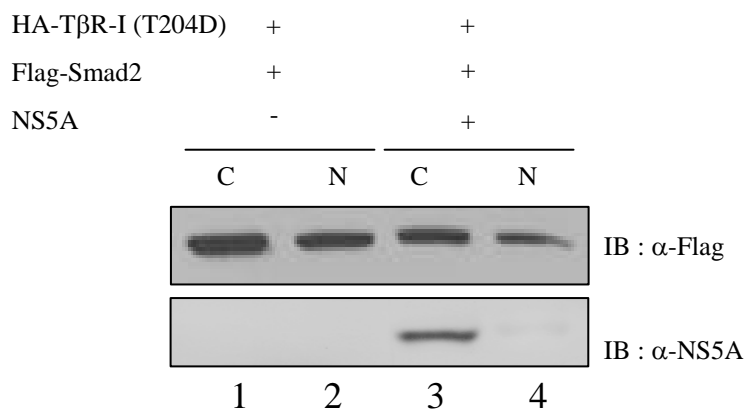
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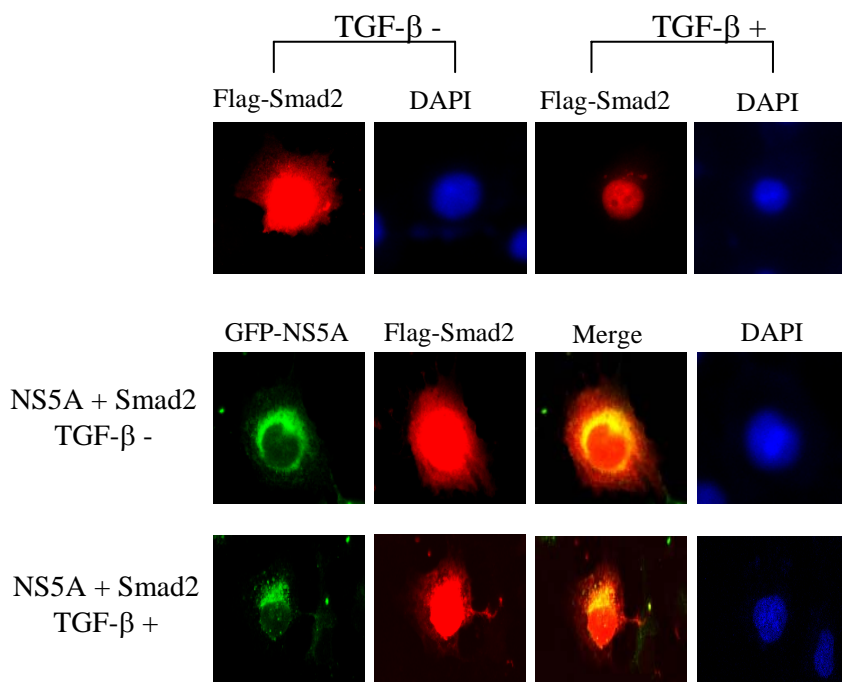
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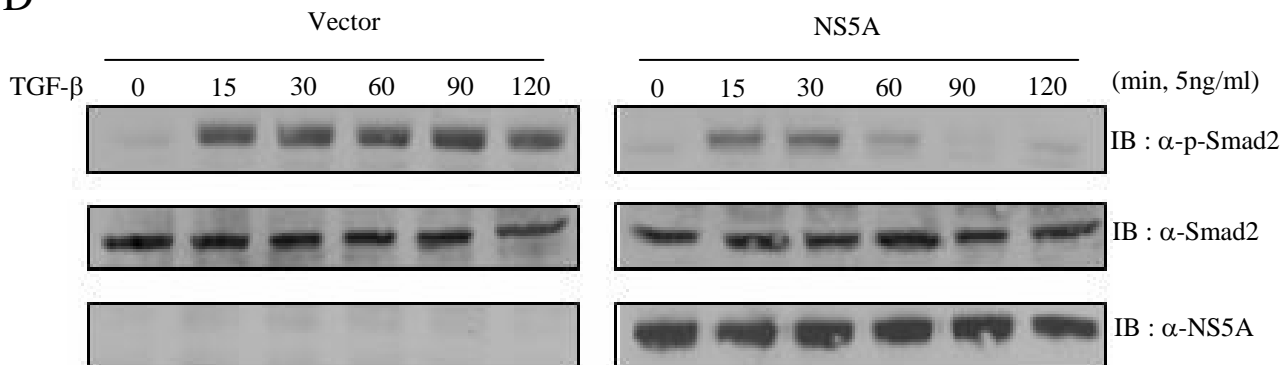
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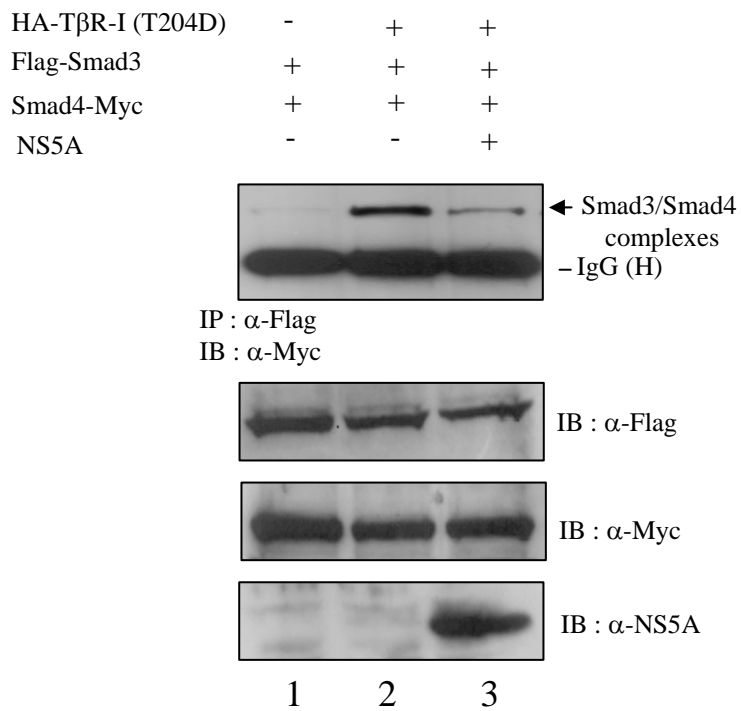


C

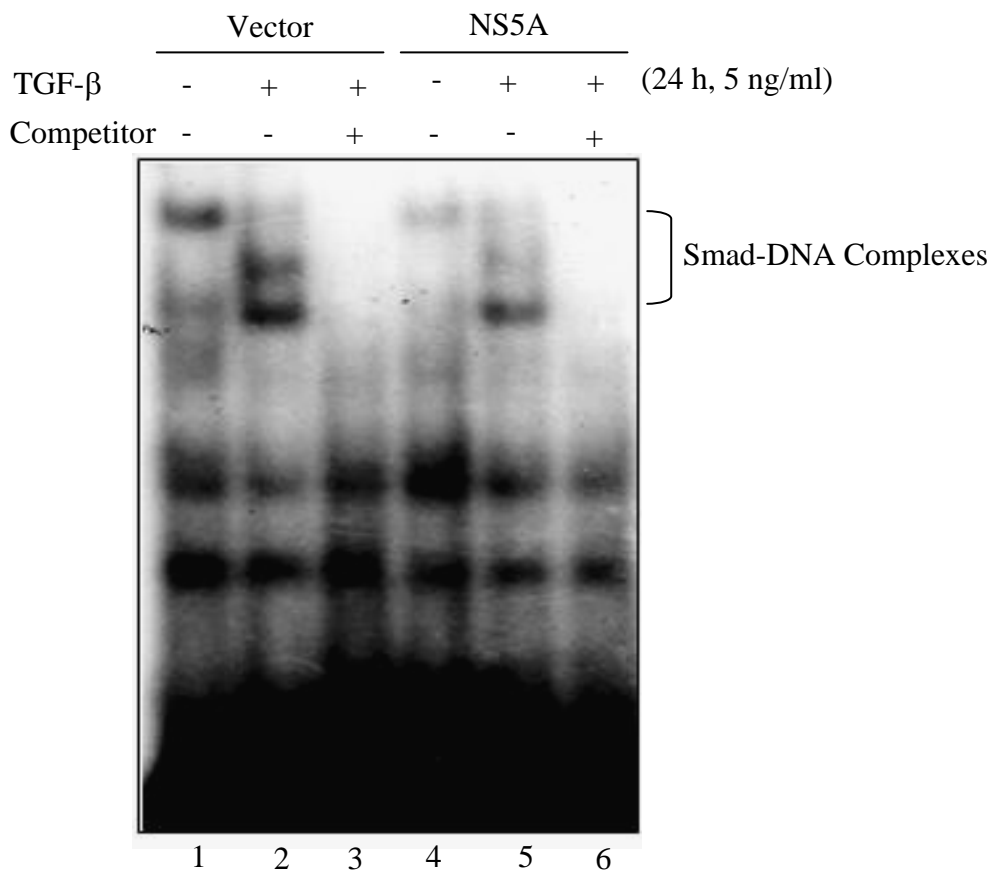


D

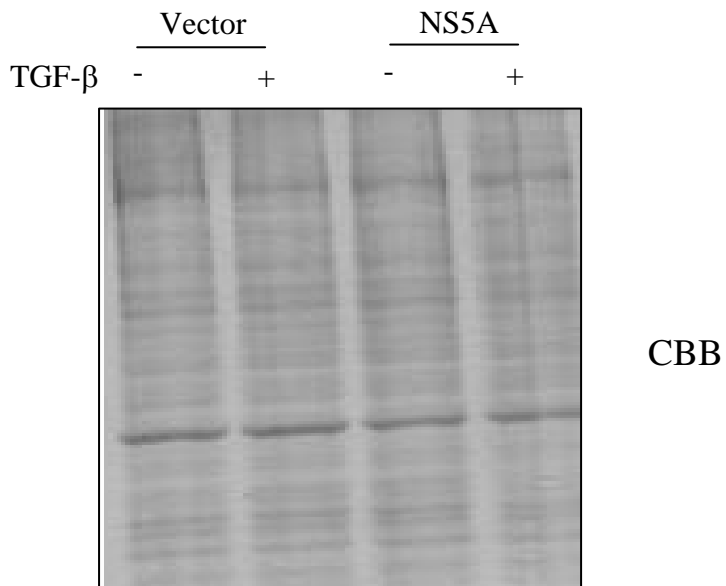




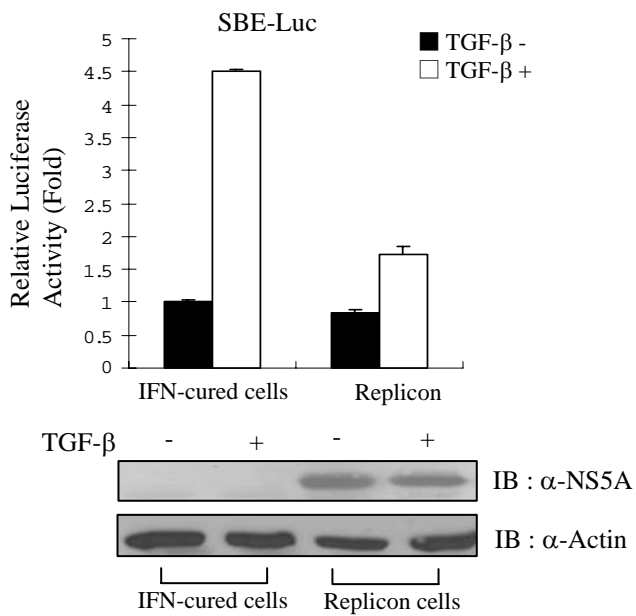
A



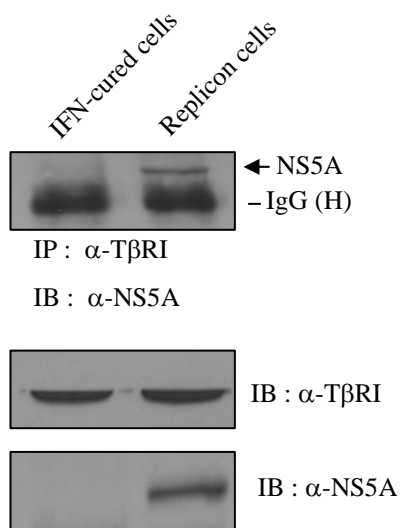
B



A



B



C

