Modulation of the Transforming Growth Factor-β Signal Transduction Pathway by Hepatitis C Virus Nonstructural 5A Protein

Received for publication, November 21, 2005, and in revised form, December 27, 2005. Published, JBC Papers in Press, January 6, 2006, DOI 10.1074/jbc.M512438200

Soo-Ho Choi and Soon B. Hwang

From the Ilsong Institute of Life Science, Hallym University, Chuncheon 200-702, Korea

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 show that NS5A protein inhibited the 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 signal transducers. Both in vitro and in vivo binding data showed that NS5A protein directly interacted with TGF-β type 1 receptor (TβR-I) in hepatoma cell lines. This interaction was mapped to amino acids 148–238 of NS5A. We also found that NS5A protein co-localized with TβR-I in the cytoplasm of HuH7 cells and inhibited TGF-β-mediated nuclear translocation of Smad2. Furthermore, we demonstrate that NS5A protein abrogated the phosphorylation of Smad2 and the heterodimerization of Smad3 and Smad4. To further explore the relevance to viral infection, we examined the effect of the HCV subgenomic replicon on the TGF-β signaling pathway. We show that the HCV subgenomic replicon also inhibited TGF-β-induced signaling cascades. These results indicate that HCV NS5A modulates TGF-β signaling through interaction with TβR-I and that NS5A may be an important risk factor in HCV-associated liver pathogenesis.

Hepatitis C virus (HCV) 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 ~9.6 kb in length and encodes a single polyprotein precursor of a little more than 3000 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 viral particles. The remainders are the nonstructural proteins with various enzymatic activities, including serine protease, RNA helicase, and RNA-dependent RNA polymerase. Nonstructural 5A (NS5A) protein is a multifunctional phosphoprotein consisting of 447 amino acid residues. NS5A protein exists in 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 the IFN sensitivity-determining region in the middle domain of the protein and functions as a repressor of PKR (8). NS5A protein has a nuclear localization signal at its 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 cell signaling pathway by selectively targeting the Grb2 (growth factor receptor-bound protein 2) adapter protein (10). Moreover, NS5A protects cells from tumor necrosis factor-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 one of the multifunctional cytokines that are involved in the regulation of cell proliferation and differentiation, apoptosis, and matrix production (16, 17). The TGF-β-dependent signaling pathway involves two transmembrane serine/threonine kinases, viz. TGF-β receptor (TβR) types I and II (18, 19). Upon ligand binding, TβR-II phosphorylates TβR-I at the Gly/Ser-rich domain. Phosphorylated TβR-I initiates the intracellular signal transduction pathway through the activation of receptor-specific Smad (R-Smad) proteins 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 this study, we examined the effects of HCV NS5A protein on the TGF-β-dependent signaling pathway. We demonstrate that NS5A physically associated with TβR-I and thereby inhibited the TGF-β-mediated signaling pathway in hepatoma cells and in HCV subgenomic replicon cells. These data suggest that the association of NS5A with TβR-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 PCR using the Korean isolate of HCV (28) as a template and subcloned into the pcDNA3 (Invitrogen) and pGEX-4T-1 (Amersham Biosciences AB, Uppsala) vectors. Hemagglutinin (HA)-TβR-I(T204D), FLAG-Smad2, FLAG-Smad3, and FLAG-Smad4 mobility shift assay.

This work was supported by Korea Research Foundation Grant KRF-2002-015-CP0308 and by Hallym University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed: Ilsong Inst. of Life Science, Hallym University, 1 Ockcheon-dong, Chuncheon 200-702, Korea. Tel.: 82-31-380-1732; Fax: 82-31-384-5395; E-mail: sbhwang@hallym.ac.kr.

1 The abbreviations used are: HCV, hepatitis C virus; NS5A, nonstructural 5A; IFN, interferon; TβR-I, transforming growth factor-β type 1 receptor; TβR-II, transforming growth factor-β receptor; R-Smad, receptor-specific Smad; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; EMSA, electrophoretic mobility shift assay.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
expression vectors were provided by Dr. Masa Kawabata (The Cancer Institute, Tokyo). Smad4-Myc and NSSA-Myc expression plasmids were subcloned into the pEF6/His-Myc vector (Invitrogen). NSSA 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’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. For transfection, ~5 × 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 cell lines stably expressing NSSA, Huh7 cells were transfected with the pcDNA3-NSSA expression plasmid. Single clones were selected in the presence of 500 μg/ml G418 in culture medium. 4–5 weeks after transfection, positive clones were selected by Western blot analysis using anti-NSSA 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’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 0.1 mM nonessential amino acids (Invitrogen), and 500 μg/ml G418 (Qbiogene, Inc., Irvine, CA). To establish IFN-cured cells, HCV replicon cells were treated with 100 units/ml IFN-α (Sigma) for 2 weeks. Elimination of HCV replicon RNA was confirmed by reverse transcription-PCR, Western blotting, and loss of resistance to G418.

**Luciferase Reporter Gene Assay**—Either HepG2 or Huh7 cells were transfected by the calcium phosphate method with 2 μg of expression plasmid, 0.1 μg of SBE4-luciferase (Luc; consisting of four Smad-binding elements in tandem) (30), 0.1 μg of 3TP-Lux reporter plasmid (consisting of TGF-β-inducible elements in the promoter of the human plasminogen activator inhibitor-1 gene) (31), the p21-Luc reporter plasmid (containing the p21 promoter region; a gift from Dr. Jae Yong Lee, Hallym University, Chuncheon, Korea), and 0.1 μg of pCH110 reference plasmid (Amersham Biosciences). Huh7 cells harboring HCV subgenomic replicons were transfected with 0.1 μg of SBE4-Luc, 0.1 μg of 3TP-Lux reporter plasmid, and 0.1 μg of pCH110 reference plasmid. The total DNA amount in each transfection was kept constant by adjustment with empty vector. At 24 h after transfection, cells were stimulated with human TGF-β (5 ng/ml) for 1 h. The cell supernatant was prepared as described above and incubated with anti-TGF-β (5 ng/ml) for 1 h. The cell supernatant was prepared as described above and incubated with anti-TβR-I monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For the co-immunoprecipitation assay, COS-7 cells were infected with the recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (32). At 2 h after infection, cells were transfected with 5 μg of the corresponding plasmids. Following incubation at 37 °C for 12 h, cells were harvested and lysed in buffer A (50 mmol/liter HEPES (pH 7.5), 150 mmol/liter NaCl, 10% glycerol, 1 mmol/liter EDTA, 1% Triton X-100, 1.5 mmol/liter MgCl₂, 10 mmol/liter sodium pyrophosphate, 100 mmol/liter NaF, 1 mmol/liter Na₃VO₄, and 1 mmol/liter phenylmethylsulfonyl fluoride). The cell lysates were trituated 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 or rabbit anti-NSSA polyclonal antibody. The samples were further incubated with 30 μl of protein A beads (Zymed Laboratories Inc.) for 1 h. The beads were washed five times with 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-Myc monoclonal antibody or anti-HA monoclonal antibody.

**Conical Microscopy**—Either Huh7 or COS-7 cells grown on chamber slides (Nunc Inc., Naperville, IL) were cotransfected with either the NSSA-Myc and HA-TβR-I(T204D) expression plasmids or the FLAG-Smad2 and green fluorescent protein-NSSA expression plasmids. At 36 h after transfection, slides were washed with 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 for 20 min at 37 °C and then incubated with anti-HA, anti-Myc, or anti-FLAG (Sigma) monoclonal antibody for 2 h at 37 °C. After being washed three times with PBS, cells were further incubated with TRITC-conjugated goat anti-mouse IgG (American Qualex, San Clemente, CA) for 1 h at 37 °C. After two washes with 0.1% Triton X-100 in PBS and three washes with PBS, cells were analyzed using the LSM 510 laser confocal microscopy system (Carl Zeiss, Inc., Thornwood, NY).

**Co-immunoprecipitation of the Smad3-Mad4 Complex**—Approximately 5 × 10⁵ cells plated on 60-mm dishes were transfected with the FLAG-Smad3, Smad4-Myc, and HA-TβR-I(T204D) expression plasmids using Lipofectamine. At 36 h after transfection, cells were lysed in buffer A and subjected to immunoprecipitation with anti-FLAG monoclonal antibody. 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 with PBS, resuspended in buffer B (10 mmol/liter HEPES (pH 7.9), 1.5 mmol/liter MgCl₂, 10 mmol/liter KCl, 0.5 mmol/liter dithiothreitol, and 0.5 mmol/liter phenylmethylsulfonyl fluoride) for 2 min on ice. Cells were harvested, vor-
texed for 5 s, and centrifuged at 10,000 rpm for 2 min. The supernatant was saved as a cytoplasmic fraction. The pellet was washed twice with buffer C (20 mmol/liter HEPES (pH 7.9), 1.5 mmol/liter MgCl₂, 420 mmol/liter KCl, 25% glycerol, 0.2 mmol/liter EDTA, 0.5 mmol/liter dithiothreitol, and 0.5 mmol/liter phenylmethylsulfonyl fluoride) and resuspended in buffer C. The samples were vortexed for 10 s, 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. The 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 anti-FLAG monoclonal antibody, anti-β-actin monoclonal antibody (Sigma), anti-B23 polyclonal antibody (Santa Cruz Biotechnology, Inc.), or rabbit anti-NS5A polyclonal antibody.

Western Blot Analysis—HepG2 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 buffer A. Equal amounts of proteins were subjected to 10% SDS-PAGE and electrotransferred to a nitro-
cellulose 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, and anti-NS5A polyclonal antibody in Tris-buffered saline/Tween (20 mmol/liter Tris-HCl (pH 7.5), 500 mmol/liter NaCl, and 0.05% Tween 20). Following two washes in Tris-buffered saline/Tween, the membrane was incubated with either horseradish peroxidase-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in Tris-buffered saline/Tween for 90 min at room temperature. Proteins were detected using an ECL kit (Amersham Biosciences).

**FIGURE 2.** NS5A protein interacts with TGF-β-I both in vitro and in vivo. A, Huh7 cells were transfected with the HA-Tg~β-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 (upper panel). Tg~β-I expression in Huh7 cells was verified using anti-HA antibody (middle panel). Both GST and GST-NS5A fusion protein (GST-5A) used for the binding analysis are shown by staining with Coomassie Brilliant Blue (CBB) (lower panel). IB, immunoblot. B, both NS5A and HA-Tg~β-I (T204D) proteins were coexpressed in COS-7 cells using recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3). At 12 h after transfection, cell lysates were immunoprecipitated (IP) with either anti-HA monoclonal antibody (α-HA) or normal mouse IgG. Bound proteins were detected by Western blot analysis using rabbit anti-NS5A polyclonal antibody (α-NS5A) (upper panel). Both HA-Tg~β-I (T204D) and NS5A proteins were verified using the same cell lysates by Western blotting with anti-HA monoclonal antibody (middle panel) and anti-NS5A polyclonal antibody (lower panel), respectively. C, the 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 (upper panel). Both NS5A and HA-Tg~β-I (T204D) proteins were verified using the same cell lysates by Western blotting with anti-NS5A polyclonal antibody (α-5A) (middle panel) and anti-HA monoclonal antibody (lower panel), respectively. D, Huh7 cells were transfected with the NS5A-Myc expression plasmid in a dose-dependent manner (0, 2, and 5 μg). 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 immunoprecipitated with anti-NS5A polyclonal antibody (α-NS5A) (upper panel). The immunoprecipitates were analyzed by Western blot analysis using anti-Myc monoclonal antibody (α-Myc). The membrane was reprobed with either anti-Tg~β-I polyclonal antibody (middle panel) or anti-Myc monoclonal antibody (lower panel). The asterisk indicates the heavy chain of IgG. E, shown is the co-localization of NS5A and Tg~β-I. Both NS5A-Myc and HA-Tg~β-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 anti-HA polyclonal antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG to detect Tg~β-I (green) and anti-Myc monoclonal antibody and TRITC-conjugated goat anti-mouse IgG to detect NS5A (red). Dual staining showed co-localization of NS5A and HA-Tg~β-I (T204D) as yellow fluorescence in the merged image. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label nuclei.
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 μl of buffer B 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 with buffer B and resuspended in 100 μl of buffer C. The sample was further incubated with agitation in a cold room for 30 min and centrifuged at 15,000 rpm for 10 min at 4°C. The protein concentration in nuclear extract was determined by the Bradford method (Bio-Rad). Nuclear extract (20 μg) was incubated with an oligonucleotide probe (34) labeled with 32P (1 × 10^6 cpm) in 15 μl of binding buffer (20% glycerol, 5 mmol/liter MgCl2, 2.5 mmol/liter EDTA, 2.5 mmol/liter dithiothreitol, 250 mmol/liter NaCl, 50 mmol/liter 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× Tris borate/EDTA buffer and detected by autoradiography. For the 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). Because we found that TGF-β was differentially expressed in hepatocellular carcinoma of NS5A transgenic mice (data not shown) and because NS5A regulates several cell signaling events, we investigated the role of NS5A in TGF-β-dependent transcriptional activation using a luciferase reporter gene assay. Human hepatoma cell lines were transiently cotransfected with the NS5A expression plasmid and either the TGF-β-responsive 3TP-Lux reporter plasmid (31) or the SBE4-Luc reporter plasmid (containing four Smad-binding elements) (30). At 24 h after transfection, cells were either left untreated or treated with human TGF-β (5 ng/ml) for 24 h, and the luciferase assay was performed. As shown in Fig. 1A, TGF-β induced a 40–60-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 ~40–60% compared with the vector control in both reporter plasmids. It is noteworthy that HepG2 cells are less responsive to reporter plasmids compared with 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 green fluorescent protein showed no effect on TGF-β-induced transcriptional activation (data not shown). These results suggest that NS5A protein inhibits the TGF-β-induced signal transduction pathway at the 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 and differentiation, apoptosis, and transformation (36, 37). Recently, Majumder et al. (12) have shown that HCV NS5A protein represses p21 expression in a p53-dependent manner. To investigate the direct effect of NS5A protein on p21 transcriptional activity, we performed a luciferase reporter gene assay using the p21-Luc plasmid. Huh7 cells were cotransfected with the NS5A expression plasmid, the TβR-I(T204D) expression plasmid, and the 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 TβR-I(T204D) was not affected by NS5A protein (Fig. 1, B and C). 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 a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed. Upon TGF-β treatment, only 40% of the cells stably transfected with empty vector remained viable (Fig. 1D). However, the majority of Huh7 cells stably expressing NS5A were viable (Fig. 1D), indicating that these cells are significantly less sensitive to TGF-β-induced cell death compared with vector control cells. These results suggest that HCV NS5A protein inhibits TGF-β-induced cell death through down-regulation of p21 activity.

NS5A Protein Specifically Interacts with TβR-I in Vitro and in Vivo—To further investigate how NS5A protein interacts with TGF-β signal transducers. Because many viral proteins regulate TGF-β signaling through Smad proteins, we first examined the interaction

FIGURE 3. NS5A interacts with HA-TβR-I(T204D) through amino acids 148–237 of NS5A. A, the schematic diagram shows both wild-type and mutant forms of NS5A (5A). aa, amino acids. B, COS-7 cells were cotransfected with HA-TβR-I(T204D) and NS5A mutant plasmids paired with the recombinant vaccinia virus vTF7-3. At 12 h after transfection, cell lysates were immunoprecipitated (IP) with anti-HA monoclonal antibody (α-HA), and bound proteins were detected by Western blotting with anti-NS5A polyclonal antibody (α-NS5A) (upper panel). Both NS5A and TβR-I(T204D) proteins were verified using the same cell lysates by Western blotting with anti-NS5A polyclonal antibody (middle panel) and anti-HA monoclonal antibody (lower panel), respectively. IB, immunoblot.
between NS5A and Smad proteins using a co-immunoprecipitation assay. The NS5A expression plasmid was cotransfected into Huh7 cells with FLAG-tagged Smad2, Smad3, and Smad4 individually. Cell lysates were immunoprecipitated with anti-NS5A antibody, and the bound proteins were detected using anti-NS5A antibody. Reciprocally, cell lysates were immunoprecipitated with anti-NS5A antibody, and the bound proteins were detected using anti-NS5A antibody. In either case, NS5A protein failed to interact with Smad proteins (data not shown). We then examined the interaction between NS5A and TβR-I in vitro. We performed a GST pull-down assay using GST and GST-NS5A fusion protein expressed in E. coli. Cell extracts containing HA-TβR-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 TβR-I, whereas GST protein failed to interact with TβR-I. To further confirm in vitro interaction between NS5A and TβR-I, we performed a co-immunoprecipitation assay. HA-TβR-I(T204D) was coexpressed with NS5A in COS-7 cells paired with a recombinant vaccinia virus (vTF7-3) system. At 12 h after transfection, cell lysates were immunoprecipitated with either mouse anti-HA monoclonal antibody or control anti-mouse IgG, and the coprecipitated proteins were detected by Western blot analysis using rabbit anti-NS5A polyclonal antibody. Indeed, NS5A interacted with TβR-I in vivo (Fig. 2B). We confirmed this result by a reciprocal experiment using anti-NS5A antibody for immunoprecipitation (Fig. 2C). Next, we investigated whether NS5A interacts with endogenous TβR-I. Huh7 cells were transfected with increasing amounts of the 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-TβR-I polyclonal antibody, and the immunoprecipitated proteins were analyzed by Western blot analysis using anti-Myc monoclonal antibody. As shown Fig. 2D, NS5A protein interacted with endogenous TβR-I regardless of TGF-β treatment. We also confirmed that NS5A interacted with TβR-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 co-localize with endogenously expressed TβR-I(T204D). To determine this possibility, Huh7 cells were cotransfected with the NS5A-Myc and HA-TβR-I(T204D) expression plasmids, and we examined the subcellular localization by confocal microscopy. It was reported previously that NS5A protein is localized to the endoplasmic reticulum and Golgi apparatus in Huh7 cells (38). Fig. 2E shows that NS5A was localized in the cytoplasm, and dual staining showed co-localization of NS5A and TβR-I(T204D) in the

**FIGURE 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 (N) fractions, respectively. B, Huh7 cells were cotransfected with constitutively activated HA-TβR-I(T204D) and FLAG-Smad2 expression plasmids in the absence or presence of the NS5A plasmid. At 36 h after transfection, cells were fractionated into cytoplasmic and nuclear fractions. Smad2 protein was detected by Western blot analysis using anti-FLAG monoclonal antibody (α-Flag) (upper panel). NS5A protein was verified by Western blotting with anti-NS5A polyclonal antibody (α-NS5A) in the same cell lysate (lower panel). IB, immunoblot. C, NS5A protein inhibits TGF-β-induced Smad2 nuclear translocation. Both FLAG-Smad2 and green fluorescent protein-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 anti-FLAG monoclonal antibody and TRITC-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 using anti-phospho-Smad2 polyclonal antibody (α-p-Smad2) (upper panels). The protein expression of both total endogenous Smad2 and NS5A was verified using the same cell lysates by Western blotting with anti-Smad2 polyclonal antibody (α-Smad2) (middle panels) and anti-NS5A polyclonal antibody (lower panels), respectively.
cytoplasm as yellow fluorescence. We further confirmed that NS5A and TβR-I(T204D) were co-localized in non-hepatic COS-7 cells (data not shown). Taken together, these results indicate that NS5A protein specifically interacts with TβR-I both in vitro and in vivo.

The Middle Region of NS5A Mediates the NS5A/TβR-I(T204D) Interaction—To determine the region in NS5A that is responsible for TβR-I binding, the interaction of TβR-I with various deletion mutants of NS5A (Fig. 3A) was determined by a transfection-based coprecipitation assay in COS-7 cells infected with the recombinant vaccinia virus vTF7-3. As shown in Fig. 3B, HA-TβR-I(T204D) interacted with an N-terminal deletion mutant of NS5A (amino acids 148–237) but not with wild type NS5A (amino acids 1–301). However, the NS5A-(238–447) mutant no longer interacted with HA-TβR-I(T204D), suggesting that the middle region (amino acids 148–237) of NS5A is required for NS5A/TβR-I(T204D) interaction.

NS5A Blocks TGF-β-mediated Nuclear Translocation and Phosphorylation of Smad2—TGF-β activation stimulates the phosphorylation and nuclear translocation of Smad proteins. In control Huh7 cells, Smad2 protein was localized mostly in the cytoplasm, as shown in Fig. 4A. To understand the molecular mechanism of the inhibitory effect of NS5A on TGF-β-mediated transactivation, we examined whether NS5A represses TβR-I(T204D)-mediated nuclear translocation of Smad2 protein. Huh7 cells were cotransfected with HA-TβR-I(T204D) and FLAG-Smad2 in the absence or presence of NS5A. Cells were fractionated into distinct cytoplasmic and nuclear fractions, and the nuclear translocation of Smad2 protein was determined. Indeed, overexpression of HA-TβR-I(T204D) induced the nuclear translocation of Smad2 protein (Fig. 4B, lane 2), whereas NS5A protein stayed in the cytoplasm. Notably, HA-TβR-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 reported previously (39), FLAG-Smad2 was dispersed in the cytoplasm and nucleus of Huh7 cells. Upon TGF-β treatment, FLAG-Smad2 accumulated in the nucleus (Fig. 4C, upper panels). However, TGF-β-induced nuclear translocation of Smad2 was inhibited by NS5A protein (Fig. 4C, middle and lower panels). These results indicate that TGF-β-dependent nuclear translocations of Smad proteins are inhibited by NS5A protein in hepatoma cell lines.

Because NS5A interacts with TβR-I and because TβR-I plays an important role in the TGF-β-dependent signaling pathway through the phosphorylation of R-Smad proteins, we examined the role of NS5A protein in Smad2 phosphorylation. To investigate whether NS5A inhibits 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 inhibited 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 TβR-I.

NS5A Inhibits TβR-I-induced Heterodimerization of Smad3 and Smad4—Ligand-activated TβR-I phosphorylates R-Smad proteins at the C-terminal SSXS motif (20, 40), which induces a conformational change in R-Smad proteins, thereby facilitating heterodimerization with Co-Smad (21). To determine the effect of NS5A on TβR-I-induced heterodimerization of Smad3 and Smad4, Huh7 cells were cotransfected with constitutively activated HA-TβR-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 the bound complex was analyzed by western blotting using anti-FLAG, anti-Myc, and anti-NS5A (α-NS5A) antibodies, respectively (lower panels). H, heavy chain; IB, immunoblot.
Western blot analysis using anti-Myc monoclonal antibody. As expected, constitutively activated TβR-I induced heterodimer formation between Smad3 and Smad4, whereas coexpression of NS5A dramatically inhibited complex formation between Smad3 and Smad4 (Fig. 5, lane 3). Because the protein expression levels of Smad3 and Smad4 were not affected by NS5A, this result may explain how NS5A modulates the TGF-β-dependent signaling pathway.

**NS5A Protein Inhibits TGF-β-induced Smad DNA-binding Activity**—To examine how the inhibitory effect of NS5A on Smad protein activities results in TGF-β-induced Smad-DNA complex, EMSA was per-

---

**FIGURE 7. HCV subgenomic replicon inhibits the TGF-β-induced signal transduction pathway.**

A, both IFN-cured and HCV subgenomic replicon cells were transfected with the SBE4-Luc reporter plasmid. At 24 h after transfection, cells were either left 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-galactosidase activities (upper panel). Total cell extracts were subjected to SDS-PAGE and immunoblotted (IB) with anti-NS5A polyclonal antibody (α-NS5A) or anti-β-actin monoclonal antibody (α-Actin) (middle and lower panels, respectively). The data shown represent three independent experiments. B, both IFN-cured and HCV subgenomic replicon cell lysates were immunoprecipitated (IP) with anti-TβR-I polyclonal antibody (α-TβR-I). Bound proteins were detected by immunoblotting with rabbit anti-NS5A polyclonal antibody (upper panel). The protein expression of both endogenous TβR-I and NS5A was verified using the same cell lysates by immunoblotting with anti-TβR-I polyclonal antibody (middle panel) and anti-NS5A polyclonal antibody (lower panel), respectively. C, both IFN-cured and HCV subgenomic replicon cells were stimulated with human TGF-β (5 ng/ml) for the indicated times, and the phosphorylation level of endogenous Smad2 was detected using anti-phospho-Smad2 polyclonal antibody (α-p-Smad2) (upper panel). The protein expression of both endogenous Smad2 and NS5A was verified using the same cell lysates by immunoblotting with anti-Smad2 polyclonal antibody (α-Smad2) (middle panel) and anti-NS5A polyclonal antibody (lower panel), respectively.
formed. At 24 h after plating, Huh7 cells stably expressing NSSA or empty vector were treated with TGF-β for 24 h, and then nuclear extracts were prepared. EMSA was performed using the TGF-β-inducible element in the plasminogen activator inhibitor-1 promoter (approximately −586 to −551). Upon TGF-β treatment, retarded bands of Smad-DNA complexes were increased in stable vector control cells (Fig. 6A, lane 2), whereas these complexes were markedly decreased in stable cells expressing NSSA protein (lane 5). It is noteworthy that TGF-β-induced Smad-DNA complexes formed distinctive triple bands, as reported previously (34). Also, the disappearance of TGF-β-responsive complexes using an unlabeled probe further suggests the specificity of Smad-DNA binding (Fig. 6A, lanes 3 and 6).

**HCV Subgenomic Replicon Suppresses the TGF-β-induced Signal Transduction Pathway**—We further asked whether the inhibitory effect of NSSA 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 particles. For this purpose, we investigated TGF-β-induced signaling cascades in an 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 BSE4-Luc reporter plasmids. At 24 h after transfection, cells were either left untreated or treated with human TGF-β for 24 h. A luciferase reporter gene assay was then performed. As shown in Fig. 7A, TGF-β-induced promoter activity in HCV subgenomic replicon cells was suppressed by ∼60% compared with IFN-cured Huh7 cells. We then examined whether NSSA protein can interact with TβR-I in the context of viral RNA replication. For this purpose, both IFN-cured and HCV subgenomic replicon cell lysates were immunoprecipitated with anti-TβR-I antibody, and then bound proteins were immunoblotted with anti-NSSA antibody. As shown in Fig. 7B, NSSA protein in HCV subgenomic replicon cells specifically interacted with TβR-I. We further asked whether TGF-β-mediated endogenous Smad2 phosphorylation is affected by the 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 using anti-phospho-Smad2 polyclonal antibody. TGFβ treatment immediately stimulated endogenous Smad2 phosphorylation in IFN-cured cells (Fig. 7C, lane 2). However, the phosphorylation level of endogenous Smad2 was inhibited by ∼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 NSSA inhibits TGF-β-mediated signaling cascades through interaction with TβR-I.

**DISCUSSION**

TGF-β is one of the multifunctional cytokines that are implicated in diverse cell phenomena, including cell growth control, cell adhesion and motility, alteration of cell 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 has demonstrated the novel regulatory role of HCV NSSA protein in the TGF-β-dependent signaling pathway of transiently transfected human hepatoma cells and in cells harboring HCV subgenomic replicons. Several viruses encode gene products that modulate the TGF-β signaling pathway through interaction with cellular proteins. For example, hepatitis B virus X protein 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 the TGF-β signaling pathway by blocking the DNA-binding activity of the Smad complexes (34). Likewise, the HCV core protein inhibits p21 promoter activity by inhibiting the TGF-β signaling pathway (47).

NSSA is a multifunctional protein and regulates various cell signaling events. In this study, we investigated the role of NSSA in TGF-β-dependent signaling cascades. NSSA inhibited TGF-β-stimulated transcriptional activities, and this inhibition was mediated through interaction with TβR-I. We have shown that NSSA directly interacted with TβR-I through the middle region (amino acids 148–237) of NSSA. NSSA/TβR-I interaction was confirmed by a co-immunoprecipitation assay and confocal microscopy. Because NSSA inhibits tumor necrosis factor-α-induced NF-κB activation by interaction with TRAF2 through the middle domain (amino acids 148–301) of NSSA (48), this region plays a key role in modulation of many cytokine-dependent cell signaling events. Although NSSA protein did not affect the autophosphorylation of TβR-I (data not shown), the phosphorylation level of Smad2 was decreased by NSSA; and hence, complex formation between R- and Co-Smad proteins was subsequently disrupted by NSSA protein.

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

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

The TGF-β-dependent signaling pathway involves liver fibrogenesis and chronic hepatitis (58, 59) and hepatocellular carcinoma (60). In this study, we have shown that NSSA is a negative regulator of TGF-β-dependent signaling intermediates through interaction with TβR-I. We have demonstrated that NSSA protein inhibits the phosphorylation of Smad2, abrogates interaction between Smad3 and Smad4, prevents the nuclear translocation of Smad proteins, inhibits the formation of Smad-DNA complexes, and down-regulates p21 expression in hepatoma cell lines. These results and our previous findings (33, 48) suggest that NSSA may play a key role in regulating cytokine signaling pathways. Indeed, we demonstrated that transgenic mice harboring the HCV NSSA gene, but not the HCV core gene, develop hepatocellular carcinoma. In conclusion, HCV regulates the TGF-β-induced signal transduction pathway, and NSSA 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., Ikuchi, 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., A. G. Wang, S. H. Choi, H. B. Moon, D. Y. Yu, and S. B. Hwang, unpublished observations.
HCV NS5A Regulates TGF-β Signaling Cascades

Johnson, R. G., Barbosa, L. H., and Nemo, G. J. (1991) *N. Engl. J. Med.* 325, 1325–1329

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 Publishers, Philadelphia

Reed, K. E., and Rice, C. C. (2000) *Curr. Top. Microbiol. Immunol.* 242, 55–84

Ide, Y., Tanimoto, A., Sasaguri, Y., and Padmanabhan, R. (1997) *Gene (Anst.)* 201, 151–158

Gale, M. Jr., Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polya, S. J., Gretch, D. R., and Katz, M. G. (1997) *Virology* 230, 217–227

Ide, Y., Zhang, L., Chen, M., Inchauspe, G., Bahl, C., Sasaguri, Y., and Padmanabhan, R. (1996) *Gene (Anst.)* 182, 203–211

Tan, S. L., Nakao, H., He, Y., Vijayari, S., Neddermann, P., Jacobs, B. L., Mayer, B. J., and Katze, M. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 5533–5538

Kim, B.-C., Lee, M.-N., Kim, J.-Y., Lee, S.-S., Chang, J.-D., Kim, S.-S., Lee, S.-Y., and Ewen, M. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 5333–5338

Newman, M. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 5253–5256

Ewen, M. E. (1994) *Cancer Metastasis Rev.* 13, 45–66

Cho, Y. G., Yoon, J. W., Jiang, K.L., Kim, C. M., Sung, Y.C. (1993) *Mol. Cell 3*, 195–202

Kim, B.-C., Lee, M.-N., Kim, Y.-J., Lee, S.-S., Chang, J.-D., Kim, S.-S., Lee, S.-Y., and Kim, J.-H. (1999) *J. Biol. Chem.* 274, 24372–24377

Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) *Mol. Cell 1*, 611–617

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) *EMBO J.* 17, 3091–3100

Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 8122–8126

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

Lee, D. K., Kim, B.-C., Kim, I. Y., Cho, E.-a., Satterwhite, D. J., and Kim, S.-J. (2002) *J. Biol. Chem.* 277, 38557–38564

Massagué, J., Blain, S. W., and Lo, R. S. (2000) *Cell* 103, 295–309

Harper, J. W., and Elledge, S. J. (1996) *Curr. Opin. Genet. Dev.* 6, 56–64

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* 75, 817–825

Shi, S. T., Polya, S. J., Tu, H., Taylor, D. R., Gretch, D. R., and Lai, M. C. M. (2002) *Virology* 292, 198–210

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

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

Guo, J. T., Bichko, V. V., and Seeger, C. (2001) *J. Virol.* 75, 8516–8523

Kim, S.-J., Im, Y. H., Markowitz, S. D., and Bang, Y. J. (2000) *Cytokine Growth Factor Rev.* 11, 159–168

Howe, P. H., Draetta, G., and Lead, E. B. (1991) *Mol. Cell. Biol.* 11, 1185–1194

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

Lee, D. K., Park, S. H., Yi, Y., Choi, S. G., Lee, C., Park, W. T., Cho, H., de Caestecker, M. P., Shaul, Y., Roberts, A. B., and Kim, S. J. (2001) *Genes Dev.* 15, 455–466

Nishihara, A., Hanai, J., Imamura, T., Miyazono, K., and Kawabata, M. (1999) *J. Biol. Chem.* 274, 28716–28723

Lee, M.-N., Jung, E. Y., Kwun, H. I., Jun, H. K., Yu, D. Y., Choi, Y. H., and Jung, K. L. (2002) *J. Gen. Virol.* 83, 2145–2151

Park, K.-J., Choi, S.-H., Lee, S.-Y., Hwang, S. B., and Lai, M. C. M. (2002) *J. Biol. Chem.* 277, 13121–13128

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. C. M. (1999) *Virology* 263, 30–41

Saltus, J. (1996) *Mol. Cell. Endocrinol.* 116, 227–232

Lyons, R. M., and Moses, H. L. (1990) *Eur. J. Biochem.* 187, 467–473

Jetten, A. M., Shirley, J. E., and Stoner, G. (1986) *Exp. Cell Res.* 167, 539–549

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 5545–5549

Qidrhi, I., Iwahashi, M., and Simon, F. (2002) *Biochim. Biophys. Acta 1592*, 193–204

Gale, M. Jr., Kwiczasiewski, B., Bossett, M., Nakao, H., and Katze, M. G. (1999) *J. Virol.* 73, 6506–6516

He, Y., Nakao, H., Tan, S. L., Polya, S. J., Neddermann, P., Vijayari, S., Jacobs, B. L., and Katz, M. G. (2002) *J. Virol.* 76, 9207–9217

Schuppam, D., Krebs, A., Bauer, M., and Hahn, E. G. (2003) *Cell Death Differ.* 10, Suppl. 1, 559–567

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

Shinizu, I. (2001) *Curr. Drug Targets Infect..Disord.* 1, 227–240

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