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Nonstructural 5A Protein of Hepatitis C Virus Modulates Tumor Necrosis Factor α-stimulated Nuclear Factor κB Activation*

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The hepatitis C virus nonstructural protein 5A (NS5A) is a multifunctional phosphoprotein that leads to pleiotropic responses, in part by regulating cell growth and cellular signaling pathways. Here we show that overexpression of NS5A inhibits tumor necrosis factor (TNF)-α-induced nuclear factor κB (NF-κB) activation in HEK293 cells, as determined by luciferase reporter gene expression and by electrophoretic mobility shift assay. When overexpressed, NS5A cannot inhibit the recruitment of TNF receptor-associated factor 2 (TRAF2) and IκB kinase (IKK)β into the TNF receptor 1-TNF receptor-associated death domain complex. In contrast, NS5A is a part of the TNF receptor 1 signaling complex. NF-κB activation by TNF receptor-associated death domain and TRAF2 was inhibited by NS5A, whereas MEKK1 and IKKβ-dependent NF-κB activation was not affected, suggesting that NS5A may inhibit NF-κB activation signaled by TRAF2. Coimmunoprecipitation and colocalization of NS5A and TRAF2 expressed *in vivo* provide compelling evidence that NS5A directly interacts with TRAF2. This interaction was mapped to the middle one-third (amino acids 148–301) of NS5A and the TRAF domain of TRAF2. Our findings suggest a possible molecular mechanism that could explain the ability of NS5A to negatively regulate TNF-α-induced NF-κB activation.

Hepatitis C virus (HCV)[1] is the major cause of non-A, non-B hepatitis worldwide (1, 2), which often leads to liver cirrhosis and hepatocellular carcinoma (3–5). HCV is an enveloped positive-sense RNA virus of the *Flaviviridae* family (6). The viral genome encodes a single polyprotein precursor of ~3,010 amino acids, which is cleaved by both host and viral proteases into three structural and seven nonstructural proteins (7–10). The N-terminally localized core and envelope proteins (E1 and E2) are the viral structural proteins, and the remainders are the nonstructural proteins with various enzymatic activities. The nonstructural protein 5A (NS5A) is a multifunctional phosphoprotein. This protein is phosphorylated at serine residues by α-catalytic subunit of cAMP-dependent protein kinase A (11) and liberates two different sizes of NS5As, p56 and p58 (12). Recent studies have shown that the hyperphosphorylated form (p58) does not exist in all HCV genotypes (13), indicating that the functional role of NS5A may be different between HCV genotypes. To date, the biological significance of phosphorylation of NS5A has not been elucidated. HCV NS5A has a functional nuclear localization signal that can target a heterologous reporter gene product to the nucleus, although native NS5A is localized in the cytoplasm (14). Both NS5A and nonstructural protein 5B bind to different domains of a human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33) (15), suggesting that NS5A may form part of the HCV RNA replication complex. It has been reported that N terminus-truncated NS5A, but not full-length NS5A, is a potent transcriptional activator in yeast and mammalian cells (16, 17). Subsequent studies demonstrated that both N- and C-terminal cleaved NS5A product was localized in the nucleus and showed transcriptional activity in the presence of α-catalytic subunit of protein kinase A (18). Whether this trans-activation activity is related to HCV RNA synthesis is not clear. NS5A contains an interferon (IFN) sensitivity-determining region in the middle domain (amino acids 237–276) of the protein (19). NS5A from some genotype 1 HCV interacts with the IFN-inducible double-stranded RNA-activated protein kinase (PKR) and functions as a repressor of PKR (19). Subsequent studies showed that cells expressing HCV NS5A blocked the PKR-dependent signaling pathways and exhibited an oncogenic phenotype (20). These functions required the PKR binding of NS5A, suggesting the involvement of NS5A in conferring IFN resistance. In addition, PKR facilitates IFN-induced transcriptional programs by participating in the activation of nuclear factor κB (NF-κB) and IFN-regulatory factor 1 (21). Recently, NS5A was shown to perturb cellular signaling by selectively targeting the growth factor receptor-bound protein 2 adapter protein (22).

Tumor necrosis factor (TNF) is a cytokine produced by many cell types in response to inflammation and infection by microorganisms. TNF elicits diverse cellular events such as induction of other cytokines, cell proliferation, differentiation, and apoptosis (23–25). Many of these TNF-induced processes are mediated by either TNFR1 or TNFR2, both of which belong to the TNF receptor superfamily (26, 27). Both receptors lack recognizable enzymatic domains, and their ability to transduce signals is dependent on the interaction with proteins associ-

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[1] The abbreviations used are: HCV, hepatitis C virus; TNF, tumor necrosis factor; GST, glutathione *S*-transferase; TNFR, tumor necrosis factor receptor; TRADD, tumor necrosis factor receptor-associated death domain; TRAF2, tumor necrosis factor receptor-associated factor 2; HA, hemagglutinin; NS5A, nonstructural protein 5A; NF-κB, nuclear factor κB; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; IFN, interferon; PKR, RNA-activated protein kinase; FADD, Fas-associated death domain; aa, amino acid(s); PBS, phosphate-buffered saline; IKK, IκB kinase.

ated with their cytoplasmic tails. TRADD has been identified as a TNFR1-associated protein in a ligand-dependent manner. Once TRADD is recruited to TNFR1, it functions as an adapter protein to recruit several structurally and functionally divergent proteins, including FADD, receptor-interacting protein, and TRAF2 (28, 29). The interaction of TRADD with FADD leads to apoptosis through the activation of a caspase cascade, which is initiated by the interaction of FADD with caspase-8. The interaction of TRADD with TRAF2 and receptor-interacting protein leads to the activation of transcription factor NF-κB (29).

The NF-κB pathway is a key component of the cellular response to a variety of extracellular stimuli, including TNF, interleukin 1, and phorbol esters (30). Cellular genes activated by NF-κB include those involved in the inflammatory and immune response and cellular growth control (30). NF-κB is sequestered in the cytoplasm by a group of inhibitory proteins known as IκBs. Many of the signals leading to the nuclear translocation of NF-κB result in the inducible phosphorylation and degradation of IκBα (31). After phosphorylation of IκBα on serine residues, IκBα is ubiquitinated and degraded by the proteasome, leading to the release of free NF-κB (31). After degradation, IκBα is rapidly replenished by NF-κB-mediated transcription of the IκBα gene, which then constitutes the autoregulatory loop of NF-κB-IκBα activation. Although NF-κB activation is tightly regulated by this autoregulatory loop, NF-κB is regulated by some viral gene products. For example, human T-cell leukemia virus type 1 Tax protein can activate NF-κB through enhancement of IKKβ phosphorylation (32).

In the present study, we examined the effects of HCV NS5A on NF-κB activation induced by TNF-α. We demonstrate that NS5A can inhibit the transcription factor NF-κB activation stimulated by TNF-α. This inhibition of NF-κB activity was contributed by the NS5A-TRAF2 protein interaction. These data suggest that the association of NS5A with TRAF2 in the TNFR1 signaling complex can negatively regulate TNF-α-induced NF-κB activation.

EXPERIMENTAL PROCEDURES

Plasmid Construction—cDNA corresponding to the NS5A coding sequence of HCV was amplified by PCR using the Korean isolate of HCV (33) and subcloned into the *Kpn*I site of pcDNA3 (Invitrogen). TNFR1, HA-TRADD, and TRAF2 expression vectors were described previously (34, 35). FLAG-IKKβ expression vector was a gift from Dr. Hiroyasu Nakano (Juntendo University). Both wild type TRAF2 and TRAF2-(1–358) were tagged with FLAG epitope at N termini by PCR. TRAF2-(1–272) and TRAF2-(314–501) were subcloned into the pcDNA3.1 expression vector. The GST-fused TNFR1 construct was described previously (36). An active form of MEKK (pFC-MEKK) expression vector was purchased from Stratagene.

Cell Culture and Transfection Experiment—HEK293 cells, HEK293T cells, and Cos7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml streptomycin, and 100 units/ml penicillin (Invitrogen). For transfection, $\sim 5 \times 10^5$ cells plated on 60-mm dishes were transfected with DNA by using either LipofectAMINE (Invitrogen) or the calcium phosphate method as described previously (37).

NF-κB-dependent Reporter Gene Assay—HEK293 cells were transfected by the calcium phosphate method with 2 μg of expression plasmid, 0.1 μg of NF-κB luciferase reporter (38), and 0.1 μg of pCH110 (Amersham Biosciences) reference plasmid, respectively. The total DNA concentration in each transfection was kept constant by adjusting with an empty vector. At 24 h after transfection, the cells were stimulated with TNF-α (10 ng/ml; Invitrogen) for 6 h. Luciferase and β-galactosidase assays were performed as described previously (37).

GST Binding Assay and Coimmunoprecipitation—cDNA corresponding to full-length NS5A was subcloned into the *Sma*I and *Eco*RI site of the pGEX-2T expression vector (Novagen). GST-NS5A fusion protein was expressed in *Escherichia coli* BL21 (DE) (Novagen) and purified with glutathione-Sepharose 4B beads (Amersham Biosciences) as specified by the manufacturer. HEK293 cells were lysed in 400 μl of cell lysis buffer A (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysate was centrifuged at 14,000 rpm for 10 min, and protein concentrations of each of the cell lysates were determined using a Bio-Rad protein assay kit.

For binding assay, TRAF2 protein was incubated with either GST or GST-NS5A fusion proteins for 2 h at 4 °C in cell lysis buffer A. Samples were washed four times with cell lysis buffer A, and the bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by immunoblot analysis using either rabbit anti-HCV NS5A antibody or HCV patients' sera.

For coimmunoprecipitation assay, Cos7 cells were infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (39). Two h after infection, cells were transfected with 4 μg of corresponding plasmids. After incubation at 37 °C for 12 h, cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and incubated in 400 μl of cell lysis buffer B (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1% Triton X-100, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were triturated by 10 passes through a 21-gauge needle on ice and centrifuged at 14,000 rpm for 10 min. The supernatant was incubated for 2 h with either anti-FLAG monoclonal antibody (Sigma-Aldrich), anti-NS5A polyclonal antibody, or anti-TRAF2 polyclonal antibody (Santa Cruz Biotechnology). The samples were further incubated with 30 μl of protein A beads (Zymed Laboratories Inc.) for 1 h. Beads were washed five times with cell lysis buffer B, and the bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by immunoblot analysis using either rabbit anti-NS5A polyclonal antibody or HCV patients' sera. The TRAF2 proteins were detected with either TRAF2 N19 or C20 polyclonal antibody.

Confocal Microscopy—Cos7 cells grown on chamber slides (Nunc) were cotransfected with NS5A and FLAG-TRAF2 plasmids as described above. At 30 h after transfection, slides were washed in PBS and fixed in 4% paraformaldehyde and 0.1% Triton X-100 for 20 min. Cells were incubated in 5% bovine serum albumin for 20 min and then incubated with rabbit anti-NS5A antibody and mouse anti-FLAG antibody for 1 h. After being washed in PBS three times, cells were further incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (American Qualex) and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (American Qualex). After two washes in PBT (0.1% Triton X-100 in PBS) and three washes in PBS, cells were analyzed using MRC 1024 laser confocal microscopy system (Bio-Rad).

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described previously (34). Protein concentrations were determined using the method of Bradford (Bio-Rad). Nuclear extracts (10 μg) were assayed for NF-κB DNA binding activity in the electrophoretic mobility shift assay by incubation with 1×10^5 cpm of a ^{32}P-end-labeled 40-mer single-stranded NF-κB oligonucleotide from immunoglobulin κB (5′-GAGAGGGGACTTTCCGATTAGCTTTCGGAAAGTCCCCTCT-3′) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA (pH 7.5), 5% glycerol, and 2 μg of poly(dI-dC) plus 5% Nonidet P-40 for 15 min at room temperature. The protein-DNA complexes were separated by electrophoresis on 5% native polyacrylamide gels using 0.25× Tris-borate EDTA buffer and detected by autoradiography. For competition analysis, unlabeled oligonucleotide was incubated with nuclear extract in binding buffer for 15 min before the addition of radiolabeled oligonucleotide.

Endogenous IKK Assay—HEK293 cells stably expressing either HCV NS5A or empty vector were plated at a density of $\sim 1 \times 10^6$ cells in a 60-mm plate. At 24 h, cells were treated with TNF for the indicated times and lysed in 400 μl of cell lysis buffer A. Cell extracts were precipitated with 1 μg of anti-IKKα antibody (Santa Cruz Biotechnology) for 2 h at 4 °C, and protein A-Sepharose was added for 1 h at 4 °C. Immune complexes were washed twice with lysis buffer A and then washed twice with kinase buffer (20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM NaF, and 1 mM dithiothreitol). Kinase assays were performed for 30 min at 30 °C in 20 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (Amersham Biosciences) and 1 μg of GST-IκBα (aa 1–54) as a substrate. The reaction mixtures were resolved by SDS-PAGE and then detected by autoradiography. Kinase activity was quantified using a densitometric scanner (Molecular Dynamics).

RESULTS

Inhibition of TNF-α-induced NF-κB Activation by NS5A—Because TNF-α plays prominent roles in the host defense

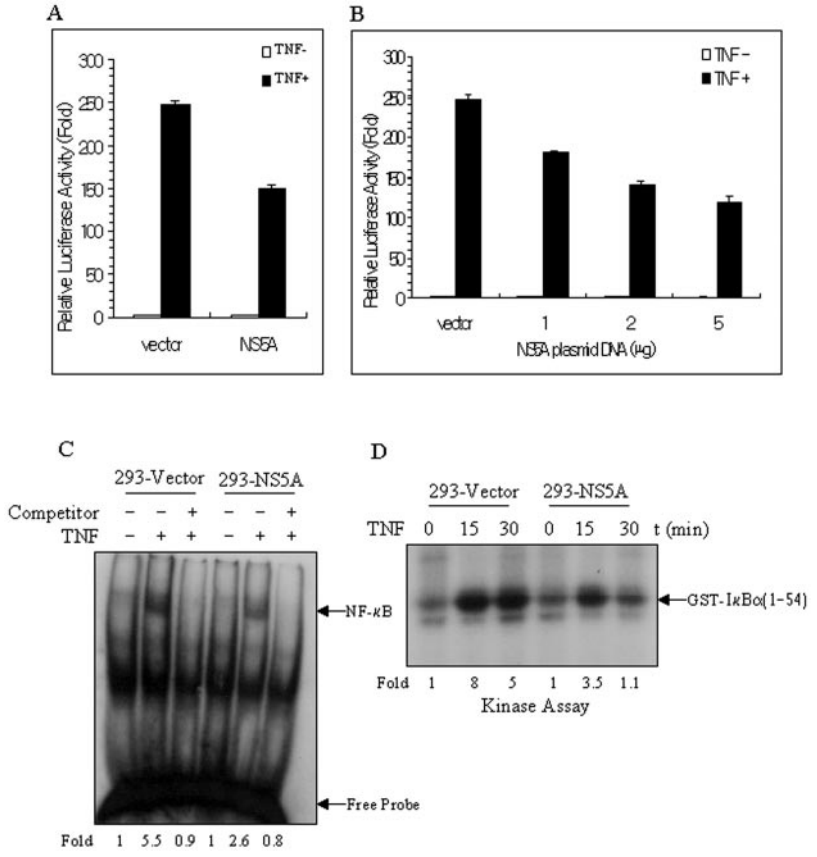

FIG. 1. **Inhibition of TNF-α-induced NF-κB activity by HCV NS5A.** *A*, HCV NS5A inhibits TNF-α-stimulated NF-κB activation. HEK293 cells were cotransfected with NF-κB-Luc and pCH110 reporter plasmids together with empty vector or NS5A expression plasmid. DNA concentration was held constant by the addition of empty vector. At 24 h after transfection, cells were either left untreated or treated with human TNF-α (10 ng/ml) for 6 h, and then reporter activity was measured as described under "Experimental Procedures." *B*, NS5A inhibits NF-κB activation in a dose-dependent manner. Reporter genes were transiently cotransfected with selected amounts (1, 2, and 5 μg) of HCV NS5A plasmid. Total DNA amount was held at 5 μg by adjusting with empty vector. The results shown are representative of three independent experiments. *C*, NS5A inhibits TNF-α-induced NF-κB DNA binding activity. 293-vector and 293-NS5A cells were incubated with TNF-α (20 ng/ml) for 30 min. Nuclear extracts were prepared and assessed for activated NF-κB by electrophoretic mobility shift assay with γ-^{32}P-labeled single-stranded immunoglobulin κB oligonucleotide as described under "Experimental Procedures." Fold of NF-κB activation was calculated based on levels in untreated cells. *D*, effects of HCV NS5A on endogenous IKKβ activity. 293-vector and 293-NS5A cells were stimulated with TNF-α for the indicated times (0, 15, and 30 min). After TNF treatments, cell lysates were precipitated with anti-IKKβ antibody, and endogenous IKKβ activity was determined using GST-IκBα-(1–54) as a substrate.

against viruses, we decided to determine the role of NS5A in the TNF-α-mediated activation of NF-κB by using a transient transfection system. HEK293 cells were cotransfected with NF-κB-luciferase reporter vectors with either empty vectors or NS5A expression plasmids. At 24 h after transfection, cells were either left untreated or treated with human TNF-α (10 ng/ml) for 6 h, and then NF-κB activity was analyzed. As shown in Fig. 1*A*, endogenous NF-κB activity was maintained at a basal level, and this activity was not affected by NS5A. TNF-α induced a ~250-fold increase of NF-κB activity as compared with the endogenous level. On the other hand, overexpression of NS5A inhibited TNF-α-induced NF-κB activation in a dose-dependent manner (~30–55% reduction in reporter gene activity), whereas NS5A itself, regardless of its protein expression level, had no effects on the endogenous NF-κB activity (Fig. 1*B*).

Gel shift experiments with nuclear extracts from NS5A- and empty vector-expressing HEK293 cells further confirmed that NS5A could inhibit TNF-α-induced NF-κB activation (Fig. 1*C*). We next determined whether NS5A could regulate TNF-α-induced IKKβ kinase activity. Upon TNF treatment, IKK kinase activity was increased 8-fold in control cells (Fig. 1*D*, *lane 2*). In contrast, overexpression of NS5A weakly activated IKKβ kinase activity (Fig. 1*D*, *lane 5*), further confirming that NS5A may be a negative regulator of TNF-α-induced NF-κB activation.

The Recruitment of TRAF2 into TNFR1-TRADD Complex Is Not Inhibited by NS5A—The TNFR1 complex, in response to TNF treatment, recruits TRAF2 as well as TRADD and IKKs. (28, 29, 40–42). To determine whether the ability of NS5A to inhibit NF-κB is mediated through the prevention of TRAF2 or IKKβ recruitments into the TNFR1 signaling complex, HEK293 cells were transfected with various combinations of expression vectors in the presence or absence of NS5A. At 36 h after transfection, cell lysates were precipitated with glutathione-Sepharose beads and analyzed on immunoblots with anti-TRAF2, anti-HA, anti-FLAG, and NS5A antibodies. As reported previously, TRADD, TRAF2, and IKKβ were recruited to the TNFR1 complex (Fig. 2, *A* and *B*, *lanes 2*). However, TRAF2 and IKKβ were not recruited to the TNFR1 complex in the absence of TRADD (Fig. 2*A*, *lane 1*) and TRAF2 (Fig. 2*B*, *lane 1*), respectively. Importantly, coexpression of NS5A did not block the recruitment of TRAF2 (Fig. 2*A*, *lane 3*) and/or IKKβ into the TNFR1 complex (Fig. 2*B*, *lane 3*). Furthermore, NS5A could be coimmunoprecipitated with the complex, suggesting that NS5A forms a complex with TNFR1, TRADD, TRAF2, and IKKβ.

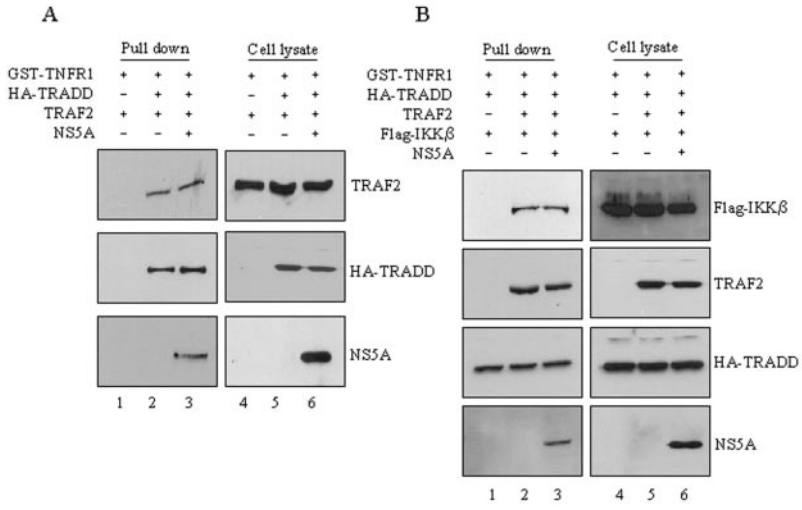

FIG. 2. **Recruitments of TRAF2 and IKKβ to the TNFR1-TRADD complex are not affected by NS5A.** *A*, NS5A-TRAF2 interaction does not block the recruitment of TRAF2 to the TNFR1 complex. HEK293T cells were cotransfected with either GST-tagged TNFR1 and TRAF2 as a negative control or GST-tagged TNFR1, HA-TRADD, and TRAF2 in the absence or presence of NS5A. At 36 h after transfection, cell lysates were pulled down by glutathione beads overnight, and proteins coprecipitated with GST-TNFR1 were detected by immunoblot with TRAF2, HA, and NS5A antibody, respectively (*left panels*). Protein expressions of TRAF2, HA-TRADD, and NS5A in the same cell lysate were verified by immunoblot analysis (*right panels*). *B*, recruitment of IKKβ to the TNFR1 complex is not inhibited by NS5A. HEK293T cells were cotransfected with the indicated combinations of expression plasmids. Cell lysates were pulled down, and coprecipitated proteins were detected as described in *A* (*left panels*). Protein expression of each plasmid in the same cell lysate was confirmed by immunoblot analysis (*right panels*).

NS5A Inhibits TRAF2-mediated but Not MEKK1- and IKKβ-mediated NF-κB Activation—Because NS5A forms a complex with TNFR1, TRADD, TRAF2, and IKKβ, thereby resulting in the inhibition of TNF-α-induced NF-κB activation, we investigated the effects of HCV NS5A expression on NF-κB activation induced by TNFR1 signaling transducers, including TRADD, TRAF2, MEKK1, and IKKβ. When overexpressed, NS5A inhibited TRADD- and TRAF2-mediated NF-κB activation (~35–60% reduction in luciferase activity) (Fig. 3). However, MEKK1- and IKKβ-mediated NF-κB activations were not affected by the overexpression of NS5A. These results suggest that NS5A acts as an inhibitor of TRAF2-mediated NF-κB activation in TNFR1 signaling cascades.

NS5A Interacts with TRAF2 in Vitro and in Vivo—To further determine how NS5A inhibits TRAF2-mediated NF-κB activation, we asked whether NS5A interacts directly with TRAF2 *in vitro* and *in vivo*. We first used a GST pull-down assay using *E. coli*-expressed GST and GST-NS5A fusion protein. Cell extracts containing TRAF2 were incubated with either GST or GST-NS5A beads. The GST-NS5A selectively bound to TRAF2, whereas GST protein failed to retain TRAF2 (Fig. 4*A*). To demonstrate the potential interaction between TRAF2 and NS5A *in vivo*, we performed a coimmunoprecipitation assay. FLAG-tagged TRAF2 was coexpressed with NS5A in Cos7 cells paired with a recombinant vaccinia virus (vTF7-3) system. Cell lysates were immunoprecipitated with either control IgG or anti-FLAG monoclonal antibody, and the coimmunoprecipitated protein was detected by immunoblot assay using anti-NS5A polyclonal antibody. In Fig. 4*B*, it can be seen that NS5A can be coprecipitated by antibody against the FLAG epitope-tagged TRAF2 protein, but not by control antisera. To further confirm this finding, the same cell lysates were immunoprecipitated with anti-NS5A polyclonal antibody, and then bound TRAF2 was detected by immunoblotting with anti-TRAF2 polyclonal antibody (Fig. 4*C*). Taken together, these results clearly demonstrate that NS5A interacts with TRAF2 both *in vitro* and *in vivo*.

Intracellular Colocalization of NS5A with TRAF2—To investigate whether NS5A colocalizes with the exogenous TRAF2, we cotransfected Cos7 cells with plasmids expressing both

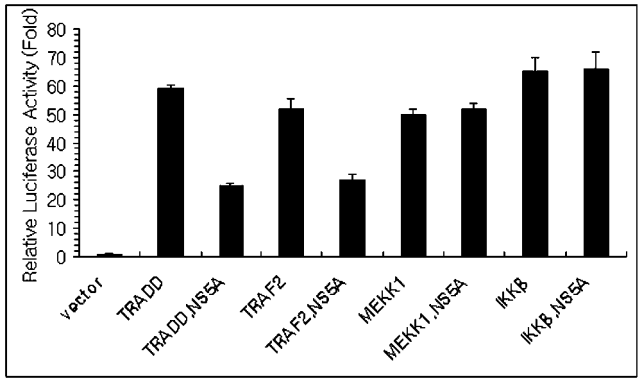

FIG. 3. **TRAF2-induced NF-κB activity is inhibited by NS5A.** The effects of NS5A on NF-κB activity in TNF signaling molecules. HEK293 cells were cotransfected with reporter plasmids and with either TNFR1 signaling molecules (TRADD, TRAF2, MEKK1, and IKKβ) alone or NS5A plasmid. NF-κB reporter gene activity was determined as described in the legend to Fig. 1. The results shown are representative of three independent experiments.

NS5A and FLAG-TRAF2 and examined the subcellular localization. Fig. 5 shows that NS5A was localized exclusively in the cytoplasm. The same cells incubated with FLAG-TRAF2 showed a prominent cytoplasmic localization. Dual staining showed colocalization of NS5A and TRAF2 as yellow fluorescence in the overlay. Moreover, we found that NS5A was also colocalized with endogenous TRAF2 in the cytoplasm (data not shown). Dual staining of the same cells with pre-immune sera showed no detectable staining (data not shown).

The Middle Domain of NS5A Interacts with the TRAF Domain of TRAF2—To delineate a region in NS5A that is required for TRAF2 binding, the interaction of TRAF2 with various truncation mutants of NS5A (Fig. 6*A*) was determined by a transfection-based coprecipitation assay in Cos7 cells infected with the recombinant vaccinia virus system. As shown in Fig. 6*B*, TRAF2 interacted with an N-terminal deletion mutant of NS5A (5A-(148–447)) and a mutant NS5A lacking the C-terminal 146 aa (5A-(1–301)), respectively, suggesting that TRAF2 interacts with NS5A through the middle domain (aa

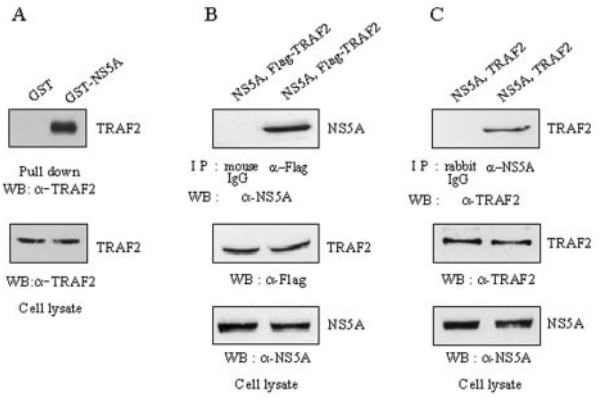

FIG. 4. **The NS5A of hepatitis C virus interacts with TRAF2 protein both *in vitro* and *in vivo*.** *A*, HEK293 cells were transiently transfected with wild type TRAF2 plasmid. Either GST or GST-NS5A purified from *E. coli* was incubated with cell lysate containing TRAF2. Bound proteins were precipitated by glutathione beads and detected by immunoblot using TRAF2 C20 antibody (*top panel*). TRAF2 expression in HEK293 cells was verified by TRAF2 C20 antibody (*bottom panel*). *B*, using recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3), both NS5A and TRAF2 proteins were coexpressed in Cos7 cells. At 12 h after transfection, cell lysates were immunoprecipitated with either control mouse IgG or anti-FLAG monoclonal antibody, and bound proteins were immunoblotted with NS5A polyclonal antibody (*top panel*). Both TRAF2 protein and NS5A were verified using the same cell lysates by immunoblotting with either FLAG monoclonal antibody (*middle panel*) or NS5A polyclonal antibody (*bottom panel*). *C,* reciprocally, cell lysates used in *B* were immunoprecipitated with either rabbit normal sera or NS5A polyclonal antibody, and bound proteins were immunoblotted with TRAF2 polyclonal antibody (*top panel*). Both TRAF2 protein and NS5A were verified using the same cell lysates by immunoblotting with either TRAF2 polyclonal antibody (*middle panel*) or NS5A polyclonal antibody (*bottom panel*). *WB*, Western blot; *IP*, immunoprecipitation.

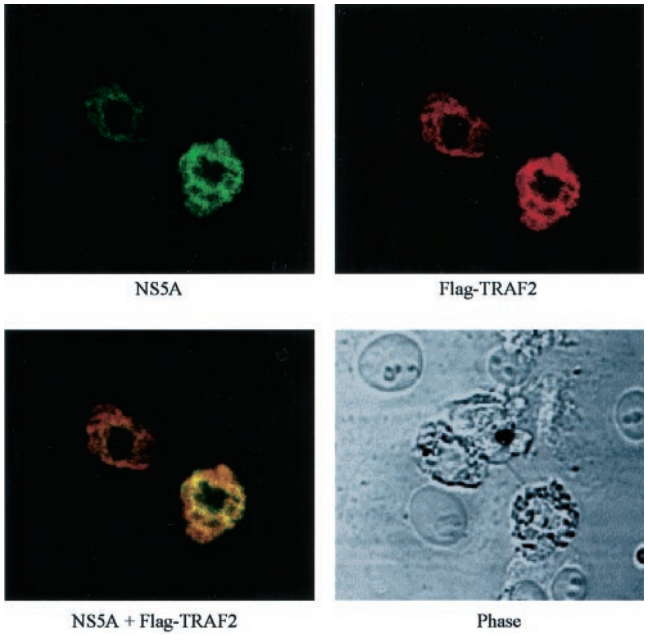

FIG. 5. **Colocalization of NS5A and TRAF2.** Both NS5A and FLAG-TRAF2 plasmids were cotransfected into Cos7 cells. At 30 h after transfection, cells were fixed in 4% formaldehyde, and immunofluorescence staining was performed using a rabbit anti-NS5A antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG to detect NS5A (*green*) and using mouse anti-FLAG antibody and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG to detect FLAG-TRAF2 (*red*). Dual staining showed colocalization of NS5A and TRAF2 as *yellow fluorescence* in the overlay.

148–301). However, the mutant consisting of aa 148–301 interacted with TRAF2 only weakly (data not shown), suggesting that neighboring sequences were required for this interaction.

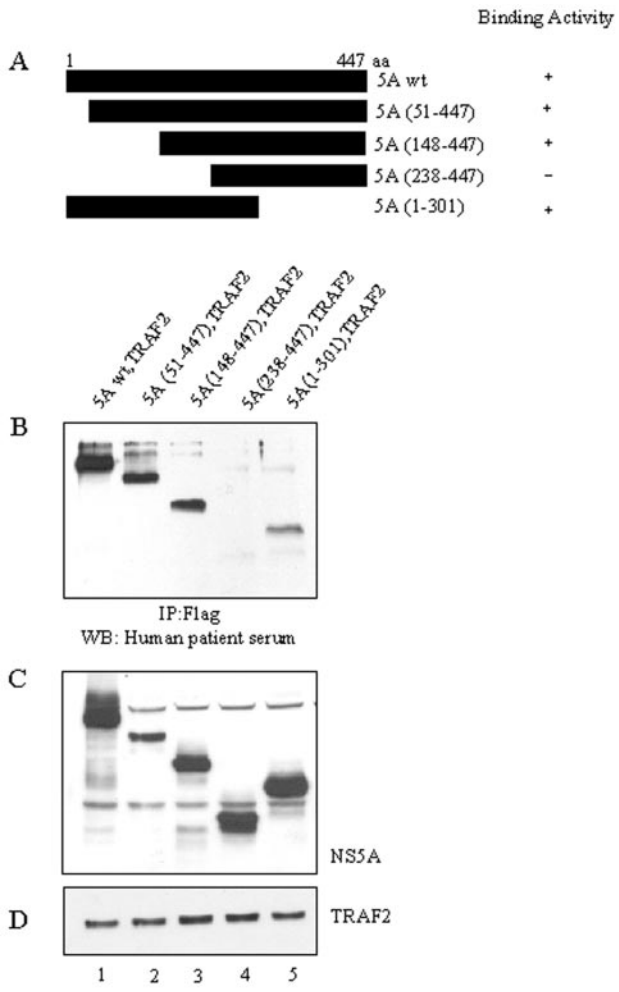

FIG. 6. **The middle domain of NS5A mediates the NS5A-TRAF2 interaction.** *A*, schematic diagram showing both wild type and mutant forms of NS5A. *B*, Cos7 cells were cotransfected with FLAG-tagged TRAF2 and NS5A mutant plasmids paired with recombinant vaccinia virus (vTF7-3). At 12 h after transfection, cell lysates were immunoprecipitated with FLAG-TRAF2 antibody, and bound proteins were detected by immunoblotting with human patient serum. *C* and *D*, cell lysates were subjected to SDS-PAGE and immunoblotted with patient serum (*C*) and anti-FLAG-TRAF2 antibody (*D*).

When NS5A mutants (Fig. 6*A*) including 5A-(148–301) were examined in the NF-κB luciferase assays, the 5A-(238–447) mutant indeed lost the ability to inhibit NF-κB activation (data not shown). Furthermore, those mutants that bound TRAF2 inhibited NF-κB activation, indicating that the inhibition of TNF-α-stimulated NF-κB activation was mediated through the interaction of TRAF2 and NS5A.

TRAF2 consists of the N-terminal RING finger domain, an intermediate region containing five zinc fingers, and a conserved TRAF domain comprised of two tandem TRAF motifs (Fig. 7*A*) (43–45). The TRAF domain is thought to mediate both heterotypic and homotypic protein-protein interactions (46). To determine the structural requirements for the interaction of TRAF2 with NS5A, we generated various mutants expressing truncated TRAF2 proteins either untagged or tagged with FLAG epitope. Coimmunoprecipitation data showed that NS5A interacted with an N-terminal deletion mutant of TRAF2 expressing TRAF domain (TRAF2-(314–501)), but failed to interact with a C-terminal deletion mutant of TRAF2 lacking the TRAF domain (TRAF2-(1–272)), suggesting that the interaction of NS5A with TRAF2 requires the TRAF domain (Fig. 7*B*).

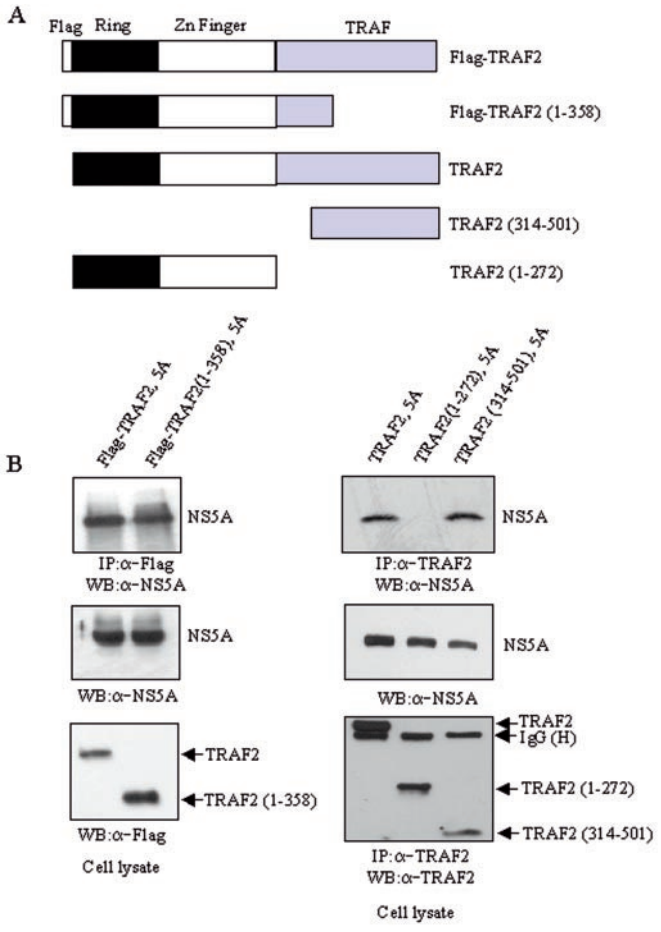

FIG. 7. **The NS5A-TRAF2 interaction requires the TRAF domain of TRAF2.** *A*, schematic diagram showing both wild type and mutant forms of TRAF2. *B*, Cos7 cells were transfected with the indicated combinations of expression plasmids paired with recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3). At 12 h after transfection, cell lysates were immunoprecipitated with either FLAG monoclonal antibody (*top left panel*) or TRAF2 polyclonal antibody (*top right panel*). The bound protein was detected by immunoblotting with rabbit anti-NS5A polyclonal antibody. Both NS5A and TRAF2 proteins were verified using the same cell lysates by immunoblotting with either NS5A polyclonal antibody (*middle panels*), FLAG-TRAF2 monoclonal antibody (*bottom left panel*), or TRAF2 polyclonal antibody (*bottom right panel*).

DISCUSSION

TNF-α is one of the most pleiotropic proinflammatory cytokines signaling a large number of cellular responses, including cytotoxicity, antiviral activity, proliferation, and the transcriptional regulation of various genes (23–25, 30). Such varied outcomes arise from the selective activation of different signal transduction pathways: the caspase cascade, the NF-κB family of transcription factors, and c-Jun N-terminal protein kinase (29, 47). TNF-α signaling events activate TNFR1 to form signaling complex with a number of proteins, one of which is TRADD (42). Moreover, the TNFR1-TRADD complex can recruit receptor-interacting protein, TRAF2, and IKKs, leading to NF-κB activation (28, 29).

In the present study, we provide several lines of evidence supporting a novel role for NS5A of HCV as a negative regulator in the TNF-α signaling cascade. First, overexpression of NS5A inhibited TNF-α-induced NF-κβ activation, as determined by luciferase reporter gene expression, gel shift assay, and IKK kinase assay. Second, NS5A binds to the TNFR1 signaling complex through its interaction with TRAF2. Third, the NS5A-TRAF2 interaction inhibits TRAF2-dependent NF-κB activation. Finally, we showed that the TRAF domain of TRAF2 is required for the interaction of the middle domain of NS5A. Thus, our work suggests a possible mechanism explaining how NS5A inhibits TNF-α-induced NF-κB activation.

To date, six members of the TRAF family have been found (44, 48). TRAF2, TRAF5, and TRAF6 activate NF-κβ by signaling via NF-κB-inducing kinase (NIK) and IKKs (48). All contain a conserved C-terminal TRAF domain that is used for interactions with the cytoplasmic regions of the TNFR superfamily (44). Therefore, it is likely that NS5A interacts with other TRAF family members through a conserved TRAF domain. However, we found that NS5A specifically interacted with TRAF2 but failed to interact with TRAF1, TRAF3, TRAF5, and TRAF6 (data not shown). It must be noted that an N-terminal portion of TRADD specifically interacts with the middle part (aa 400–471) of the TRAF domain in TARF2 (49). Likewise, NS5A exhibits more specificity for the TRAF domain (aa 272–358) close to the coiled-coil domain of TRAF2 (Fig. 7). Because the NS5A binding site in TRAF2 is distinct from the TRADD binding site, NS5A may inhibit NF-κB through the formation of TNFR1-TRADD-TRAF2-NS5A complex.

Recent evidence indicates that NF-κβ activation can block TNF-α-induced apoptosis via induction of antiapoptotic molecules including cellular inhibitor of apoptosis protein (cIAP1), cIAP2, TRAF1, and Bfl (40, 50, 51). Thus, the inhibition of TNF-α-induced NF-κB activation prompts TNF-α-induced apoptosis (52–55). The fact that NS5A inhibits TRAF2-dependent NF-κβ activation suggests that NS5A overexpression also sensitizes the cells to TNF-α-induced cytotoxicity. However, the sensitivity of cells expressing NS5A to TNF-α exposure was not affected (data not shown). It is possible that the recruitment of NS5A in the TNFR1 signaling complex inhibits TRADD-FADD interaction, which is responsible for the induction of cell death. When overexpressed, NS5A severely diminished TRADD-FADD interaction (data not shown). Although TRAF2 and FADD do not compete for binding to TRADD (29), it appears that the recruitment of NS5A in TRADD-TRAF2 complex may provide the structural restraints to inhibit the TRADD-FADD interaction. Thus, NS5A may act as a negative regulator in TNF-α-induced NF-κB activation without the enhancement of cytotoxicity.

Although the molecular mechanisms of HCV persistence and pathogenesis are not yet clear, these processes clearly involve avoidance of the cellular immune responses through the evolution of viral quasispecies (56, 57) and alteration of host signaling pathways by interaction with specific viral proteins (58). Several viruses encode gene products that modulate cellular responses. For example, Tax protein of the human T-cell leukemia virus type I activates NF-κB. Adenovirus E1A protein enhances TNF-α-induced cell death. Similarly, the core protein of HCV inhibits or enhances TNF-α-induced cell death, depending on the cell type. Recently, it has been also reported that NS5A induces oxidative stress and activates signal transducers and activators of transcription 3 and NF-κB via reactive oxygen species in Huh7 cells (59). However, NS5A itself had no effect on TNF-independent NF-κB activation in our system. This discrepancy may be due to differences in the type of cells and the method of detecting NF-κB activation.

We propose here that NS5A is a novel regulator of TNF-α signaling events, specifically inhibiting NF-κB activation through interaction with TRAF2. However, the precise effects of NS5A on the cells likely vary with the conditions of cells because NS5A also exerts a competing effect on the FADD-mediated apoptotic pathway. Because NF-κB regulates a large number of genes involved in inflammatory response, innate immune response, cell proliferation, and apoptosis, the association of NS5A with TRAF2 in the TNFR1 signaling complex

may lead to a detrimental effect on these biological functions. Thus, the down-regulation of TNF-α-induced NF-κB activation by NS5A may confer the ability to escape immune suppression to the virus, leading to persistent infection.

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Nonstructural 5A Protein of Hepatitis C Virus Modulates Tumor Necrosis Factor α -stimulated Nuclear Factor κB Activation

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