The nonstructural 5A (NS5A) protein of hepatitis C virus (HCV) is a phosphoprotein possessing various functions. We have previously reported that the HCV NS5A protein interacts with tumor necrosis factor (TNF) receptor-associated factor (TRAF) domain of TRAF2 (Park, K.-J., Choi, S.-H., Lee, S. Y., Hwang, S. B., and Lai, M. M. C. (2002) J. Biol. Chem. 277, 13122–13128). Both TRAF2 and TRAF2-mediated nuclear factor-κB (NF-κB) activations were inhibited by NS5A-TRAF2 interaction. Because TRAF2 is required for the activation of both NF-κB and c-Jun N-terminal kinase (JNK), we investigated HCV NS5A protein for its potential capacity to modulate TRAF2-mediated JNK activity. Using in vitro kinase assay, we have found that NS5A protein synergistically activated both TNF-α- and TRAF2-mediated JNK in human embryonic kidney 293T cells. Furthermore, synergism of NS5A-mediated JNK activation was inhibited by dominant-negative form of MEK kinase 1. Our in vivo binding data show that NS5A does not inhibit interaction between TNF receptor-associated death domain and TRAF2 protein, indicating that NS5A and TRAF2 may form a ternary complex with TNF receptor-associated death domain. These results indicate that HCV NS5A protein modulates TNF signaling of the host cells and may play a role in HCV pathogenesis.

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis worldwide (1, 2), which often leads to cirrhosis and an increased risk of hepatocellular carcinoma (3–5). HCV is a single-stranded, positive-sense RNA virus belonging to 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 to generate putative structural proteins (core, E1, and E2/p7) and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (7–10). The nonstructural protein 5A (NS5A) is a phosphoprotein consisting of 447 amino acid residues. NS5A exists in two forms of polypeptide, p56 and p58, which are phosphorylated mainly at serine residues both in vivo and in vitro (11). NS5A protein is localized exclusively in the cytoplasm (12). Both NS5A and NS5B (RNA-dependent RNA polymerase) bind to human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33) (13), suggesting that NS5A may form a part of the HCV RNA replication complex. NS5A of HCV genotypes 1a and 1b interacts with the interferon-inducible double-stranded RNA-activated protein kinase and inhibits its activity (14). Because RNA-activated protein kinase is responsible for the inhibition of cellular and viral growth by phosphorylating eIF2α, the down-regulation of RNA-activated protein kinase by NS5A may be one mechanism of viral resistance against the antiviral action of interferon. In addition, NS5A binds to the growth factor receptor-bound protein 2 (Grb2) adapter protein in vivo and perturbs Grb2-mediated signaling pathways by selectively inhibiting phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (15). Furthermore, NS5A regulates cellular growth by interacting with cellular transcription factor Snf2-related CBP activator protein (16) and modulates cellular activities by binding with karyopherin β3 protein (17).

Tumor necrosis factor-α (TNF-α) is a potent cytokine produced by many cell types in response to inflammation, infection, and other environmental stimuli (18). TNF-α elicits diverse cellular responses, including lymphocyte and leukocyte activation, cell proliferation, differentiation, and apoptosis (19, 20). These processes are mainly mediated by either TNFR1 or TNFR2, both of which belong to the TNF receptor superfamily (21, 22). The binding of TNF-α to TNFR1 and TNFR2 induces receptor trimerization and recruitment of TNFR1-associated death domain protein (TRADD). TRADD then recruits three additional mediators: receptor-interacting protein 1, Fas-associated death domain protein (FADD), and TNF-receptor-associated factor 2 (TRAF2). TRAF2 plays a central role in early events that leads to NF-κB and mitogen-activated protein kinase (JNK and p38) activation (23, 24). Overexpression of TRAF2 is sufficient to activate NF-κB and AP-1 (25, 26). JNKs, also known as stress-activated protein kinases, belong to the family of mitogen-activated protein kinases, which are regul-
Fig. 1. Activation of TNF-α-induced JNK activity by HCV NS5A protein. A, NS5A protein potentiates TNF-α-induced JNK activation in cells transiently expressing HCV NS5A protein. 293T cells were transfected with either pcDNA3 vector (lanes 1 and 3) or plasmid expressing HCV NS5A protein (lanes 2 and 4). At 24 h after transfection, cells were either untreated (lanes 1 and 2) or treated with 10 ng/ml human TNF for 15 min (lanes 3 and 4). Using cell lysates, JNK activity was determined (top panel) as described under “Experimental Procedures.” Both HCV NS5A...
lated by phosphorylation of threonine and tyrosine residues. JNK is activated by MEKs, stress-activated protein kinase/ERK kinase-1 (SEK1), also called MKK4 and MKK7 (27, 28). Mitogen-activated protein kinase kinase kinase (MEKK1) (also known as JNK kinase kinase) can activate the JNK upstream regulators SEK1/MKK4 and MKK7 (29). In this study, the effects of HCV NS5A protein on JNK activation induced by TNF and TRAF2 were investigated. NS5A protein synergistically activated JNK by interacting with TRAF2. Our results indicate that NS5A acts as a positive regulator of TNF-α-mediated JNK signaling pathway that may play a key role in HCV pathogenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—HCV NS5A, HA-TRADD, TRAF2, and FLAG-TRAF2 expression vectors were described previously (30, 31). The active form of MEKK (pFC-MEKK) expression vector was purchased from Stratagene (La Jolla, CA). Dominant negative mutant form of MEKK1 (EECMV/MEKK1-KR) was kindly provided by Dr. D. J. Templeton (Case Western Reserve University).

**Cell Culture and Transfection Experiment**—HEK 293T 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). Approximately 1 × 10⁶ cells plated on 60-mm dishes or 2 × 10⁶ cells plated on 100-mm dishes were transfected with 4–6 μg of DNA using LipofectAMINE (Invitrogen) as described previously (32). To make stable cells, human embryonic kidney (HEK) 293 cells were transfected with pcDEF-NS5A plasmid. Stable cell clones were selected in the presence of 0.5 mg/ml G418 (Invitrogen) in culture medium. 3–4 weeks after transfection, positive clones were selected by immunoblot analysis using sera from HCV patients. Stable cells transfected with empty vector were also selected as described above.

**In Vivo Binding Assay**—HEK 293T cells were cotransfected with DNA on 60-mm dishes. At 36 h after transfection, cells were harvested and lysed in buffer containing 1% Nonidet P-40, 250 mM NaCl, 50 mM HEPES, pH 7.6, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were immunoprecipitated with anti-HA antibody (Santa Cruz Biotechnology) for 1.5 h and further incubated with protein A beads (Zymed Laboratories Inc.) for 1 h. After five washes with the cell lysis buffer, the bound proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Proteins were detected by immunoblot analysis using either anti-FLAG monoclonal antibody (Sigma) or anti-NS5A polyclonal antibody (31) or anti-TRAF2 polyclonal antibody (Santa Cruz Biotechnology).

**c-Jun N-terminal Kinase Assay**—For the JNK kinase assay, subconfluent HEK 293T cells were incubated for 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and then stimulated with 10 ng/ml TNF-α for 15 min. Total cell lysates were prepared as described previously (30), and JNK activity was determined using a JNK assay kit according to the manufacturer’s instruction (New Eng-

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**Fig. 2.** TRAF2-mediated JNK activation is synergistically elevated by NS5A protein. A, 293T cells were transfected with either vector, NS5A and TRAF2, individually or NS5A and TRAF2 together. The amount of total DNA transfected in each lane was adjusted to 3 μg with an empty vector DNA: lane 1, 3 μg of vector; lane 2, 2 μg of NS5A and 1 μg of vector; lane 3, 1 μg of TRAF2 and 2 μg of vector; and lane 4, 2 μg of NS5A and 1 μg of TRAF2. JNK activity was determined using glutathione S-transferase-c-Jun (1–89) as a substrate (top panel). B, HEK 293 cells stably expressing either NS5A (lanes 2 and 4) or vector (lanes 1 and 3) were transfected with TRAF2, and JNK activity was assayed. Protein levels of HCV NS5A, TRAF2, and β-actin in the same cell lysates were determined by immunoblotting.

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protein (middle panel) and β-actin protein (bottom panel) levels were determined by immunoblotting. **B** and **C**, NS5A protein potentiates TNF-α-induced JNK activation in stable cells expressing HCV NS5A protein. Stable cells expressing either HCV NS5A (lanes 2 and 4) or vector alone (lanes 1 and 3) were either untreated (lanes 1 and 2) or treated with TNF (lanes 3 and 4), and JNK activity was assayed (top panels). Both HCV NS5A protein (middle panels) and β-actin protein (bottom panels) levels were determined by immunoblotting. **B**, HEK 293 stable cells. **C**, HepG2 stable cells. **D**, endogenous levels of c-Jun were determined in five individual clones of stable cells expressing either vector (lanes 1–5) or HCV NS5A (lanes 6–10). Protein levels of HCV NS5A (top panel), c-Jun (middle panel), and β-actin (bottom panel) in the same cell lysates were determined by immunoblotting. Fold of c-Jun level was calculated based on protein levels of actin. The arrowhead indicates a nonspecific band. **E**, TNF-α-induced AP-1 DNA binding activity in control and NS5A expressing cells. **F**, HEK 293T cells transfected with either vector or NS5A plasmid DNA were cotransfected with TNF-α (20 ng/ml) for the indicated times. Nuclear extracts were assessed for AP-1 DNA binding activity by electrophoretic mobility shift assay as described under “Experimental Procedures.”
HCV NS5A Modulates JNK

**RESULTS**

**NS5A Protein Potentiates TNF-α-induced JNK/STRESS-activated Protein Kinase Activation in HEK 293T Cells**—Previous studies have shown that TNF-α induces activation of both transcription factor NF-κB (34) and the JNK (23, 35) through TNFR1. To investigate whether NS5A can affect TNF-α-mediated JNK activation, HEK 293T cells were transfected with either an empty vector or NS5A expression plasmid. At 24 h after transfection, cells were either untreated or treated with human TNF-α (10 ng/ml) for 15 min and then JNK activity was analyzed by *in vitro* kinase assay using glutathione S-transferase-c-Jun-(1–89) fusion protein as a substrate. NS5A protein itself does not induce JNK activation (Fig. 1A, lane 2). However, TNF-α-stimulated JNK activation was significantly increased by HCV NS5A protein (Fig. 1A, lane 3 versus lane 4). To further substantiate this finding, we performed a similar experiment using stable cells. Stable cells transfected with vector alone activated JNK slightly with TNF treatment (Fig. 1B, lane 3). However, NS5A in stable cells significantly activated JNK with TNF treatment (Fig. 1B, lane 3 versus lane 4).

**Electrophoretic Mobility Shift Assay**—HEK 293 cells stably expressing NS5A or empty vector were plated at a density of 5 × 10⁴ in 60-mm dishes. At 36 h after plating, stable cells were stimulated with TNF-α (20 ng/ml) for the indicated times and then nuclear extracts were prepared as described previously (31). For the transient transfection experiment, HEK 293T cells were transfected with either vector or NS5A encoding DNA by using LipofectAMINE (Invitrogen) and treated with TNF-α for the indicated times and nuclear extracts were prepared as described above. Protein concentrations were determined using the method of Bradford (Bio-Rad). Nuclear extracts (10 μg) were assayed for AP-1 DNA binding activity in the electrophoretic mobility shift assay by incubation with 1 × 10⁵ cpm of a [³²P]-end-labeled 21-mer double-stranded AP-1 oligonucleotide (Promega) in binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.25 mg/ml poly(C·C)) for 20 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 20 min before the addition of radiolabeled oligonucleotide.

**MTT Assay**—Approximately 2 × 10⁵ cells stably expressing either NS5A or empty vector were plated on 6-well plates and incubated for 24 h. Cells were treated with 10 ng/ml human TNF (Invitrogen) and 1 μg/ml actinomycin D for 20 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 determined as described previously (33).

**Western Blot Analysis of Poly(ADP-ribose) Polymerase (PARP)**—HEK 293 cells stably expressing NS5A were treated with 20 ng/ml TNF-α and 500 ng/ml actinomycin D (ActD) for 16 h. Cells were washed twice with cold phosphate-buffered saline, treated with lysis buffer (50 mM Tris-HCl, pH 6.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylnitrile fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate) for 10 min on ice, and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was collected, and the protein concentration was determined using the Bradford (Bio-Rad). The same amount of protein from each lysates was subjected to electrophoresis on 5% native polyacrylamide gels using 0.25× Tris borate EDTA buffer and detected by autoradiography.
cells (Fig. 1C). Because the protein expression level of NS5A was not affected by TNF (Fig. 1, B and C, lanes 2 and 4), JNK activation was not the result of overexpression of NS5A protein. To test whether the endogenous protein expression level of c-Jun is increased in a certain clone of stable cells, we determined the c-Jun levels in five individual cell lines of stable cells expressing either NS5A or vector. We found that all of the individual cell lines show the similar levels of c-Jun protein (Fig. 1D). This result indicated that NS5A protein potentiates TNF-α-induced JNK activation. To examine whether c-Jun phosphorylation is necessarily leading to activity, electrophoretic mobility shift assay was performed using AP-1 sequences. In a time course experiment, AP-1 was activated at 30 min after TNF treatment and reached a maximum by 90 min in the vector control cells (Fig. 1E). The disappearance of AP-1 binding using an unlabeled probe further suggested the specificity of AP-1 activation. On the other hand, AP-1 was activated in the presence of the NS5A protein prior to exposure to TNF. Ap-1 DNA binding activity was minimally enhanced by the prolonged exposure to TNF. These data suggest that cells expressing NS5A have increased AP-1 activity compared with control cells. In a reporter gene assay, we found that NS5A itself could activate AP-1 activity and TNF-α-induced AP-1 activity was synergistically activated by NS5A protein (data not shown). These results indicate that NS5A protein acts as a positive regulator of TNF-α-induced JNK signaling pathway.

**TRAF2-induced JNK Activity Is Synergistically Elevated by HCV NS5A Protein**—TRAF2 is required for the activation of both transcription factor NF-κB and JNK through TNFR1 (24, 36). Since NS5A protein directly interacts with the TRAF domain of TRAF2 (31), we have examined whether this interaction could affect TRAF2-mediated JNK activation. For this purpose, we transfected HEK 293T cells with either an empty vector, NS5A, TRAF2 individually or NS5A and TRAF2 together. As reported previously (25, 37), TRAF2 is an efficient activator of JNK (Fig. 2A, lane 3). Furthermore, TRAF2-mediated JNK activation was synergistically activated by NS5A (Fig. 2A, lane 4). To confirm this finding, we performed a similar experiment using stable cells. HEK 293 cells stably expressing either NS5A (Fig. 2B, lanes 2 and 4) or vector (Fig. 2B, lanes 1 and 3) were transfected with TRAF2 and JNK activity was determined. Likewise, TRAF2 alone activated JNK (Fig. 2B, lane 3), and TRAF2-stimulated JNK activity was synergistically activated by the HCV NS5A protein in stable cells (Fig. 2B, lane 4).

**TRADD-TRAF2 Interaction Was Not Inhibited by HCV NS5A Protein**—To further investigate how NS5A can activate TRAF2-mediated JNK, we examined whether NS5A-TRAF2 binding might interfere with the interaction between TRADD and TRAF2. To demonstrate this, we cotransfected 293T cells with HA-TRADD and FLAG-TRAF2 in the absence or presence of NS5A. At 36 h after transfection, cell lysates were immunoprecipitated with either HA mouse antibody or control mouse IgG and coprecipitated proteins were detected by immunoblot assay. As shown in Fig. 3, the protein level of TRAF2 was not affected by the expression of NS5A protein, indicating that NS5A did not inhibit TRADD-TRAF2 interaction. This result further suggests that NS5A protein may form a ternary complex with TRADD and TRAF2.

**MEKK1-mediated JNK Activation Was Not Affected by NS5A Protein**—MEKK1 preferentially activates the JNK pathway (38, 39), and JNK activation via TNFR1 involves TRADD/TRAF2/MEKK1/SEK1 (29). To verify whether MEKK1-mediated JNK activation is affected by NS5A, we used a constitutively active form of MEKK1 (amino acids 360–672) whose expression is driven by a cytomegalovirus promoter. For this purpose, we transfected 293T cells with either an empty vector, NS5A or MEKK1, individually or NS5A and MEKK1 together. As expected, JNK was prominently activated by MEKK1 (Fig. 4, lane 3). Meanwhile, MEKK1-induced JNK activation was not further elevated by NS5A (Fig. 4, lane 4), indicating that JNK activation was mainly mediated by NS5A-TRAF2 interaction.

**NS5A-TRAF2-mediated JNK Activation Was Inhibited by Dominant-negative Mutant of MEKK1**—It has been also reported that dominant-negative form of MEKK1 (DNMEKK1) could inhibit TNF-mediated JNK activation in many cell types (38). To investigate whether JNK activation mediated by TRAF2 and NS5A could be affected by DNMEKK1, 293T cells were transfected with an empty vector, TRAF2, individually or cotransfected with TRAF2 and DNMEKK1 or NS5A and TRAF2 or NS5A, TRAF2, and DNMEKK1. It is clear that DNMEKK1 inhibits TRAF2-mediated JNK activation (Fig. 5, lanes 2 and 3, top panel). As shown previously (Fig. 2, lane 4), JNK activity was synergistically elevated by NS5A-TRAF2 interaction (Fig. 5, lane 4, top panel). However, synergism of JNK activation mediated by NS5A was significantly inhibited by DNMEKK1 (Fig. 5, lane 5, top panel). This result further shows that HCV NS5A protein potentiates JNK activation by interacting with TRAF2 adapter protein.

**NS5A Overexpression Inhibits TRADD-FADD Interaction**—Since one of the consequences of JNK activation is the induc-
tion of apoptosis, the activation of JNK may promote TNF-α-induced cell death. This led us to examine the effects of NS5A overexpression on TNF-α-induced cell death. To test this possibility, apoptosis was induced in HEK 293 cells by treatment with TNF and ActD for 16 h. As shown in Fig. 6A, coadministration of TNF-α and ActD to HEK 293 cells induced apoptosis as determined by the cleavage of PARP. However, PARP degradation was not altered by NS5A protein, indicating that these cells were not protected by NS5A from apoptosis. To confirm this result, MTT assay was performed using cells treated with both TNF and ActD. HEK 293 cells were transfected with either CMV-β-galactosidase and empty vector or CMV-β-galactosidase and NS5A. Following TNF-α and ActD treatment, cells were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopiranoside) and positive blue cells were counted. Similarly, we found that NS5A overexpression had no effect on TNF-α-induced cell death (data not shown). To investigate the cause of this phenomenon, we examined the effects of NS5A on the recruitment of FADD to the TNFR1-TRADD complex. As shown in Fig. 6B, FADD is truly being precipitated by the FLAG antibody (bottom left panel) and TRADD is also coprecipitated by the FADD protein (middle left panel). However, there is no direct interaction between NS5A and FADD (Fig. 6B, top left panel). Overexpressed NS5A protein significantly inhibited TRADD-FADD interaction (Fig. 6C, top left panel), thereby defining a mechanism to explain why JNK activation by NS5A cannot prompt TNF-α-induced cell death.

**DISCUSSION**

TNF plays an important role in diverse cellular events such as septic shock, induction of other cytokines, cell proliferation, differentiation, and cell death (19, 20). These responses are elicited by TNF-induced trimerization of two distinct cell surface receptors, TNFR1 (p55) and TNFR2 (p75). In 293 cells, TNFR1 is constitutively expressed, whereas endogenous TNFR2 is barely detected (26, 40). TNF binding to the TNFR1

![Fig. 6. Overexpression of NS5A inhibits TRADD-FADD interaction. A. NS5A protein does not affect on TNF- and actinomycin-induced apoptosis. HEK 293 cells stably expressing NS5A were treated with TNF and ActD for 16 h. Apoptosis was determined by immunoblotting cellular proteins with anti-PARP monoclonal antibody (top panel) as described under “Experimental Procedures.” Both NS5A protein (middle panel) and β-actin protein (bottom panel) levels were determined by immunoblotting. B. NS5A protein does not interact with FADD. COS-7 cells were transfected with the indicated combinations of expression plasmids paired with recombinant vaccinia virus (vTF7-3). At 12 h after transfection, cell lysates were immunoprecipitated with FLAG monoclonal antibody (top left panel), anti-HA antibody (middle left panel), or FADD antibody (bottom left panel). Protein expression level of each plasmid was verified using the same cell lysates by immunoblotting with rabbit anti-NS5A polyclonal antibody (top right panel), anti-HA antibody (middle right panel), or FADD antibody (bottom right panel). C. NS5A protein inhibits TRADD-FADD interaction. HEK 293T cells were cotransfected with HA-TRADD, TRAF2, and FLAG-FADD in the absence or presence of NS5A as indicated. At 36 h after transfection, cell lysates were immunoprecipitated with HA antibody and proteins coprecipitated with HA-TRADD were detected by immunoblotting with FLAG, TRAF2, and NS5A antibody, respectively (left panels). Protein expression levels of each plasmid in the same cell lysates were determined by immunoblot analysis (right panels).](http://www.jbc.org/content/early/2018/07/17/jbc.M117007201/Fig6)
promotes receptor trimmerization and recruitment of the TRADD (40, 41). TRADD also stimulates TRAF2 to be associated with TNFR1 complex (37). TRAF2 mediates rapid activation of NF-κB, JNK, and p38 kinase in response to TNF (25, 36, 37). We have previously demonstrated that NS5A inhibits TNF-α-induced NF-κB activation by interacting with TRAF2 (31). We found that NS5A protein specifically interacted with TRAF domain (amino acids 272–358) close to coiled-coiled domain of TRAF2 (31). However, NS5A binding site in TRAF2 was distinct from the TRADD binding site. Overexpression of NS5A protein hence did not inhibit the recruitment of TRAF2 into TNFR1/TRADD complex. In this study, we found that HCV NS5A protein did not inhibit the recruitment of TRAF2 to TRADD-FADD interaction. Nevertheless, cells expressed NS5A protein functions as a multifunctional protein. HCV, like TNF-induced JNK activation is not linked to TNF-mediated JNK activation, whereas TNF-α-induced NF-κB activation was inhibited by NS5A. It is not clear as to why NS5A shows pleiotropic responses to signaling pathways during viral infection.

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