Hepatitis C Virus NS5A and Subgenomic Replicon Activate NF-κB via Tyrosine Phosphorylation of IκBα and Its Degradation by Calpain Protease*

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This article has been withdrawn by the authors. Antonia Livolsi, Véronique Imbert, and Jean-François Peyron participated in the study by providing IκBα mutant plasmids. An investigation by the Journal determined the following. A band was pasted over in the NS5A immunoblot in Fig. 2A. The actin immunoblot from Fig. 2A was reused as actin in 2B, NS5A in 4A, NS5A in 4B, actin in 7D, and actin in 9A. Additionally, this actin immunoblot was reused in the following publications representing different experimental conditions: Waris et al. (2005) J. Virol. 79, 1569-1580, Waris and Siddiqui (2005) J. Virol. 79, 9725-9734, Waris et al. (2007) J. Virol. 81, 8122-8130, Nasimuzzaman et al. (2007) J. Virol. 81, 10249-10257, Burdette et al. (2010) J. Gen. Virol. 91, 681-690, Burdette et al. (2012) J. Gen. Virol. 93, 235-246, Presser et al. (2013) PLOS One 8, e56367, and McRae et al. (2016) J. Biol. Chem. 291, 3254-3267. The EMSA shown in Fig. 3D was reused in Waris et al. (2005) J. Virol. 79, 1569-1580 representing different experimental conditions. Lanes 3 and 5 of the IκBα immunoblot in Fig. 9A were duplicated. The authors who performed the experiments were unable to clarify the Journal’s concerns with original data. Gulam Waris does not agree that the actin immunoblot was reused in other publications. Aleem Siddiqui disagrees with the determination of the reuse of actin immunoblots in publications other than the JBC paper being withdrawn. The authors sincerely apologize to the readers.
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FIG. 1. Organization of the HCV RNA genome. A, the 5'- and 3'-non-coding regions (NCRs) are drawn with the structural and non-structural proteins located in the N-terminal portion of the polyprotein, and the remainder encoding the non-structural (NS) proteins (NS2-NS5B). B, organization of the HCV subgenomic replicon. The HCV 5'-NCR fused to a small portion of the neomycin phosphotransferase gene (Neo'). These sequences are followed by the EMCV IRES, and the HCV 3'-NCR, terminating at the 3'-NCR (9).

MATERIALS AND METHODS

Plasmids and Oligonucleotides—Plasmid p8x-kB-Luc (luciferase reporter driven by the minimal fos promoter with three upstream NF-κB binding sites from MHC class I) was a generous gift of J. Martin (University of Colorado, Boulder). The HCV NS5A expression vector (pCNS5A) and its mutant (pCNSM4) were generated from pCMV 729/(University of Colorado, Boulder). The HCV NS5A expression vector (pCNSM4) and its mutant (pCNSM4) were generated from pCMV 729/NS3/NS4A/NS4B/NS5A/NS5B were harvested and cell extracts were prepared with 50 units/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 μg/ml b-mercaptoethanol, and 10% fetal calf serum.

Preparation of Nuclear Extracts—Nuclear lysates were prepared from untransfected and Hu7 cells transfected with 2–3 μg of indicated plasmid DNA by Lipofectin (Invitrogen) followed by treatment with PDTC (100 μM) for 4 h, piceatannol (150 μM) for 30 min, herbimycin A (2 μM) for 12 h, genistein (74 μM) for 2 h, AG490 (20 μM) for 2 h, H7 (25 μM) for 2 h, BAIPA-AM (50 μM) for 2 h, ruthenium red (30 μM) for 45 min, Ru 360 (50 μM) for 45 min, BAY 11–7085 (10 μM) for 1 h, MG132 (20 μM) for 12 h, ALLM (100 μM) for 24 h, and E64-D (50 μM) for 45 min before harvesting the cells at 42 h post-transfection. Cells were lysed in hypotonic buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM Na2VO4, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 3 mg/ml aprotinin, 1 mg/ml pepstatin, 20 mM NaF, and 1 mM dithiothreitol with 0.2% Nonidet P-40) on ice for 10 min. After centrifugation at 4°C (15,000 rpm) for 1 min, the nuclear pellet was resuspended in high salt buffer (hypotonic buffer with 20% glycerol and 420 mM NaCl) at 4°C by rocking for 30 min following centrifugation. The supernatant was collected and stored in aliquots at ~80°C.

EMSA—The oligonucleotide NF-κB was radiolabeled at 5' end with [γ-32P]ATP by T4 polynucleotide kinase. About 20,000 cpm of gel purified probe was incubated with nuclear lysates from FCA4 cells and Hu7 cells untransfected or transfected with pCNS5A and pCMV729/3010 expression vectors respectively in EMSA buffer (20 mM Tris-HCI, pH 7.9, 10 mM MgCl2, 50 mM KCl, 16.7 μg/ml poly dIdC, 1 mM EDTA, 1 mM dithiothreitol, and 1 μM leupeptin) for 20 min on ice. Competition analyses were carried out in the presence of a 100-fold excess of an unlabeled competitor oligonucleotide that was preincubated for 20 min on ice prior to the addition of radiolabeled probe. The DNA-protein complexes were resolved by 5% polyacrylamide gel electrophoresis in 0.5x TBE buffer. The gels were dried and subjected to autoradiography.

Immunoprecipitation and Western Blot Analysis—Exponentially growing FCA4 and Hu7 cells transfected with pCNS5A or pCMV729/3010 expression vectors were harvested and cell extracts were prepared...
**Fig. 2.** HCV replicon and NS5A protein induce tyrosine phosphorylation of IκBα.

A) Western blot analysis of unphosphorylated and phosphorylated IκBα in NS5A expressing cells. Lane 1, untreated lysates; lane 2, NS5A transfected lysates; lane 3, transfected with pCMV729/3010 and NS5A expression vector respectively; lane 4, transfected with pCMV729/3010 and mutant (NSM4) and NS5B expression vectors respectively; lane 5, transfected with pCMV729/3010 and calcium chelator, BAPTA-AM (50 μM) for 4 h, and treated with H2O2 for 2 h. Lane 6, NS5B transfected cells treated with H2O2 for 2 h. Lane 7, transfected with pCMV729/3010 and NS5B expression vectors respectively; lane 8, transfected with pCMV729/3010 and NS5B expression vectors respectively.

B) Western blot analysis of unphosphorylated and phosphorylated IκBα in NS5A expressing cells. Lane 1, untreated lysates; lane 2, NS5A transfected lysates. Immunoblots were probed with monoclonal anti-phosphotyrosine monoclonal antibody. This analysis shows that the cells expressing NS5A alone or in the context of all nonstructural proteins, and subgenomic replicon contained p-tyrosine-IκBα gene under the control of SV40 promoter/enhancer to serve as an internal control and used to adjust the variations during transient transfections. The expression of p-galactosidase activity was measured using the 0-nitrophenyl-β-D-galactosidase (ONPG) as a substrate (Stratagene kit). The tyrosine kinase inhibitors (piceatanol, genistein, herbimycin A, AG490), calcium chelator (BAPTA-AM, TMB-8, RR, Ru360), IKK inhibitor (BAY 11-7085), proteasomal inhibitor (MG 132), and calpain inhibitors (ALLM and E64D) were added at various times before cells were harvested for luciferase activity.

C) Western blot analysis of unphosphorylated and phosphorylated IκBα in NS5A expressing cells. Lane 1, untreated lysates; lane 2, NS5A transfected lysates. Immunoblots were probed with monoclonal anti-phosphotyrosine monoclonal antibody. This analysis shows that the cells expressing NS5A alone or in the context of all nonstructural proteins, and subgenomic replicon contained p-tyrosine-IκBα gene under the control of SV40 promoter/enhancer to serve as an internal control and used to adjust the variations during transient transfections. The expression of p-galactosidase activity was measured using the 0-nitrophenyl-β-D-galactosidase (ONPG) as a substrate (Stratagene kit). The tyrosine kinase inhibitors (piceatanol, genistein, herbimycin A, AG490), calcium chelator (BAPTA-AM, TMB-8, RR, Ru360), IKK inhibitor (BAY 11-7085), proteasomal inhibitor (MG 132), and calpain inhibitors (ALLM and E64D) were added at various times before cells were harvested for luciferase activity.

**RESULTS**

NS5A Induces Tyrosine Phosphorylation of IκBα—To initiate these studies, we sought to determine the ability of NS5A alone or in the context of all the HCV nonstructural proteins (NS) to induce the tyrosine phosphorylation of IκBα. Cellular lysates from cells expressing NS5A, all the NS proteins (pCMV729/3010) and subgenomic replicon lysates from cells expressing NS5A, all NS proteins (pCMV729/3010) and subgenomic replicon were immunoprecipitated with anti-IκBα monoclonal antibody. This analysis shows that the cells expressing NS5A alone or in the context of all nonstructural proteins, and subgenomic replicon contained...
tyrosine-phosphorylated IκBα (Fig. 2A, lanes 2–4). Neither untransfected (Fig. 2A, lane 1), nor the NS5A deletion mutant, which does not target to the ER (22) (Fig. 2A, lane 5) and nor the NS5B (RNA-dependent RNA polymerase)-expressing cells (Fig. 2A, lane 6) induced tyrosine phosphorylation of IκBα. The involvement of ROS and Ca^{2+} signaling in the IκBα tyrosine phosphorylation induced by HCV NS5A was further evaluated by using antioxidant PDTC and calcium chelator BAPTA-AM. NS5A expressing cells treated with these reagents effectively inhibited tyrosine phosphorylation of IκBα (Fig. 2A, lanes 7 and 8). H2O2 has been previously shown to induce tyrosine phosphorylation of IκBα in immune cells (31). Here we reproduce this effect in Huh7 cells. H2O2-treated Huh7 lysates were immunoprecipitated with anti-IκBα serum and immunoblotted with anti-phosphotyrosine antibody. The results described in (Fig. 2B, lanes 1 and 2) show that H2O2 was able to induce tyrosine phosphorylation of IκBα. To confirm the identity of phosphorylated IκBα, lysates from Huh7 cells expressing NS5A were also immunoblotted with anti-IκBα serum. The NS5A expressing cells contain both the phosphorylated and the unphosphorylated forms of IκBα (Fig. 2C, lane 2) whereas untransfected Huh7 cells show the presence of only the unphosphorylated band (lane 1). These observations together demonstrate that NS5A and HCV subgenomic replicons are capable of constitutively phosphorylating IκBα at tyrosine residues in the absence of a cytokine.

Tyrosine Kinase-mediated Activation of NF-κB—To determine whether protein-tyrosine kinase (s) (PTK) is involved in the NF-κB activation induced by NS5A and HCV replicon, several known inhibitors of PTK were employed in the EMSA. A distinct DNA-protein complex was formed between NF-κB p65 subunit and its cognate oligonucleotide in NS5A expressing cells (Fig. 3A, lanes 2) which was abrogated in the presence of PTK inhibitors (Fig. 3A, lanes 4–6). However, BAY11–7085, an inhibitor of IKK did not affect the appearance of the NF-κB DNA-protein complex (Fig. 3A, lane 7). The specificity of DNA-protein interaction was confirmed by supershift of DNA-protein complex in the presence of an antibody to the NF-κB subunit p65 (Fig. 3B, lane 3) and using unlabeled competitor oligonucleotides representing the NF-κB sequences (Fig. 3B, lane 4). We observed similar inhibition of DNA-protein complexes by PTK inhibitors in HCV subgenomic replicon expressing cells that were either stably (FCA4) or transiently transfected with BM4–5 RNA (Fig. 3C, lanes 3 and 4; D, lanes 4–6). Huh7 cellular lysates subjected to EMSA produced a modest level of NF-κB protein-DNA interaction (Fig. 3A, lane 2; B, lane 1; C, lane 1; D, lane 2;
FIG. 4. Protein tyrosine kinase inhibitors inhibit NF-κB controlled luciferase activity. Huh7 cells were cotransfected with NF-κB luciferase plasmid along with (A) pCNSS5A (B) pCMV728/3010 expression vectors. Huh7 cells were also transfected with NF-κB luciferase plasmid along with pRC/CMV parental vector used as a control. Transfected cells were treated with piceatannol (150 μM), herbimycin A (2 μM), AG490 (20 μM).
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Fig. 5. HCV NS5A and NS proteins expression did not induce NF-κB activation in ZAP-70 knock-out Jurkat cells. A. EMSA was carried out in the presence of γ-[32P]-labeled NF-κB cognate nucleotide probe and the nuclear lysates prepared from Jurkat cells and ZAP-70 knock-out Jurkat cells. Lane 1, probe alone; lanes 2 and 5, equal amounts of nuclear lysates from untransfected Jurkat cells (JE6) and ZAP-70 deficient Jurkat cells (P116) respectively; lanes 3 and 6, JE6 and P116 cells transfected with pCNS5A; lanes 4 and 7, JE6 and P116 cells transfected with pCMV 729/3010 respectively. B, lanes 1 and 2, equal amounts of untransfected and NS5A transfected JE6 cells nuclear lysates; lane 3, DNA-protein complex formed in lane 2 incubated with anti-NF-κB p65 subunit; lane 4 is same as lane 2 but treated with 100-fold excess of unlabeled NF-κB oligonucleotide. C, Huh7 lysates were analyzed by Western blot assay using anti-ZAP-70 antibody.

Fig. 6. Treatment of untransfected Huh7 nuclear lysates with PTK inhibitors did not seem to have any dramatic effect on the intensity of the DNA-protein complex (Fig. 3). Our results with cell-based reporter assays, which confirm the results shown in Fig. 3, are described in Fig. 4. The cell-based controlled reporter gene activity was similarly reduced in the presence of these tyrosine kinase inhibitors (Fig. 4A), pCMV729/3010 respectively, in contrast to the effect of inhibitors of PTK and JAK kinase (Fig. 4C). Our results show that cells expressing NS5A along with the wild-type IκBα and the IκBα tyrosine individual and double mutants (Y42F and Y305F), and serine double mutant (S32A/S36A) were immunoprecipitated with anti-IκBα serum and Western-blotted with anti-phosphotyrosine monoclonal antibody. Lane 1, untransfected Huh7 lysates; lanes 2, 3, and 4, Huh7 lysates transfected with wild-type IκBα, IκBα Y42F, and Y305F expression vectors respectively; lanes 5 and 6, Huh7 lysates transfected with IκBαY42, 305F, and IκBαS32A/S36A double mutants, respectively.

E, lane 1. Treatment of untransfected Huh7 nuclear lysates with PTK inhibitors did not seem to have any dramatic effect on the intensity of the DNA-protein complex (Fig. 2E). Our results with cell-based reporter assays, which confirm the results shown in Fig. 3, are described in Fig. 4. The cell-based controlled reporter gene activity was similarly reduced in the presence of these tyrosine kinase inhibitors (Fig. 4A), pCMV729/3010 respectively, in contrast to the effect of inhibitors of PTK and JAK kinase (Fig. 4C). Our results show that cells expressing NS5A along with the wild-type IκBα and the IκBα tyrosine individual and double mutants (Y42F and Y305F), and serine double mutant (S32A/S36A) were immunoprecipitated with anti-IκBα serum and Western-blotted with anti-phosphotyrosine monoclonal antibody. Lane 1, untransfected Huh7 lysates; lanes 2, 3, and 4, Huh7 lysates transfected with wild-type IκBα, IκBα Y42F, and Y305F expression vectors respectively; lanes 5 and 6, Huh7 lysates transfected with IκBαY42, 305F, and IκBαS32A/S36A double mutants, respectively.

Since piceatannol, a specific ZAP-70/Syk inhibitor, eliminated NF-κB activation in a ZAP-70 knock-out Jurkat cell line, we further investigated the activation of NF-κB in a ZAP-70 knock-out cell line. Nuclear lysates from Jurkat (JE6) and ZAP-70 knockout (P116) cells (35) expressing NS5A or HCV nonstructural proteins (729/3010) were incubated with NF-κB consensus oligonucleotide probe during EMSA. Expression of NS5A or the nonstructural proteins (729/3010) activated NF-κB in Jurkat (JE6) cells (Fig. 5A, lanes 3 and 4) but no activation was observed in ZAP-70 deficient (P116) cells (Fig. 5A, lanes 6 and 7). The specificity of DNA-protein complex formation was confirmed by a supershift of the complex in the presence of anti-p65 serum (Fig. 5B, lane 3) and by unlabeled NF-κB consensus competitor oligonucleotide (Fig. 5B, lane 4). We further show that ZAP-70 tyrosine kinase is expressed in Huh7 cells as evidenced by Western blot assay (Fig. 5C). Together, these results unambiguously rule out the involvement of IKK and suggest the possible functional role of ZAP-70 kinase as one of the kinases responsible in the NS5A-induced NF-κB activation.

Next, we examined the status of IκBα tyrosine phosphorylation in cells co-expressing NS5A along with the tyrosine (Y42F and Y305F), and serine (S32A/S36A) IκBα mutants. Cellular lysates from these cells were immunoprecipitated with anti-IκBα serum and Western-blotted with anti-phosphotyrosine monoclonal antibody. The results show that cells expressing wild-type IκBα and Ser32/36 mutant contained tyrosine phosphorylated IκBα (Fig. 6, lanes 2–4 and lane 6). Similarly, individual tyrosine (Y42F and Y305F) mutant IκBα-expressing cells displayed presence of tyrosine phosphorylated IκBα. Cells

μM, genistein (74 μM), BAY 11-7085 (10 μM), and H7 (25 μM) at various time periods before preparing lysates for luciferase activity. C and D, FCA4 and GS4.3 cells were transfected with NF-κB luciferase plasmid and treated with PTK inhibitors, PDTC and BAPTA-AM as described in A and B. E, Huh7 cells (control) transfected with NF-κB luciferase plasmid were treated with PTK inhibitors and IKK inhibitor. F, TNF-α-mediated stimulation of NF-κB. Huh7 cells transfected with NF-κB luciferase plasmid and were treated with TNF-α (10 ng/ml) for 1 h before preparing lysates for luciferase activity. Bottom panel in A and B represents the expression level of NS5A protein.
co-expressing NS5A and IκBα tyrosine double mutant (Y42F/ Y305F), however failed to show tyrosine-phosphorylated IκBα (lane 5). These data suggest that HCV NS5A induced phosphorylation of IκBα requires both Tyr$_{42}$ and Tyr$_{305}$ residues but not the Ser$_{32,36}$ residues. The fact that IκBα tyrosine mutation at either Y42F or Y305F map positions retained the tyrosine phosphorylation suggests that either residue is sufficient for tyrosine phosphorylation of IκBα and subsequent activation of NF-κB.

Tyrosine 42 and 305 Residues of IκBα Are Involved in HCV NS5A-induced IκBα Degradation—To determine the mechanism of NF-κB activation by HCV NS5A, we analyzed the degradation of IκBα serine and tyrosine mutants. Cellular lysates from cells co-expressing NS5A and Myc-tagged IκBα (wild type) or IκBα Tyr$_{42}$ and Tyr$_{305}$ mutants were analyzed by Western blot analysis using anti-Myc monoclonal antibody. As shown in Fig. 7A, wild-type IκBα was degraded in NS5A-expressing cells (lane 3), whereas individual Tyr$_{42}$ and Tyr$_{305}$ mutant IκBαs were not degraded (Fig. 7A, lanes 5 and 7). Similarly, the wild-type IκBα was also degraded in FCA4 cells (Fig. 7B, lane 2) but IκBα Tyr$_{42}$ and Tyr$_{305}$ mutants were not degraded (Fig. 7B, lanes 3 and 4). Cytokine-induced phosphorylation of IκBα at Ser$_{32,36}$ residues is an essential step for subsequent proteasome-mediated degradation (30). To examine whether IκBα phosphorylation at these sites is required for HCV NS5A-induced IκBα degradation, cellular extracts from cells co-expressing IκBα Ser$_{32,36}$ double mutant and NS5A were immunoblotted with anti-IκBα serum. The IκBα Ser$_{32,36}$ was degraded by HCV NS5A (Fig. 7C, lane 3), indicating the lack of involvement of serine phosphorylation of IκBα in the NS5A-induced NF-κB activation. In summary, our results clearly identify Tyr$_{42}$ and Tyr$_{305}$ residues of IκBα as key amino acids in the HCV NS5A and replicon-induced IκBα tyrosine phosphorylation, followed by its degradation and subsequent NF-κB activation. Tyr$_{305}$ residue has not been previously implicated in the NF-κB activation. To examine the expression levels of IκBα, lysates from Huh7 cells expressing wild-type IκBα, and various IκBα mutants were immunoblotted with anti-IκBα serum. The results show similar levels of IκBα expression (Fig. 7D). The differential migration pattern of these IκBα proteins reflects different conformations of the IκBα due to Myc tag addition to the IκBα gene.

HCV NS5A Induces the Mitochondrial Ca$^{2+}$ Uptake—We have previously shown that NS5A expression induces alteration of Ca$^{2+}$ homeostasis (22). Ca$^{2+}$ released from the ER is readily taken up by mitochondria, located near Ca$^{2+}$ release channel SERCA (36). To determine whether mitochondrial uptake of Ca$^{2+}$ is involved in the NF-κB activation, we employed
Calcium chelators inhibit the subgenomic HCV replicon and NS5A-induced NF-κB activity. A, EMSA was carried out using the presence of γ-32P-labeled NF-κB cognate nucleotide probe and nuclear lysates prepared from FCA4 cells. Lane 1, probe; lane 2, probe with BAPTA-AM; lane 3, probe with TMB-8 (50 μM) for 2 h; lane 4, probe with ruthenium red (30 μM) for 45 min. B, reporter gene activity. Huh7 cells were cotransfected with p3X-κB-Luc and pCMV-729/3010 and pBzC/CMV and pNCNSA expression vectors. NF-κB luciferase plasmids were used as a control. Transfected cells were treated with BAPTA-AM (100 μM) and/or Ru360 (45 μM) for 1 h, preparing lysates for luciferase assays. BAPTA-AM and TMB-8 reduced the intensity of the DNA-protein complex (Fig. 8A, compare lane 3 with lane 6) and in cell-based luciferase assays (Fig. 8B). The chelation of intracellular calcium with BAPTA-AM and TMB-8 reduced the intensity of the DNA-protein complex (Fig. 8A, compare lane 3 with lane 4 and 5) and affected the NF-κB activity in cell-based assays in both NS5A and pCMV729/3010 (expresses all NS proteins)-transfected cells (Fig. 8B). Treatment of untransfected Huh7 cells with BAPTA-AM, RR, and Ru360 (Fig. 8B) did not affect the marginal NF-κB driven reporter gene activity. Similar effects were observed in GS.4 cells treated with calcium chelators (Fig. 4D). These results demonstrate that Ca2+ signaling is an important aspect of NS5A-induced NF-κB activation and that the mitochondrial uptake of calcium is an important step in the pathway.

Calpain-mediated IκBα Degradation—Since activation of NF-κB is associated with the degradation of IκBα, we examined the effect of proteasome inhibitor, MG132 on IκBα degradation. In cells expressing NS5A, the degradation of IκBα occurred despite the presence of proteasome inhibitor, MG132 (Fig. 9A, lane 5). Because Ca2+ signaling has been implicated in the present NF-κB activation process (22), we investigated whether calpain protease, which is activated by Ca2+, is involved in the IκBα degradation. Treatment of cells with inhibitors of calpain protease, E64D and ALLM (39, 40), blocked the IκBα degradation in NS5A-transfected (Fig. 9A, lane 4) and replicon-expressing FCA4 cells (Fig. 9C, lanes 2 and 3). The IκBα Ser72,73 mutant was degraded (Fig. 9B, lanes 2 and 3) in the presence of NS5A just like the wild-type IκBα (Fig. 9A, lane 3). The observed degradation was blocked in the presence of calpain inhibitors (Fig. 9B, lanes 4 and 5). Luciferase reporter assays were also used to examine the effect of calpain inhibitors on IκBα degradation and subsequent NF-κB activation. The activation of NF-κB was blocked when NS5A and FCA4 cells were treated with calpain inhibitors (data not shown). These results together implicate a potential role of calpain in the IκBα degradation induced by NS5A alone or in the context of subgenomic replicon. Under normal conditions, calpain and calpastatin exist in a heteromeric complex (41). Ca2+ apparently binds calpain, which leads to its dissociation from the complex (42). Once dissociated, calpastatin is degraded by calpain protease. We examined the status of calpain and calpastatin in NS5A expressing cells. Western blot analysis shows stable expression of calpain in untransfected and NS5A expressing cells (Fig. 9D, lanes 1 and 2), whereas calpastatin appears to be rapidly degraded in the NS5A expressing cells (Fig. 9D, lane 2). Cells expressing calpain and calpastatin proteins in the 293T cell line were used as a control. These data support that IκBα degradation induced by NS5A is in contrast to proteasome-mediated degradation of IκBα that involves tyrosine phosphorylation and calpain protease-mediated degradation of IκBα. These activities of NS5A were also examined in the context of all the NS proteins in cells transfected with an expression vector (pCMV729/3010) or in subgenomic replicon expressing cells. A striking observation made in this work is K. D. Tardif and A. Siddiqui, unpublished results.
the non-requirement of the Ser32,36 and Ser33 residues and 26 S proteasome-mediated degradation of IκBα during NF-κB activation. However, the integrity of Tyr42 and Tyr305 is required for the IκBα degradation during NS5A or replicon-induced NF-κB activation. Involvement of Tyr42 phosphorylation in the NF-κB activation under oxidative stress conditions has been reported previously (31, 32), whereas the role of Tyr305 in this process is being reported for the first time. We also observed that antioxidant PDTC prevented IκBα phosphorylation (Fig. 2A), which support the previous observations that tyrosine phosphorylation of IκBα occurs under conditions of oxidative stress (46). Our results demonstrate that both Tyr42 and Tyr305 residue are important for the IκBα phosphorylation and subsequent NF-κB activation.

In the present analysis, we observed that HCV replicon and NS5A induced-NF-κB activation is sensitive to piceatannol, herbimycin A, AG490, and genistein. The blocking of JAK2 with AG490 abolished the NF-κB activation (Fig. 4). Although JAK2 is typically associated with the STAT pathway, Siebenlist (47) in a recent report suggested a possible cross-talk between STATs and NF-κB pathways and identified JAK2 to be an essential player in this process. This cross-talk may involve the activation of IκB kinases, as well as the phosphorylation of IκBα at tyrosine residues. In the present study we were able to make use of an available ZAP-70 knock-out cell line, since piceatannol, a ZAP-70/Syk specific inhibitor was effective in eliminating NF-κB activation. In the ZAP-70 knock-out cell line expressing NS5A and other NS protein, a complete failure of HCV nonstructural protein mediated-NF-κB activation was observed (Fig. 5). This is consistent with an earlier report that T cell-specific p56 LCK and ZAP-70 kinases are involved in pervanadate-induced IκBα phosphorylation and NF-κB activation (31). Since HCV does not replicate in Jurkat cells, we could not use the HCV subgenomic replicon expression scheme in transient transfections. ZAP-70 kinase is expressed in Huh7 cells and we could not use the HCV subgenomic replicon expression scheme in transient transfections. ZAP-70 kinase is expressed in Huh7 cells (Fig. 5E).

Previously, it was reported that pervanadate induces NF-κB activation without degradation of IκBα (46). However, our results indicate that HCV NS5A protein and subgenomic replicons induce activation of NF-κB via IκBα tyrosine phosphorylation and its degradation. This discrepancy may be attributed to the kind of stimulus for IκBα phosphorylation and the cell type being used. The ability of HCV replicon and NS5A to activate NF-κB without serine phosphorylation of IκBα correlates with similar observations obtained in cells treated with UV (UV-C) or pervanadate (48, 49).

Previous studies have shown that calpain is involved in NF-κB regulation (50, 51). Normally calpain and calpastatin exit in an inactive heteromeric complex (41). Calpain is activated by Ca2+ binding to its catalytic domain, which stimulates its protease activity (52). Once stimulated, calpain degrades calpastatin (Fig. 9D) (53).

It has been shown that mitochondria accumulate Ca2+ from intracellular microdomains of high Ca2+ such as ER in the
diseased or damaged cells (54). However, the physiological role of 
Ca\(^{2+}\) transport into mitochondria is not clearly understood. 
In our model, Ca\(^{2+}\) is effluxed from the ER as a result of HCV 
NS5A expression either alone or in the context of subgenomic 
replicon (22). The elevated cytosolic Ca\(^{2+}\) levels can potentially 
activate calpain protease, which can degrade the tyrosine-phos-
phorylated I protein alone or in the context of subgenomic 
replicon involves 
activation of I protein. These studies relate to the mechanism(s) of 
calpain activation are currently under intense study, Ca\(^{2+}\) 
release after exposure to hydrogen peroxide together with calp-
ain activation under oxidative stress conditions is well docu-
mented (55, 56). Several groups have reported that the PEST 
sequence is essential for oxidative stress mediated degradation 
of I protein (57–59). However, we found that I protein containing 
PEST sequence mutation was degraded when expressed in the pre-

ever of HCV NS5A (data not shown).

In summary, we show that NF-kB activation by HCV NS5A 
protein alone or in the context of subgenomic replicon involves 
tyrosine phosphorylation of I protein and its subsequent degra-
dation by calpain proteases. Ca\(^{2+}\) signaling via elevation of ROS 
in mitochondria provides the pivotal link in this pathway of ER-
nucleus signal transduction (22). These studies provide important 
clues to the understanding of the mechanisms of 
chronic liver disease induced by ER stress and other intracel-

ular events associated with HCV gene expression.

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