The hepatitis C virus non-structural NS5A protein inhibits activating protein-1 function by perturbing Ras-ERK pathway signaling

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Hepatitis C virus (HCV) can establish a persistent infection, often leading to chronic liver disease and cirrhosis. There is considerable interest in HCV, because chronic infection is strongly associated with development of hepatocellular carcinoma. Indeed, HCV infection is the leading indicator for liver transplantation in the United States. HCV is a small, enveloped Hepacivirus classified within the family Flaviviridae, most closely related to the Pestiviruses (e.g. bovine viral diarrhea virus). The genome is a single-stranded positive sense RNA molecule of ~9.5 kilobases in length with a single large open reading frame encoding for a polyprotein of ~3000 amino acids (2). The open reading frame is flanked by 5' and 3' untranslated regions, which have been shown to be essential in both initiation of translation and viral replication (3). Processing of the precursor polyprotein requires both host and viral proteases to produce the structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (4).

The mature NS5A protein is generated by the action of the NS3/4A serine protease. NS5A is localized to cytoplasmic and perinuclear regions of the cell and exists in a basal or hyperphosphorylated state (p56 or p58), with the degree of serine/threonine phosphorylation accounting for the difference in size (5). The exact function of NS5A is unknown, although it is speculated to form part of a multiprotein replication complex located on the cytosolic face of the endoplasmic reticulum membrane. An increasing body of evidence has demonstrated that NS5A interacts with a number of cellular proteins and may also interfere with host cell signaling pathways; for example, NS5A was shown to interact directly with the interferon-induced double-stranded RNA-activated protein kinase PKR, and this interaction seems to correlate with inhibition of PKR function (6). NS5A and NS5B form a complex with a human membrane-associated protein, hVAP (7), indicating the membrane localization of the replication complex. Recent reports suggest that NS5A may also regulate cell cycle progression, resulting in a reduced S phase and an increase in the G2/M phase (8).

One indicator that NS5A may have a role in cell signaling is the presence of polyproline (PXXP) motifs, which are highly conserved throughout a range of HCV genotypes. These motifs form extended helical structures and are found in a number of viral and cellular proteins involved in signal transduction. They bind to Src homology 3 (SH3) domains found in a diverse extracellular signal-regulated kinase kinase; MEKK, MEK kinase; AP1, activating protein 1; EGF, epidermal growth factor; IL-6, interleukin-6; CRE, cAMP-response element; SRE, serum-response element; PKC, protein kinase C; PAK, p21-activated kinase; FCS, fetal calf serum; HGF, hepatocyte growth factor; CREB, CRE-binding protein; ERK, extracellular signal-regulated kinase; DA, dominant active; DN, dominant negative; PI3-kinase, phosphatidylinositol 3-kinase.

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group of signal-transducing molecules (9), and indeed it was previously shown that one of these PXXP motifs in NS5A interacts directly with the SH3 domains of the growth factor receptor-bound protein 2 (Grb2) (10), a component of the mitogen-activated protein kinase (MAPK) cascade (see below).

The MAPK cascades are among the best characterized of intracellular signaling pathways. These cascades consist of a three-kinase module that includes a MAPK/ERK kinase (MEK) kinase (MEKK), which activates a MEK that in turn activates a MAPK (11). Three distinct groups of MAPK have been discovered in mammalian cells, including the mitogen-responsive ERK (extracellular signal-regulated kinases), the stress-activated c-Jun N-terminal kinase (JNK)/stress-activated protein kinases, and the less understood p38 MAPKs (12). Phosphorylation on both threonine and tyrosine residues is required to activate MAPKs. Once activated, MAPKs translocate to the nucleus, where they phosphorylate and activate transcription factors and other target proteins (13, 14).

Activating protein-1 (AP1) is a collective term referring to homo- and heterodimeric transcription factors composed of Jun, Fos, or ATF2 subunits that bind to a common DNA site, the AP1 site (15). MAPKs contribute to the activation of AP1 activity in response to a diverse array of extracellular stimuli, including cytokines, growth factors, T-cell activators, neurotransmitters, and UV irradiation. Each of the three types of MAPK kinases affects AP1 activity through phosphorylation of a different substrate. Whereas the ERKs phosphorylate TCF/Elk1 and thereby induce c-Fos synthesis, they do not phosphorylate c-Jun. In addition, the ERKs do not appear to be involved in ATF2 phosphorylation. The JNKs, on the other hand, phosphorylate the stimulatory sites of both c-Jun and ATF2 but generally do not phosphorylate c-Fos (15). Activated AP1 triggers transcription of many genes involved in the cell response to external stimuli.

Grb2 acts as an adaptor in the ERK pathway linking growth factor receptors to the MEKK cascade via SOS and Ras. Although it has been shown that in cells expressing NS5A phosphorylation of ERK resulting from EGFR stimulation was reduced (16), the exact mechanism for this is unclear, and that study failed to observe a reduction in Grb2 interactions with Sox. Given the importance of mitogenic signaling pathways for cell survival and proliferation, we set out to determine the mechanism and downstream consequences of the previously observed inhibition of ERK phosphorylation by NS5A. Using a luciferase reporter containing an AP1-responsive promoter sequence, in transient NS5A expression systems, we demonstrate that a C-terminal PXXP motif within NS5A is required for the inhibition of AP1. We show that this inhibition acts directly through the ERK MAPK pathway and not the JNK or p38 pathways. Phorbol 12-myristate 13-acetate stimulation and expression of dominant active Ras, Raf, and PKC isoforms abrogated inhibition, implying that NS5A interferes with the ERK MAPK cascade at a very early stage. In both Huh-7 cells harboring a subgenomic HCV replicon and stable osteosarcoma cells expressing NS5A alone, we also observed an inhibition of phosphorylation of members of the ERK MAPK pathway and a concomitant reduction in levels of the c-Fos transcription factor. Most significantly, we find a reduction in AP1 reporter activity in cells expressing the full-length HCV genome under the control of an authentic HCV IRES. Thus, our data provide new insight into the signaling pathways modulated by the HCV NS5A protein and may account for the oncogenic and immunomodulatory effects observed during a HCV infection.

**EXPERIMENTAL PROCEDURES**

**DNA Manipulations and Plasmids**—NS5A sequences from HCV genotype 1a (H77) (16) (kindly supplied by Dr. Jens Bukh, National Institutes of Health, Bethesda, MD) were amplified by PCR with appropriate sequence-specific primers (sequences available on request) and Pfu polymerase (Promega). PCR-amplified fragments were subcloned into the mammalian expression vector, pSG5 (17). A mutant of NS5A in which proline residues 350, 353, and 354 were substituted for alanine (PA2) was generated by the PCR overlap method (18) using an appropriate template and overlapping internal oligonucleotide primers. Briefly, internal forward and reverse primers containing the modified sequence were used in conjunction with flasking primers to create two primary PCR products. The primary amplification products were agarose gel-purified and used together as template in a secondary round of amplification using flasking primers to produce full-length product containing the required mutation. All constructs were verified by DNA sequencing. The pSG5/NS5A construct has been previously described (19). The IL-6 luciferase reporter constructs (20) were kindly provided by Dr. Derek Mann (University of Southampton). Camp-response element (CRE)-luciferase and serum-response element (SRE)-luciferase constructs were obtained from Stratagene; Sp1-luciferase was previously described (19). A luciferase reporter construct regulated by a promoter sequence responsive to AP1 (pAP1-luc) and dominant active Ras, Raf, PKC, and p21-activated kinase (PAK) expression constructs were previously described (21–23).

**Cell Culture**—COS-7 (African Green Monkey kidney cells) and HEK 293T (human embryonic kidney cells) were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) were cultured in minimal essential medium supplemented with 10% FCS, 1% nonessential amino acids, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. To generate a replicon, cell line transscripts were produced from the ScaI-linearized plasmid pF-Kmneo/NS3-2/17.1 (kindly provided by Dr. Ralf Bartenesch, University of Heidelberg, Germany) (24) using T7 RNA polymerase (New England Biolabs) according to the manufacturer’s recommendations, treated with DNase (Promega), extracted with acid phenol/chloroform, and spun through a mini-QuickSpin column (Roche Applied Sciences). 5 μg of transcript was transcribed into 400 μl of Huh-7 cells (1 x 10^6 cells/ml in phosphate-buffered saline) using a MaxiScript kit (Ambion, Austin, TX). Cells were allowed to recover for 24 h before the addition of 1 mg/ml G418 to the culture medium. Selection with G418 was maintained for 16 days, during which time the medium was changed every 3 or 4 days. G418-resistant colonies formed were maintained as a polyclonal cell line in medium supplemented with 250 μg/ml G418. Western analysis confirmed that the cell line expressed NS3, NS5A, and NS5B, and Northern analysis demonstrated the presence of a replicon transcript. All cell lines were incubated at 37 °C in a humidified 5% CO₂ incubator.

**Transfection of Plasmid DNA and Luciferase Assays**—One day prior to transfection, cells were seeded into six-well dishes (2 x 10^5 cells/dish). Cells were transfected using the commercially available Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Briefly, 1 μg of expression vector was transfected together with 1 μg of luciferase reporter construct. As an internal control for transfection efficiency, an expression plasmid encoding the Renilla luciferase gene driven by the herpes simplex virus thymidine kinase promoter (pRLTK) was also transfected (0.85 pg). For co-transfections, the final DNA concentration in all groups was kept constant by the addition of empty expression vector as appropriate. Cells were incubated with transfection reagent for 6 h, after which the transfection reagent was removed, and the cells were overlaid with growth medium. For most experiments, transfected cells were maintained under low serum conditions (0.5% serum) prior to stimulation with 10% serum containing growth medium. Where appropriate, cells were treated with 100 ng/ml phorbol 12-myristate 13-acetate, 10 μM 2’-amino-3’-methoxyflavone (PD98059), or 10 μM 2-(4-chlorophenyl)-4-(4fluorophenyl)-5-pyridin-4-yl-1,2-dihydro-2-propyl-2-phenyl analog of p38 MAPK inhibitor) (all obtained from Calbiochem) 1 h prior to growth factor stimulation. EGF and hepatocyte growth factor (HGF) were obtained from Sigma and were used at a final concentration of 20 ng/ml.

For luciferase reporter assays, cells were harvested in 200 μl of passive lysis buffer (Promega). Quantitation of relative light units was determined using the dual luciferase Stop & Glo reagent (Promega) and a Berthold luminometer (EG & G Berthold) with a dual injector system. All assays were performed in triplicate, and each experiment was repeated a minimum of three times.

Western Blotting—To analyze the activity of specific MAPK pathway proteins, cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibi-
Fig. 1. Inhibition of AP1 by NS5A is cell line-independent and requires a C-terminal Class II polyproline motif within NS5A. Cells were transfected with 1 μg of plasmid pAP1-luc, which contains the luciferase reporter under the transcriptional control of three tandem AP1 binding sites and a minimal TATA box promoter, with or without co-transfection of pSG5-NS5A(wt) or pSG5-NS5A(PA2), and placed in low serum (0.5%) growth medium for 18 h. Cells were stimulated by the addition of serum to 10% and harvested 6 h later. The level of expression of the luciferase reporter was assayed using a luminometer and normalized for transfection efficiency by using a co-transfected Renilla luciferase control plasmid. Results are the average of three independent experiments. The three graphs show results from COS-7 (a), 293-T (b), and Huh-7 (c). The expression of the different NS5A proteins from the 6-h time points was verified by immunoblotting using a sheep polyclonal antiserum (d). The faint bands co-migrating with NS5A in lanes 4 and 7 are the result of spill-over from adjacent lanes.

RESULTS

Inhibition of AP1 by NS5A Is Cell Line-independent and Requires a C-terminal Polyproline Motif within the NS5A Protein—A number of studies have shown that NS5A is able to transcriptionally regulate cellular genes and to thereby modulate cell growth (8, 26). Because the predicted amino acid sequence of NS5A contains no DNA binding motifs, it is likely that NS5A regulates the transcription of cellular genes either by direct interaction with cellular transcription factors or through modulation of cellular signaling pathways (27).

NS5A has been shown previously to interact with the SH3 domains of the mitogenic adaptor protein Grb2 via a highly conserved PXXP motif within the C terminus of the NS5A protein (10). Furthermore, our recent data have shown that NS5A also interacts with the SH3 domains of members of the Src family of tyrosine kinases.2 Both Src kinases and Grb2 are involved in regulating MAPK cascades that ultimately lead to activation of members of the AP1 family of transcription factors.

To further understand the mechanisms by which NS5A modulates mitogenic signaling cascades, we utilized a transient reporter assay in COS-7 cells. NS5A-expressing plasmids were co-transfected with a reporter construct in which expression of the luc gene was regulated by three tandem sequences corresponding to the binding site for the AP1 transcription factor (pAP1-luc) (28). Incubation of cells in medium containing 10% serum resulted in a 5-fold stimulation of the AP1 luciferase reporter construct (Fig. 1a), compared with cells maintained in low serum (0.5%) growth conditions. In cells expressing the NS5A protein, however, both basal and serum-stimulated levels of luciferase were reduced by ~60% (Fig. 1a). To identify the region(s) of NS5A responsible for mediating the inhibition of AP1 activity in COS-7 cells, we analyzed levels of luciferase in cells expressing a NS5A derivative (NS5A(PA2)), in which the highly conserved C-terminal PXXP motif was mutated to abrogate binding to SH3 domains (10).2 Fig. 1a clearly illustrates that NS5A(PA2) failed to inhibit AP1 reporter activity, implicating a role for this motif in NS5A-mediated inhibition of AP1.

To ensure that the results obtained were not an artifact of the COS-7 cell line, we repeated these experiments in two other cell lines, 293T and the human hepatoma cell line Huh-7 (Fig. 1, b and c, respectively). Expression of NS5A(wt), but not NS5A(PA2), resulted in a similar inhibition of AP1 reporter activity in both cases.

NS5A Expression Does Not Result in a Global Inhibition of Transcription—Expression of NS5A in transformed cell lines resulted in an inhibition of an AP1 luciferase reporter construct; however, the mode of inhibition was unknown. It was therefore crucial to rule out the possibility of a global inhibition of cellular transcription. To investigate this possibility, we analyzed the activity of luciferase reporter constructs responsive to the cAMP-responsive element-binding protein (CREB) or the transcription factor Sp1. COS-7 cells were transfected with NS5A expression plasmids and either CREB-luc or Sp1-luc. In this case, all cells were incubated in the presence of 10% FCS prior to analysis of luciferase activity. Levels of both CREB and Sp1 reporter activity were not significantly affected by expression of either NS5A(wt) or NS5A(PA2) (Fig. 2). Co-transfection of a plasmid expressing the HCV NS3/4A protein

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complex, however, impaired CREB reporter activity by up to 50%, in agreement with our previously published data (19). As expected, CREB was also inhibited by incubation of cells with a well-characterized inhibitor of cAMP-dependent protein kinase, a myristoylated peptide corresponding to residues 14-22 of the heat stable PKA inhibitor protein. Appropriate expression of NS5A or NS3/4A was confirmed by immunoblot analysis (data not shown). These data confirmed that NS5A did not mediate a global inhibition of transcription and therefore acted to specifically inhibit AP1 activity. This encouraged us to further investigate the mechanism of AP1 inhibition.

**Repression of an IL-6 Reporter Construct by NS5A Is Mediated by AP1 Promoter Sequences**—The above experiments were performed using a reporter construct containing three tandem repeats of the AP1 binding sequence. It was important to demonstrate that AP1 inhibition occurred when the binding site was present in a more physiologically relevant setting. To this end, we utilized a reporter expressing luciferase under the control of the IL-6 promoter. The IL-6 promoter has a single AP1 binding sequence and is known to be responsive to a variety of transcription factors including AP1 (29). Therefore, IL-6 was a suitable candidate to study the downstream effects of AP1 inhibition. Two IL-6 reporter constructs were used: a parental plasmid in which a 651-bp fragment of the IL-6 gene including the full-length promoter sequence was cloned into the parental plasmid in which a 651-bp fragment of the IL-6 gene.

COS-7 cells were transiently transfected with combinations of NS5A expression plasmids and IL-6-luc reporters and incubated in low serum growth conditions. Following stimulation with 10% FCS, the parental IL-6 reporter construct exhibited 40% inhibition in the presence of NS5A(wt) but was unaffected by NS5A(PA2) (Fig. 3a, gray bars). Although this level of inhibition is less than that of the AP1 luciferase reporter, it is consistent with previous results (Fig. 1) indicating that NS5A(wt) inhibits AP1 activity. In order to define these pathways more precisely, cells were transiently transfected with NS5A(wt)- or NS5A(PA2)-expressing plasmids together with pAP1-luc, incubated in low serum growth medium, and stimulated with EGF or HGF. These potent mitogens for a variety of cell lines including epithelial and mesenchymal cells. They have been shown to be effective activators of AP1 stimulation via the ERK MAPK pathway (30), exerting their influence through the EGF receptor and c-Met receptor, respectively. Transfected cells were stimulated with a 20 ng/ml concentration of either EGF or HGF for 2 min, and cells were harvested 2, 4, and 6 h poststimulation. In COS-7 cells, treated over the 6-h time period, NS5A(wt) reduced levels of AP1 reporter activity by up to 40% in comparison with control cells transfected with empty vector (Fig. 4a). Conversely, the NS5A(PA2) mutant failed to inhibit AP1 activity in EGF-stimulated cells. Interestingly, HGF-induced AP1 reporter activity was also inhibited in the presence of NS5A(wt) (Fig. 4b), although the extent of the inhibition was not as pronounced. In cells stimulated with HGF, inhibition by NS5A was ~25%. The experiments were also performed in Huh-7 cells transfected with NS5A-expressing vectors. Similar results were obtained with both EGF and HGF treatment (Fig. 4, c and d). In all cases, the increase in luciferase activity over time was a result of growth factor stimulation, since control untreated cells did not exhibit a time-dependent increase.

These data confirm that NS5A-induced inhibition of AP1 reporter activity in both COS-7 and Huh-7 cells could be ob-
served following growth factor stimulation implicating the ERK pathway. Indeed, the data observed followed a similar trend to that obtained from serum stimulation.

**Involvement of the Ras-ERK Pathway in NS5A-mediated AP1 Inhibition**—Stimulated EGF receptor activates multiple downstream targets including Grb2 and Src kinases. Formation of multiprotein complexes at the membrane leads to activation of Ras, Raf1, and MEKK1 (31, 32). Raf1, in turn, activates the ERK1 and ERK2 MAPK through MEK1 and MEK2 (33, 34). To evaluate the role of the ERK pathway in the NS5A-mediated inhibition of AP1 reporter activity, we transfected COS-7 cells with pAP1-luc, pSG5-NS5A(wt), and plasmids expressing dominant active (DA) forms of Ras, Raf1, and PKC. Both DA-Ras and DA-Raf1 significantly increased the EGF-stimulated AP1 reporter activity (Fig. 5, b and c, gray bars), indicating that these DA proteins acted synergistically with growth factor stimulation to up-regulate AP1 activity. Expression of DA-PKC isofoms α and β also led to an increase in AP1 reporter activity, although to a lesser extent than DA-Ras and DA-Raf1 (Fig. 5, d and e). Consistent with PKC-mediated up-regulation of AP1, stimulation of cells with phorbol 12-myristate 13-acetate (a PKC activator) also led to a similar increase in AP1 reporter activity (Fig. 5f). The observation that expression of each of these dominant active constructs abrogated the NS5A-mediated inhibition of AP1 reporter activity (Fig. 5, b-f, checkered bars) strongly suggested that the NS5A-mediated inhibition of AP1 activity occurred upstream of Ras and Raf1. To confirm that inhibition of ERK activity can lead to reduced AP1 activity, we employed a specific MEK1/2 inhibitor, PD98059 (35). Incubation with 10 μM PD98059 led to a 70% decrease in AP1 reporter activity in the absence of NS5A, demonstrating that specific perturbation of the ERK1/2 pathway was capable of down-regulating AP1 activity to levels seen in NS5A-expressing cells (Fig. 5g).

In addition to the Raf1 cascade, ligand stimulation can also activate the MEKK1 signaling cascade (36). In this pathway, PAKs are activated by the small membrane-bound GTPases, Rac1 and Cdc42 (21), and in turn stimulate MEKK1. MEKK1 activates MEK4, which activates JNK and p38 MAPKs (37). To evaluate whether the NS5A mediated inhibition was transduced through alternative MAPK pathways, we co-transfected cells with pAP1-luc, pSG5-NS5A(wt), and plasmids expressing either DA-PAK2 or a dominant negative form of PAK2 (DN-PAK2) (38). Co-transfection of DA-PAK2 with AP1 did not significantly increase basal AP1 reporter activity and had no effect on the inhibition mediated by NS5A(wt) (Fig. 5h), suggesting that NS5A-mediated inhibition does not function via PAK2-mediated pathways. Expression of DN-PAK2 did not decrease serum stimulated AP1 reporter activity and did not alter the NS5A mediated AP1 inhibition (Fig. 5i). To provide further evidence for our hypothesis that only the ERK MAPK pathway was being inhibited, we transfected cells with pAP1-luc with or without pSG5-NS5A(wt) and incubated these cells in the presence of a specific p38 MAPK inhibitor (2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one). Incubation of cells with this compound did not significantly reduce serum-stimulated AP1 levels, and importantly, the NS5A-mediated AP1 inhibition was maintained in the presence of the inhibitor (Fig. 5j). Appropriate expression of the DA or DN proteins and NS5A was confirmed by immunoblot analysis (Fig. 5, a–j, lower panels). In all of these experiments, cells were growth factor-stimulated to maintain consistency throughout the study, and similar data were obtained in non-stimulated cells (data not shown).

**Phosphorylation of Ras-ERK Pathway Members Is Inhibited in HCV Replicon Cells**—The data obtained from the use of specific mitogens and mutant signaling proteins suggested that the NS5A-mediated inhibition of AP1 involved only the ERK pathway. To test this hypothesis more directly, we investigated the expression and phosphorylation of individual components of the ERK and JNK pathways. This analysis was undertaken in three types of cells: stably transfected human osteosarcoma...
cells (UTA6) in which expression of NS5A was driven by the tetracycline-responsive promoter, stably transfected Huh-7 cells expressing NS5A alone, and also Huh-7 cells harboring the culture-adapted FK5.1 subgenomic replicon (i.e. expressing the HCV nonstructural proteins NS3 to NS5B). We show here data obtained from a comparison of naive Huh-7 cells and replicon FK5.1 cells (Fig. 6); however, identical data were obtained from cells expressing only NS5A (data not shown).

Naive Huh7 and Huh7-FK5.1 cells were stimulated with EGF and lysed at various points over a 4-h time course. Samples were analyzed by immunoblotting with a range of commercially available antibodies to detect both overall expression of ERK1/2, c-Fos, and c-Jun and the phosphorylated forms of MEK1/2, ERK1/2, and c-Jun. Fig. 6a shows that, following EGF stimulation, phosphorylation of MEK1/2 by Raf was significantly reduced in FK5.1 cells in comparison with naive Huh7. Similarly, immunoblotting with an antibody directed against the dually phosphorylated, activated form of ERK1/2 revealed that phosphorylation of these proteins was also reduced in FK5.1 cells (Fig. 6b), although the time course of phosphorylation was similar in both cell lines (maximal ERK1/2 phosphorylation occurred within 30 min of EGF stimulation). Blotting with a phosphorylation state-independent antibody showed similar levels of ERK1/2 in both cell lines (Fig. 6c), confirming that the reduction in ERK1/2 phosphorylation was not due to a reduction in the abundance of ERK1/2. Once phosphorylated, ERK1/2 can enter the nucleus and phosphorylate the Elk1 transcription factor; Elk1 then binds to the SRE of the c-fos promoter and activates expression of c-Fos. Fig. 6d shows that levels of c-Fos induction were dramatically reduced in FK5.1
Appropriate expression of NS5A was determined by immunoblot with antibodies specific to phosphorylated MEK (SDS-PAGE (12%) and subjected to immunoblot analysis with antibodies 4 h post-EGF treatment for analysis. Cell lysates were resolved by treatment with 20 ng/ml EGF for 2 min (0-h time point) and at 0.5, 2, and 4 h post-EGF treatment for analysis. Cell lysates were resolved by SDS-PAGE (12%) and subjected to immunoblot analysis with antibodies specific to phosphorylated MEK (a), total ERK1/2 (b), phosphorylated ERK1/2 (c), c-Fos (d), c-Jun (e), and phosphorylated c-Jun (f). Appropriate expression of NS5A was determined by immunoblot with a sheep polyclonal antiserum (g).

cells in comparison with naive Huh7. In the latter, maximal c-Fos expression occurred at 4 h post-EGF treatment, whereas, interestingly, in FK5.1 cells c-Fos expression peaked at 2 h post-EGF and declined thereafter.

In parallel, we also examined the expression and phosphorylation of c-Jun in naive Huh7 and FK5.1 cells. Overall levels of c-Jun remained stable throughout the 4-h time course, and there were no differences between the two cell lines in the kinetics or amounts of c-Jun phosphorylation following EGF stimulation. This result is consistent with our previous observation that transfection of DA- or DN-PAKs had no effect on NS5A-mediated inhibition of AP1 activity (Fig. 5, h and i). Appropriate expression of NS5A in FK5.1 cells was confirmed by immunoblot analysis (Fig. 6g).

NS5A Inhibits Activation of the Serum-response Element of the c-Fos Promoter—The c-Fos gene promoter contains an SRE that is bound by the phosphorylated ETS family transcription factor Elk1 (39). To provide further evidence that the reduction in c-Fos expression was the result of a reduction in SRE-dependent transcription, COS-7 cells were transiently transfected with NS5A expression plasmids together with a reporter construct containing the SRE upstream of luciferase (pSRE-
luc) and maintained under low serum growth conditions. As expected, EGF stimulation caused an increase in SRE-driven luciferase expression; however, EGF-stimulated levels of luciferase were reduced by ~60% in the presence of NS5A(wt) (Fig. 7). This reduction was not observed in the presence of NS5A(PA2). We conclude, therefore, that the down-regulation of ERK1/2 phosphorylation mediated by NS5A results in decreased transcription from the SRE of the c-fos promoter, with a concomitant reduction in the expression of c-Fos.

Inhibition of AP1 by Full-length HCV in Hepatocyte-derived Cell Lines—A major hindrance to HCV research is the lack of an efficient and convenient culture system. Although this has, in part, been overcome by the advent of subgenomic HCV replicons (40, 41), use of this system has the disadvantage of only expressing the HCV nonstructural proteins NS3-NS5B. Given that the HCV core protein has recently been reported to up-regulate all three MAPK pathways (42), we considered it of importance to determine whether AP1 activity was modulated in the context of the entire HCV polyprotein. To address this possibility, we took advantage of a baculovirus delivery system capable of expressing the full-length HCV genome in hepatocyte-derived cell lines, under the control of a tetracycline-responsive promoter, that has been developed in our laboratory (25).

Huh-7 cells were transduced with baculoviruses expressing the tTA tetracycline transactivator protein (BACtTA) together with a second virus encoding either β-galactosidase (BACIND-LacZ\textsuperscript{\textsc{tet}}), or the full-length HCV genome (BACH77(H6V)\textsuperscript{\textsc{tet}}), under the control of the tetracycline-responsive promoter. Cells were maintained in the presence of absence of tetracycline to either repress or activate expression from the tetracycline-responsive promoter, respectively. The transduced cells were subsequently transfected with pAP1-luc and serum-stimulated. At 6 h poststimulation, cells were harvested, and the levels of luciferase were determined for each sample. Fig. 8a illustrates that transduction of cells with BACtTA and BACIND-LacZ\textsuperscript{\textsc{tet}} did not significantly affect levels of luciferase expression. Cells infected with BACtTA and BACH77(H6V)\textsuperscript{\textsc{tet}},
in the presence of tetracycline, exhibited luciferase levels similar to control. However, in cells where the tetracycline was removed, thus allowing HCV genome transcription and polyprotein expression, levels of luciferase were significantly reduced. The accompanying Western blot illustrates that NS5A was expressed in the cells lacking tetracycline but not in those treated with the antibiotic (Fig. 8b). This observation clearly demonstrates that AP1 activity is reduced in hepatoma cells expressing the entire HCV polyprotein. Taken together with the transient expression data, we conclude that even in the presence of the core protein, expression of the complete HCV polyprotein is able to mediate inhibition of the AP1 pathway.

**DISCUSSION**

The lack of a robust *in vitro* replication system for HCV has meant that the exact role of the nonstructural NS5A protein in HCV infection is as yet unknown. To date, information about potential NS5A functions has derived, almost exclusively, from the use of *in vitro* transient or constitutive expression systems. In one such previous study, HeLa cells stably expressing NS5A exhibited a reduction in the levels of EGF-activated ERK activity compared with control cells (10). However, the mechanism of this reduction and the concomitant effect of NS5A on the activity of mitogenically modulated transcription factors were not analyzed. We therefore set out to dissect the effects of NS5A on both mitogenic and stress-stimulated transcription factors using a variety of cell lines and expression systems, including in the context of a full-length HCV genome delivery vector in hepatocyte-derived cell lines.

The ability of viruses to modulate transcription factors involved in cell proliferation, differentiation, and stress response has been studied in great depth. AP1 transcription factor activity is regulated in a complex manner involving different patterns of Fos and Jun protein expression. The type of AP1 complex formed partly accounts for the conflicting cell functions associated with induction of the *fos* and *jun* genes, which range from proliferation and transformation to differentiation and growth arrest (43). We therefore characterized the signaling pathways and the mechanism by which NS5A inhibits AP1 and the specific family members involved.

The main conclusion from the data presented here is that NS5A inhibition of AP1 transcription factor activity is mediated via specific perturbation of the Ras-ERK pathway and does not involve the other MAPK pathways (JNK and p38). The effect of NS5A was abrogated by expression of dominant active forms of proteins in the Ras-ERK pathway (Ras, Raf-1, and PCK) but not the JNK pathway (PAK2). Our data both confirm and significantly extend previous observations pertaining to mitogenic signaling via ERK (10, 44) but appear to contradict a further study by the same group showing inhibition of p38 MAPK signaling by NS5A (44). Since the latter study exclusively used HeLa cells, it is possible that cell type differences in the MAPK cascades may account for this discrepancy. However, our data did not formally exclude an effect of NS5A on p38 signaling but do demonstrate that any inhibition of p38 does not impact on AP1 activity. The observation that the p38 inhibitor has no effect on NS5A-mediated AP1 inhibition suggests that if NS5A does indeed perturb p38 signaling it must target the pathway below p38 itself (i.e. in a very different manner from the effect on the ERK pathway). This is consistent with the observations that NS5A inhibits PKR (6) and PKR phosphorylates p38 MAPK (45).

Our data show that although the effect of NS5A on AP1 activity was significant, it was clear that activity of the transcription factor was never completely inhibited. This may be due to the inability of NS5A to completely overcome the strong stimulation of AP1 activity achieved with growth factor stimulation, a hypothesis supported by the observation that NS5A inhibition of basal AP1 activity (Fig. 1) was more marked than inhibition of serum-stimulated activity. One other possible explanation may lie in the specific inhibition of c-Fos by NS5A. The active AP1 transcription factor is composed of either a Jun-Jun homodimer or a Fos-Jun heterodimer. Interestingly, the specificity of biological effects induced by upstream stimuli is determined by the composition of AP1 DNA binding complexes in a particular signal transduction cascade. The reasons for specific targeting of c-Fos are unclear. It is known that Fos-Jun heterodimers bind DNA elements containing AP1 consensus sequences up to 50 times more efficiently than Jun-Jun homodimers (43). It has also been observed that JunB isoforms can act as negative regulators of AP1 function and inhibit transcription by a negative interaction with AP1 consensus sequences (46). Thus, by inhibiting c-Fos expression, NS5A may shift the AP1-mediated response to external stimuli by modulating the composition of specific AP1 complexes. The exact composition of AP1 dimers in hepatoma cells is currently unknown but requires clarification in order to fully understand the effect of NS5A on AP1 transcription factor activity. The precise mechanism of AP1 inhibition by NS5A remains to be elucidated; at this point, it is perhaps pertinent to note that we do not observe an increase in NS5A phosphorylation following serum or growth factor stimulation (data not shown). Thus, it is unlikely that NS5A functions as a substrate for one of the kinases in the Ras-ERK pathway, thereby sequestering specific members of the pathway.

Why should HCV possess a mechanism for inhibition of AP1 signaling via the Ras/ERK pathway? Several potential explanations can be proposed. As well as contributing to AP1 activity, ERK has a number of independent functions including regulation of the STAT3 transcription factor (47), which itself plays a key role in interferon signaling and cell proliferation. Phosphorylation by ERK negatively regulates STAT3 by inhibiting STAT3 tyrosine phosphorylation (48); thus, it would be expected that inhibition of ERK activation by NS5A might stimulate STAT3. In this regard, our data are consistent with two recent reports: activation of STAT3 by both the HCV core...
protein and another (as yet undefined) HCV gene product (49) and a study demonstrating activation of STAT3 by NS5A (50). However, an earlier report (51) showed inhibition of STAT3 DNA binding activity in human osteosarcoma cells expressing the entire HCV genome, so clearly this discrepancy must be addressed in the future.

Growth factor-mediated activation of AP1 is critical in the control of hepatocyte proliferation; indeed, HGF is the most potent hepatocyte mitogen, mediating the transition from G1 phase through to S phase and mitosis. One plausible scenario, therefore, is that NS5A-mediated inhibition of AP1 might slow the exit of infected hepatocytes from the G1 phase. Induction of entry into cell cycle, followed by a block in cell cycle progression, is a common mechanism utilized both by viruses that establish chronic infections (52) and more acute viruses, such as Reovirus (53, 54). Presumably, this is because the G1 phase provides the optimal environment for viral replication. In the case of Reovirus, cell cycle arrest in G1 has been reported to occur as a result of inhibition of the Ras-MAPK pathway (55).

Two other hepatotropic viruses, hepatitis B and hepatitis E, both encode proteins that deregulate MAPK signaling pathways (56, 57). Although both the hepatitis B HBx protein and hepatitis E virus ORF3 target the Ras-ERK cascade, unlike NS5A, they lead to an increase in ERK kinase activity. The effects of ORF3 on AP1 signaling have not been described; however, there is a wealth of literature describing an HBx-mediated increase in AP1 activity (58–60). It is thought that, in comparison with other chronic viruses, HBx up-regulates AP1 in order to stimulate cell proliferation and rapid progression through the cell cycle in order to aid viral replication (61). Other viral gene products that target the Ras-ERK pathway include the human immunodeficiency virus Nef protein (62, 63), the human T-cell leukaemia virus Tax (64), and the Epstein-Barr virus latent membrane protein 1 (65). In all three cases and that of HBx, evidence for a role of Src kinases in mediating the activation of the Ras-ERK pathway has been presented. In this regard, it is pertinent to note that our recent data have addressed in the future.

Infection with HCV is increasingly linked to the development of hepatocellular carcinoma, although, unlike hepatitis B, no direct link between viral replication and tumorigenesis has been unambiguously identified. Intriguingly, a naturally occurring transforming mutant of the EGFR receptor, EGF-RvIII, down-regulates the ERK MAPK pathways while simultaneously activating PI3-kinase (66). PI3-kinase plays a key role in regulating both cell proliferation and apoptosis, and several studies have indicated that ERK acts as a negative regulator of PI3-kinase (67). While this manuscript was in preparation, He et al. (68) published data suggesting that NS5A was capable of up-regulating PI3-kinase phosphorylation in stable cell lines. Down-regulation of the ERK MAPK pathway by NS5A may therefore be a mechanism of up-regulating PI3-kinase activity in HCV-infected cells, thus affording these cells protection against apoptotic stimuli. We are currently addressing this question by investigating the physiological consequences of NS5A-mediated Ras-ERK and concomitant AP1 inhibition.

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