The Hepatitis C Virus NS5A Protein Activates a Phosphoinositide 3-Kinase-dependent Survival Signaling Cascade*

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Hepatitis C virus (HCV) establishes a persistent infection, with up to 80% of infected individuals proceeding to chronic hepatitis, which in many cases may result in liver cirrhosis and hepatocellular carcinoma (HCC); indeed HCV infection is increasingly associated with the development of HCC. The long time period (up to 30 years) between primary infection and the onset of HCC implies that HCV is not directly oncogenic but in some way predisposes patients to develop tumors, though the mechanism for this is unclear as yet. We report here that NS5A binds directly to the Src homology 3 domain of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), and this interaction is mediated by a novel (non-proline-rich) motif within NS5A. Coimmunoprecipitation analysis revealed that NS5A bound native heterodimeric PI3K and enhanced the phosphotransferase activity of the catalytic (p110) subunit both in vitro and in human cell lines harboring a subgenomic HCV replicon or expressing NS5A alone. NS5A-mediated activation of PI3K resulted in increased phosphorylation and activity of Akt/protein kinase B and concomitantly provided protection against the induction of apoptosis in both replicon-harboring cells and cells stably expressing NS5A alone. These data suggest that stimulation of PI3K by NS5A may represent an indirect mechanism for development of HCC in HCV-infected patients and further suggests potential therapeutic strategies to counteract the occurrence of HCV-related HCC.

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The abbreviations used are: HCV, hepatitis C virus; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Grb, growth factor receptor-bound; GST, glutathione S-transferase; HA, hemagglutinin; PI3K, phosphoinositide-3 kinase; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PIP5, 1,4,5trisphosphate; SH2 and SH3, Src homology domain 2 and 3, respectively.
9. An increase in the phosphorylation of p85, Akt, and Bad was demonstrated in NS5A-expressing HeLa cells (9).

Dysregulation of PI3K, by either overexpression or constitutive activation, has been linked with the malignant transformation of cells (11). Additionally, a number of viruses that establish chronic infections express proteins that interact with and activate PI3K to promote cell survival and block apoptosis thus enhancing viral replication. Examples include the hepatitis B virus X protein (12), the Epstein-Barr virus latent membrane protein 1 (13), and human immunodeficiency virus type 1 Nef (14, 15). Given that HCV establishes a chronic infection and is increasingly associated with the development of hepatocellular carcinoma we set out to define more precisely the interaction between NS5A and PI3K and to investigate the potential functional consequences in a relevant cellular context: the human hepatoma Huh7 cell line harboring a subgenomic HCV replicon (3). We demonstrate that NS5A is able to interact with the SH3 domain of the p85 subunit via a novel, non-polyproline-containing motif located in the center of NS5A. This interaction mediates association of NS5A with the native p85-p110 complex in mammalian cells, and this interaction results in stimulation of the lipid kinase activity of PI3K both in vitro and in vivo. In Huh7 cells harboring a subgenomic replicon, or stably expressing NS5A alone, phosphorylation and activation of Akt are stimulated, and this correlates with a reduction in the levels of apoptosis induced by serum deprivation. Our data are consistent with a role for NS5A in blocking apoptosis in HCV-infected cells by activation of PI3K and imply that NS5A contributes not only to the persistence of HCV but also to the propensity for the development of hepatocellular carcinoma in HCV-infected patients.

**EXPERIMENTAL PROCEDURES**

**DNA Manipulations**

NS5A sequences from HCV genotypes 1a (H77) (16), 1b (J4) (17), and 2a (J6) (18; kindly supplied by Dr. Jens Buhk, NIH, Bethesda, MD), as well as truncation mutants of NS5A(1-1a), were amplified by PCR with appropriate sequence specific primers (sequences available on request) and Pfu polymerase (Stratagene). PCR products were ligated as blunt ended fragments, using T4 ligase (New England Biolabs) into the pCRBlunt vector (Invitrogen) before verification by dideoxy sequencing and subcloning into either pSG5 (19) or pcDNA3. An N-terminal HA-tagged PI3K p85 subunit expression construct was kindly donated by Dr. Andreas Baur (University of Erlangen, Germany) (15).

**Cell Culture**

COS-7 (African green monkey kidney) and 293T (human embryonic kidney) cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM t-glutamine, 100 mM penicillin, and 100 μg/ml streptomycin. Huh7 and HepG2 (human hepatoma) cells were cultured in minimal essential medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 2 mM t-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Mammalian cell lines were incubated at 37 °C in a humidified 5% CO2 incubator. Spodoptera frugiperda (Sf9) insect cells were cultured in TC101 supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 26 °C in an unhumidified incubator. For the generation of stable Huh7 cells expressing neomycin phosphotransferase alone or with the HCV genotype 1b isolate of NS5A, cells were transfected with pcDNA3 alone or pcDNA3.NS5A, and polyclonal cell populations were selected with 250 μg/ml G418. Replicon cell lines were derived as described previously (20). Briefly, 5 μg of replicon transcript was electroporated into Huh-7 cells, and transfected cells were allowed to recover for 24 h before the addition of 1 mg/ml G418 to the culture medium. G418-resistant colonies formed were maintained as a polyclonal cell line in medium supplemented with 250 μg/ml G418.

**Expression of NS5A from Recombinant Mammalian Expression Vectors**

COS-7 cells were seeded at 2 × 10^5 cells in 60-mm dishes and transfected with 2 μg of the appropriate expression vectors using Lipofectin (Invitrogen). At 24 h post-transfection cells were washed thoroughly in ice-cold phosphate-buffered saline and then lysed in Glasgow lysis buffer (10 mM PIPES-NaOH, pH 7.2, 120 mM KCl, 30 mM NaCl, 5 mM MgCl2, 1% Triton X-100, and 10% glycerol containing protease inhibitors (Roche Applied Science)). The lysate was clarified by centrifugation at 16,000 × g for 5 min at 4 °C then aliquoted and stored at −80 °C before use.

**Expression and Purification of GST Fusion Proteins**

The pGEX vectors for bacterial expression of GST-N-SH2(p85), GST-C-SH2(p85), GST-SH3-N-SH2(p85), and GST-SH3(p85) were obtained from Dr. Yves Collette (INSERM U119, Marseilles, France). Expression and purification of the GST fusion proteins in Escherichia coli XL-1-blue or BL21 cells were carried out by standard methods (21). Briefly, bacterial cultures were grown to mid-log phase, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated at 37 °C for 3 h. Cells were pelleted, resuspended in phosphate-buffered saline containing 1% (v/v) Triton X-100 and 1 mM dithiothreitol, and sonicated briefly to release soluble proteins. GST fusion proteins were bound to pre-equilibrated glutathione-agarose (GA) beads for 1 h at 4 °C, beads were washed extensively prior to elution in 50 mM Tris-HCl, pH 8.0, containing 20 mM reduced glutathione. After dialysis in 50 mM Tris-HCl, pH 8.0, overnight at 4 °C the concentration and integrity of the proteins were verified by BCA micro™ assay (Pierce), SDS-PAGE, and Western blotting with a GST-specific antibody.

**In Vitro Binding Assays**

The appropriate GST fusion proteins were bound to GA beads at 4 °C for 1 h. Aliquots of lysates from pSG5.NS5A-transfected COS-7 cells were incubated with beads in lysis buffer. After a 3-h incubation, beads were washed extensively in lysis buffer, and bound proteins were analyzed by SDS-PAGE followed by immunoblotting using NS5A-specific antiserum. GST alone was used as a negative control.

**Coimmunoprecipitations**

For coimmunoprecipitations, magnetic protein G beads (Dyna BioTech) were utilized according to the manufacturer’s protocol. Briefly, the beads were washed in phosphate-buffered saline, and 1 μg of antibody was bound to the beads for 2 h followed by washing and resuspension in Glasgow lysis buffer. Cell lysates from transfected cells containing 500 μg of protein were added to the bead-antibody complex, and the mixtures were incubated at 4 °C on a blood mixer for 3–4 h. The beads were washed three times with lysis buffer, and bound proteins were analyzed by SDS-PAGE followed by immunoblotting with appropriate antiserum.

**Western Blot Analysis**

Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked for 1 h in 10% (w/v) lowfat dried milk, 0.1% Tween 20 (Merck) in Tris-buffered saline and probed with either an in-house polyclonal anti-serum to NS5A, anti-PI3K p85 or p110 (Upstate Biotechnology, Inc.), anti-Akt (New England Biolabs), anti-phospho-Akt (New England Biolabs), anti-Bad (New England Biolabs), and anti-HA (Santa Cruz) antibodies according the manufacturers’ recommendations. Bound antibody was detected with the appropriate conjugated secondary antibody (Sigma) and ECL reagent (Amersham Biosciences).

**In Vitro PI3K Assays**

PI3K activity was measured using an immunoprecipitation kinase assay. Briefly 1 × 10^7 cells were lysed in 20 mM HEPES, pH 7.4, 150 mM KCl, 2 mM NaVO4, 50 mM NaF, 5 mM EDTA, 20 mM CHAPS containing protease inhibitors (Roche Applied Science) and immunoprecipitated using an anti-p65 antibody (Upstate Biotechnology, Inc.) and protein A beads (Sigma). After formation of the immune complexes, the beads were washed twice in lysis buffer and once in Nonidet P-40 PAN (20 mM PIPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40) and resuspended in 500 μl of PAN buffer (20 mM PIPES, pH 7.4, 100 mM NaCl). The reactions were started by the addition of 1 mM phosphatidylinositol and phosphatidylserine (Sigma), 10 μM ATP, and 5 μCl of [γ-32P]ATP and were performed at 37 °C. After 30 min the reactions were stopped by the addition of 50 μl of 2.5 M HCl and the lipids extracted using a chloroform/methanol mix. The labeled phosphatidylinositol was separated using thin layer chromatography and visualized by autoradiography. Quantification was performed using a phosphorimag (Fuji, UK).
**NS5A activates PI3K**

Cells were grown in either 0.5% reduced serum medium or growth medium supplemented with 10% serum until 90% confluent. Cells were lysed in 0.5 ml of radiolabeled precipitation buffer (20) containing 100 mM calyculin, 500 mM cantharidin, 1 mM NaF, and 1 mM Na<sub>2</sub>VO<sub>4</sub> plus a protease inhibitor mixture. Protein concentrations in the supernatants were quantified using the BCA protein assay (Pierce). For each assay, cell lysates (1 mg of total protein) were immunoprecipitated with 4 µg of an anti-pleckstrin homology domain Akt antibody conjugated to protein G beads (Upstate Biotechnology, Inc.) for 90 min at 4 °C. After washing, samples were resuspended in Akt kinase reaction buffer containing 10 mM MOPS, pH 7.2, 1.33 mM EGTA, 0.5 mM dithiothreitol, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM NaF, 500 mM cantharidin, 10 mM calyculin, 15 mM MgCl<sub>2</sub>, and 100 µM ATP. To this reaction buffer 3 µg of purified recombinant Bad fusion protein was added (Upstate Biotechnology, Inc.) together with 3 µCi of [γ-<sup>32</sup>P]ATP. After incubation for 15 min at 30 °C, the reactions were terminated by the addition of Laemmli loading buffer, and samples were analyzed by 10% SDS-PAGE. Levels of Akt kinase activity were determined by detection of [γ-<sup>32</sup>P] labeled Bad substrate by autoradiography. Equal loading of recombinant Bad fusion protein was confirmed by immunoblot with a rabbit anti-Bad antibody (New England Biolabs). Levels of Akt phosphorylation were determined by immunoblot analysis of Akt immunoprecipitates with an antibody specific for Ser-473-phosphorylated Akt (New England Biolabs). Overall levels of Akt were confirmed by immunoblotting with a rabbit anti-Akt antibody (New England Biolabs).

**Apoptosis Assays**

**Caspase 3 Activity Assay**—Caspase 3 activity was determined using the ApoAlert fluorescent assay kit (Clontech) according to the manufacturer’s protocol. Briefly, lysates from 2 × 10<sup>6</sup> cells were incubated with the caspase 3 substrate DEVD-7-amino-4-trifluoromethyl coumarin for 1 h at 37 °C. Cleavage of the substrate was measured using a fluorometer with a 400 nm excitation filter and 505 nm emission filter.

**DNA Fragmentation**—The number of cells undergoing nuclear fragmentation was quantitated after staining with Hoechst 33342 (Molecular Probes). Briefly, 1 × 10<sup>5</sup> cells were seeded into 12-well plates containing glass coverslips and allowed to settle for 24 h. To induce apoptosis cells were treated with 1 µg/ml etoposide for 36 h. Cell monolayers were fixed in 1% paraformaldehyde and permeabilized in 0.1% Triton X-100 in phosphate-buffered saline before staining with Hoechst 33342. Randomly chosen fields were observed under fluorescence microscopy for cells with fragmented nuclei, which were considered to be apoptotic. Data were expressed as a percentage of apoptotic cells.

**DNA Laddering**—Analysis of DNA laddering was performed using a modification of a method described previously (22). Briefly, cells were incubated for 4 days in serum-free medium prior to lysis in 500 µl of cell lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 20 mM EDTA). Cell debris and intact nuclei of non-apoptotic cells were removed by centrifugation at 1,600 × g for 5 min. Apoptotic DNA fragments were isolated by extraction of the supernatant with phenol/chloroform/isoamyl alcohol (25:24:1) followed by precipitation with 3 M sodium acetate, pH 5.2, and ethanol. After centrifugation at 20,000 × g for 15 min, pellets were resuspended in 10 µl of molecular grade water containing 1 mg/ml RNase A and incubated at 37 °C for 2 h. Apoptotic DNA fragments were resolved by 1.8% agarose gel electrophoresis at 50 volts for 3 h followed by staining with ethidium bromide and visualization under UV light.

**RESULTS**

**NS5A Binds to the SH3 Domain of the p85 Regulatory Subunit of PI3K in Vitro**—a, schematic of the structure of the p85 regulatory subunit of PI3K, p110, region required for binding to the p110 catalytic subunit. BCR, breakpoint cluster region homology domain. b, binding of NS5A to GST fusion proteins. GST alone (lane 6) or fusions of GST to the N-terminal (lane 2) or C-terminal (lane 3) SH2 domains, a fragment spanning the SH3, BCR, and N-SH2 domains (lane 4), or the SH3 domain alone (lane 5) of p85 were purified, immobilized on GA beads, and used to precipitate lysates from COS-7 cells transiently transfected with pSG5.NS5A(1a). Bound NS5A was detected by immunoblotting with a sheep polyclonal anti-NS5A antiserum (upper panel) and equal loading of GST fusion proteins was verified by Coomassie staining (lower panel). Lane 1 contains an amount of lysate from transfected cells corresponding to 20% of the input into each binding assay. A proteolytic degradation product of approximately 30 kDa also bound to GST-SH3(p85). WB, Western blot. c, binding of NS5A from different HCV genotypes to GST-SH3(p85). COS-7 cells were transfected with pSG5 vectors expressing NS5A from HCV genotype 1a, 1b, or 2a, and lysates were precipitated with GST-SH3(p85) immobilized on GA beads as above. The upper panel shows the bound protein; lane 1, 20% of the input material; equal loading of GST-SH3(p85) was verified by Coomassie staining (lower panel).

**NS5A Binds to the SH3 Domain of the p85 Subunit of PI3K via a Novel Motif**—Recent findings have shown that NS5A is capable of interacting with the mitogenic adaptor protein Grb2 (7) and members of the Src family of tyrosine kinases (8) via a highly conserved class II polyproline motif situated within the C terminus of NS5A. It has also been reported that NS5A interacts with the p85 subunit of PI3K (9); however, although that report speculated about the involvement of an N-terminal polyproline motif in NS5A and the p85 SH3 domain, the sites of interaction within both NS5A and p85 have not been formally identified.

The p85 subunit contains two SH2 domains and a SH3 domain, as well as other protein interaction domains and a region involved in binding to p110 (Fig. 1a). To map more precisely the site of interaction within p85 we obtained a panel of GST fusion proteins containing either of the two p85 SH2 domains, the p85 SH3 domain and a fragment spanning the SH3 domain and the N-terminal SH2 domain. These were used as affinity matrices to precipitate lysates from COS-7 cells transiently transfected with a pSG5.NS5A(1a) vector. Precipitated proteins were detected by immunoblotting with a sheep polyclonal NS5A antiserum. As shown in Fig. 1b, NS5A interacted with the p85 SH3 domain of PI3K (lane 5) but failed to bind either of the two SH2 domains (lanes 2 and 3). NS5A also interacted with the p85 SH3-SH2 fragment with approximately equal efficiency of binding compared with the SH3 domain alone (lane 4). Coomassie staining verified the equal loading and integrity of the various fusion proteins.

To determine whether the ability to bind the p85 SH3 domain was conserved among different genotypes of HCV, we

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**FIG. 1.** NS5A binds to the SH3 domain of the p85 regulatory subunit of PI3K in vitro. a, schematic of the structure of the p85 regulatory subunit of PI3K, p110, region required for binding to the p110 catalytic subunit. BCR, breakpoint cluster region homology domain. b, binding of NS5A to GST fusion proteins. GST alone (lane 6) or fusions of GST to the N-terminal (lane 2) or C-terminal (lane 3) SH2 domains, a fragment spanning the SH3, BCR, and N-SH2 domains (lane 4), or the SH3 domain alone (lane 5) of p85 were purified, immobilized on GA beads, and used to precipitate lysates from COS-7 cells transiently transfected with pSG5.NS5A(1a). Bound NS5A was detected by immunoblotting with a sheep polyclonal anti-NS5A antiserum (upper panel) and equal loading of GST fusion proteins was verified by Coomassie staining (lower panel). Lane 1 contains an amount of lysate from transfected cells corresponding to 20% of the input into each binding assay. A proteolytic degradation product of approximately 30 kDa also bound to GST-SH3(p85). WB, Western blot. c, binding of NS5A from different HCV genotypes to GST-SH3(p85). COS-7 cells were transfected with pSG5 vectors expressing NS5A from HCV genotype 1a, 1b, or 2a, and lysates were precipitated with GST-SH3(p85) immobilized on GA beads as above. The upper panel shows the bound protein; lane 1, 20% of the input material; equal loading of GST-SH3(p85) was verified by Coomassie staining (lower panel).
confirmed that NS5A from genotypes 1b and 2a bound GST-SH3(p85) at levels comparable with NS5A(1a) (Fig. 1c). All three NS5A proteins were expressed at equal levels, and equal loading and integrity of the GST-SH3(p85) were confirmed by Coomassie staining.

SH3 domains bind to polyproline motifs with the consensus sequence Pro-Xaa-Xaa-Pro (23). As mentioned above, NS5A(1a) contains three such motifs, an N-terminal class I polyproline motif and two class II polyproline motifs near the C terminus (Fig. 2a), referred to as FP1 (class I), FP2.1 (class II motif only present in HCV genotypes 1a and 1b), and FP2.2 (a highly conserved motif shown previously to bind Grb2). To define the role played by each of these motifs in binding to the p85 SH3 domain, three NS5A(1a) mutants were generated in which specific proline residues were replaced by alanine: PA1 (P29A/P32A), PA2.1 (P343A/P345A/P346A), and PA2.2 (P350A/P353A/P354A) (Fig. 2a). Somewhat surprisingly, none of these mutations had any effect on the ability of NS5A to interact with GST-SH3(p85) (Fig. 2b), suggesting that NS5A was able to interact with the SH3 domain of the PI3K p85 subunit via a novel binding site.

To map the p85 SH3 domain binding site within NS5A we generated a series of N- and C-terminal truncations of NS5A(1a) (Fig. 2c). Deletion of the C-terminal 28 (Δc280) or 108 (Δc340) residues of NS5A had no effect on the binding to GST-SH3(p85) (Fig. 2d). The Δc340 mutant removes both the FP2.1 and FP2.2 motifs and abolished binding to all other SH3 domains tested, including those of Grb2 and Src-like kinases (data not shown). In contrast, a deletion of 178 residues from the C terminus (Δc270) abrogated binding to GST-SH3(p85). Analysis of deletions from the N terminus demonstrated that it was possible to delete the N-terminal 270 residues of NS5A (Δn270, lane 5) without abrogating binding to GST-SH3(p85). As expected, the Δn270 mutant bound to all other SH3 domains tested, including those of Grb2 and Src-like kinases (data not shown). Equal loading of GST-SH3(p85) was confirmed by Coomassie staining (data not shown).

These data suggested that the p85 SH3 domain binding site lay between residues 270 and 340 of NS5A; to map this site more precisely, two further truncations from the C terminus were generated. As shown in Fig. 2e, a deletion of the C-terminal 148 residues (Δc300) retained the ability to bind to GST-SH3(p85). When this deletion was extended to 163 residues (Δc285), a significant reduction in the binding efficiency was observed. These data suggest that the critical region required for binding to the p85 SH3 domain lies between residues 270 and 285, and binding is enhanced by the presence of residues between 285 and 300. The region from 270 to 300 does not contain a polyproline motif (Fig. 2f), and we conclude therefore that NS5A binds to the PI3K p85 SH3 domain via a novel, non-proline-rich, motif.

To verify that this result was not the result of overexpression of HA-p85 we performed a further experiment to investigate the interaction of NS5A with the endogenous p85-p110 heterodimeric complex. Lysates from COS-7 cells either mock-transfected or transfected with pSG5.NS5A were immunoprecipitated with a monoclonal antibody to the p110 subunit. Precipitated proteins were analyzed by immunoblotting with antibodies to p110, p85, or NS5A. As expected, p85 coprecipitated with p110 (Fig. 3b); in addition, NS5A could be clearly detected in the p110 precipitate, confirming that NS5A is able to associate with the native PI3K complex in mammalian cells.

NS5A Up-regulates PI3K-kinase Activity In Vivo and In Vitro—To confirm further the physiological significance of the NS5A-PI3K interaction we next examined whether NS5A was able to modulate the activity of PI3K in vitro. PI3K was immunoprecipitated from the human hepatoma cell line HepG2, and the immunoprecipitates were subjected to an in vitro kinase assay in the presence of [γ-32P]ATP and phosphatidylinositol as a substrate. His6-tagged NS5A, NS3/4A (24), or Nef (25) was purified from baculovirus-infected S/9 cells and added to the immunoprecipitate prior to the assay. As shown in Fig. 4a the addition of 1 μg (36 nM) NS5A resulted in a 10-fold increase in the phosphotransferase activity of PI3K, whereas 1.5 μg (36 nM) NS3/4A or 0.5 μg (36 nM) Nef had little effect. As controls we confirmed that the activity of PI3K was abolished by the addition of the specific inhibitor LY294002 and was unaffected by the mitogen-activated protein kinase inhibitor PD98059. Fig. 4b shows a dose-response curve; the phosphotransferase activity of PI3K was assayed in the presence of increasing concentrations of NS5A. PI3K activity increased in proportion to the concentration of NS5A with a 50% maximal stimulation at ~4 nM NS5A.

To determine whether NS5A stimulated PI3K activity within cells we generated a stable polyclonal population of human hepatoma Huh7 cells harboring the FK5.1 culture-adapted subgenomic HCV replicon (i.e. expressing all of the HCV nonstructural proteins from NS3–5B) (26). PI3K was immunoprecipitated from either naïve Huh7 cells expressing neomycin phosphotransferase (Huh7-neo) or FK5.1 cells and assayed for phosphotransferase activity as described above. Fig. 5a demonstrates that PI3K in FK5.1 cells exhibited ~10-fold greater activity than PI3K in Huh7-neo cells. The immunoprecipitates were immunoblotted with an anti-p85 antibody to confirm that this increase in PI3K phosphotransferase activity was not caused by an increase in the expression of PI3K in replicon cells. Immunoblotting with an anti-NS5A monoclonal antibody confirmed that NS5A associated with PI3K in replicon cells.

For reasons that remain obscure we were unable to establish stable Huh-7 cells expressing the NS5A Δc270 mutant (which failed to bind to GST-SH3(p85)) (Fig. 2c). Therefore, to confirm that the stimulation of PI3K activity observed in replicon cells was caused by NS5A we established stable COS-7 cell lines expressing either wild type NS5A(1a) or the Δc270 mutant. PI3K was again immunoprecipitated from these cells and assayed for phosphotransferase activity. Fig. 5b demonstrates that COS-7 cells expressing wild type NS5A exhibited a 2-fold increase in PI3K activity compared with control cells. In contrast, COS-7 cells expressing NS5A Δc270 showed no increase in PI3K activity, consistent with the observation that Δc270 failed to bind to GST-SH3(p85). Furthermore, Δc270 could not be detected in p85 immunoprecipitates (data not shown), although appropriate expression of both NS5A and p85 was verified by immunoblotting (lower two panels). Although the NS5A-mediated stimulation of PI3K activity in COS-7 cells is not as dramatic as that observed in Huh-7 cells, this can be explained by the high basal level of
PI3K activity in COS-7 cells compared with Huh-7 cells (compare Fig. 5a, lane 1, with Fig. 5b, lane 1).

Akt Phosphorylation Is Stimulated in Huh7 Cells Expressing NS5A—A key component of the signaling pathway induced by PI3K activation is the serine/threonine kinase Akt. This kinase is activated by binding to PI(3,4,5)P3 and is phosphorylated by
phosphoinositide-dependent kinase 1, also recruited to the membrane by interaction of a pleckstrin homology domain with PI(3,4,5)P3. To determine the downstream functional consequences of NS5A-mediated PI3K activation we analyzed the levels of Akt phosphorylation in three cell lines: Huh7-neo, FK5.1, and an Huh7 cell line stably expressing NS5A alone (Huh7-NS5A). Because the replicon was established from the 1b genotype of HCV we used the corresponding NS5A(1b) allele in these experiments. Akt was immunoprecipitated (IP) with a monoclonal antibody to NS5A followed by immunoblotting (WB) for either the HA tag (i) or NS5A (ii). The reciprocal experiment was also performed. Lysates were immunoprecipitated with a polyclonal antibody to p110 before immunoblotting for p110 (i), p85 (ii), or NS5A (iii). Panel iv demonstrates appropriate expression of NS5A in the lysates.

NS5A activates PI3K

Fig. 3. Binding of NS5A to native PI3K in vivo. a, binding of NS5A to HA-tagged p85 regulatory subunit. COS-7 cells were mock-transfected (lane 1) or transiently transfected with pSG5.NS5A(1a) alone (lane 2), a plasmid driving expression of HA-tagged p85 (lane 3), or pSG5.NS5A(1a) and the HA-p85 plasmid (lane 4). Lysates were immunoprecipitated (IP) with a monoclonal antibody to NS5A followed by immunoblotting (WB) for either the HA tag (i) or NS5A (ii). The reciprocal experiment was also performed. Lysates were immunoprecipitated with a monoclonal antibody to the HA tag followed by immunoblotting for either NS5A (iii) or p85 (iv). b, binding of NS5A to the endogenous PI3K p85-p110 complex. COS-7 cells were either mock-transfected (lane 1) or transiently transfected with pSG5.NS5A(1a) (lane 2), and lysates were immunoprecipitated with a polyclonal antibody to p110 before immunoblotting for p110 (i), p85 (ii), or NS5A (iii). Panel iv demonstrates appropriate expression of NS5A in the lysates.

Fig. 4. NS5A activates PI3K in vitro. a, PI3K was immunoprecipitated (IP) from lysates of HepG2 (human hepatoma) cells. Immunoprecipitates were subjected to an in vitro kinase assay in the presence of [γ-32P]ATP and phosphatidylinositol as substrate. Reaction products were extracted as described under “Experimental Procedures” before separation by thin layer chromatography and visualization by autoradiography. Lane numbers are indicated at the top, the numbers at the bottom of the figure refer to the relative levels of phosphorylated phosphatidylinositol product, as measured by phosphorimaging. The position of the phosphorylated product is indicated by an arrowhead on the left. Lanes 1 and 8 are negative controls; both contain no immunoprecipitate, but lane 8 contains 1 μg of purified NS5A. Lanes 2–7 contain equal amounts of the immunoprecipitate (confirmed by immunoblotting for p85; data not shown) with the indicated additions. b, dose-response of PI3K activation by NS5A. PI3K activity was assayed as described in a above, with the addition of increasing amounts of purified NS5A. Activity was measured by assessing the amount of phosphorylated phosphatidylinositol product by phosphorimaging and is expressed relative to activity in the absence of NS5A (lane 1). The top panel shows the autoradiograph of phosphorylated phosphatidylinositol product, and the bottom panel shows an immunoblot (WB) for NS5A of aliquots of each reaction.

One of the target substrates of Akt is the pro-apoptotic Bcl2
Fig. 5. NS5A up-regulates PI3K activity in vitro. a, Huh7 cells expressing either neomycin phosphotransferase alone (Huh7-Neo) or harboring the culture-adapted HCV subgenomic replicon FK5.1 (25) (FK5.1) were lysed and immunoprecipitated (IP) with an anti-p85 antibody (Upstate Biotechnology, Inc.). Immunoprecipitates were subjected to an in vitro kinase assay in the presence of [γ-32P]ATP and phosphatidylinositol as substrate. Reaction products were extracted as described under “Experimental Procedures” before separation by thin layer chromatography and visualization by autoradiography. The numbers at the bottom of the figure refer to the relative levels of phosphorylated phosphatidylinositol product, as determined by phosphorimaging. The position of the phosphorylated product is indicated by an arrowhead on the left. Immunoprecipitates also were immunoblotted (WB) with polyclonal antibodies to either p85 or NS5A. b, PI3K activity in COS-7 cells stably transfected with vectors expressing either wild type NS5A(1a) or the Δc270 mutant, or control cells transfected with empty vector, was analyzed as described above. Appropriate expression of p85 and NS5A in cell lysates was verified by immunoblotting with polyclonal antibodies to either p85 or NS5A (bottom panel).

homolog Bad; phosphorylation of Bad inactivates the protein, thereby blocking the apoptotic response. To confirm that the increase in Akt phosphorylation in NS5A-expressing cells correlated with an increase in Akt activity we immunoprecipitated Akt and subjected the precipitates to an in vitro kinase assay in the presence of [γ-32P]ATP and recombinant Bad protein as a substrate. Consistent with the levels of phospho-Akt shown in Fig. 6(i), in the reduced serum samples no Bad phosphorylation could be detected in kinase assays from Huh7-neo cells, whereas very low levels of Bad phosphorylation were observed in the FK5.1 or Huh7-NS5A cells (Fig. 6(iii)). After growth in 10% serum phosphorylation of Bad was stimulated in kinase assays from all three cells, and again levels were significantly higher in FK5.1 or Huh7-NS5A cells, consistent with the increased Akt activity in these cells. We conclude from these data that NS5A-mediated stimulation of PI3K activity results in a concomitant stimulation of Akt phosphorylation and phosphotransferase activity in human hepatoma cells.

NS5A Blocks the Induction of Apoptosis in Human Hepatoma Cells—Many studies have demonstrated that activation of Akt via the PI3K pathway results in the induction of an anti-apoptotic cell survival response (27). To determine whether NS5A-mediated up-regulation of PI3K resulted in increased resistance of hepatoma cells to pro-apoptotic stimuli we analyzed three distinct parameters of apoptosis in FK5.1 and Huh7-NS5A cells and compared these with the control Huh7-neo cells. First, apoptosis was induced by serum starvation, and cell lysates were assayed for levels of caspase 3 activity using a standard fluorometric assay. Fig. 7a demonstrates that in the presence of 10% serum levels of caspase 3 activity were similar in all three cell lines. Upon serum starvation caspase 3 activity was elevated 5-fold in Huh7-neo cells; however, in comparison both Huh7-NS5A and FK5.1 cells exhibited an approximate 50% reduction in caspase 3 activation. We subsequently quantitated the absolute numbers of cells undergoing apoptosis by counting those cells exhibiting nuclear fragmentation after treatment with etoposide and staining with Hoechst 33342. Fig. 7b shows that in the absence of etoposide low levels of apoptosis (2–4%) were observed in all three cell populations. After etoposide treatment a dramatic increase in the number of apoptotic cells was observed in the control cells (Huh7-neo); however, as shown for caspase 3 activation, compared with control cells the numbers of apoptotic cells in both the Huh7-NS5A and FK5.1 populations were reduced by ~50%. Lasty, we examined the induction of DNA laddering after cleavage of chromosomal DNA at the nucleosomal boundaries. Again, after serum starvation both Huh7-NS5A and FK5.1 cells exhibited less DNA laddering than the control Huh7-neo cells (Fig. 7c), although in this case the reduction was less marked than that observed for both caspase 3 activation and nuclear fragmentation (densitometry of the agarose gels revealed that DNA laddering was ~70% of control levels in both cases).

As mentioned above we were unable to establish Huh-7 cells expressing the NS5A Δc270 mutant. We therefore examined the induction of DNA laddering by serum starvation in COS-7 cells expressing either wild type NS5A(1a) or the NS5A Δc270 mutant. Fig. 7d demonstrates that, as in Huh-7 cells, NS5A blocked the induction of DNA laddering (in this case by ~60%); however, levels of DNA laddering in cells expressing the Δc270 mutant were similar to control cells. Taken together these data suggest that NS5A, by binding to and activating PI3K, is able to induce a cell survival response.

**DISCUSSION**

The data presented in this paper demonstrate that the HCV NS5A protein interacts with the SH3 domain of the PI3K p85 regulatory subunit via a novel binding site. The results of both truncation analysis and site-directed mutagenesis clearly show that this binding does not involve any of the three canonical polyproline (SH3 binding) motifs within NS5A. A number of recent studies have demonstrated specific binding of non-polyproline peptide ligands to SH3 domains. Although there appear to be no global rules governing the primary amino acid
sequence of these non-polyproline peptide ligands, a range of consensus patterns has been identified. Some, such as RK [28]; however, others such (derived from proteins that bind to the Eps8 SH3 domain)

binding to p85 (9). However, our experiments were performed in vitro, whereas the previous study involved coimmunoprecipitation of NS5A and p85 from transiently transfected HeLa cells. Given that it has been shown previously (30) that deletion of an N-terminal amphipathic helix that targets NS5A to the endoplasmic reticulum membrane resulted in predominant localization of NS5A to the nucleus, the observation that an N-terminal deletion of NS5A failed to coprecipitate with p85 could reflect a defect in subcellular targeting.

The interaction of NSSA with the p85 regulatory subunit stimulated the phosphotransferase activity of the p110 catalytic subunit both in vitro and in vivo. The mechanism for this up-regulation is unknown. Activation of p110 can result from translocation to the membrane, thus bringing it into close contact with substrate lipids; this has been shown to be mediated through protein-protein interactions, e.g. binding of p110 directly to Ras or binding of the p85 SH2 domains to receptor tyrosine kinases.

The observation that NS5A stimulates p110 activity in vitro suggests that this mechanism is not important in this case. Under resting conditions p85 inhibits the catalytic activity of p110; binding of ligands to the regulatory subunit can activate the catalytic subunit, for example binding of tyrosine-phosphorylated peptides to the N-terminal SH2 domain of p85 relieves the inhibitory activity of p85. A tyrosine at residue 688 (within the C-terminal p85 SH2 domain) can also relieve the inhibition by an intramolecular interaction with the N-terminal SH2 domain (31). Because p110 activity can be stimulated in vitro by NS5A, this implies that NS5A binding to the p85 SH3 domain is able to relieve the inhibitory effect of p85 via a mechanism that does not involve tyrosine phosphorylation. However, we cannot exclude the possibility that NS5A might also activate PI3K indirectly by activating a tyrosine kinase that copurifies with PI3K, resulting in p85 tyrosine phosphorylation.

PI3K activation leads to a wide range of signaling events within the cell; some of these are ubiquitous and others cell type-specific. The latter include aspects of the inflammatory response in macrophages and neutrophils, e.g. cell motility, phagocytosis, and respiratory burst. Arguably the most fundamental biological process regulated by PI3K is apoptosis; cell survival is ensured by a constant inhibition of apoptotic signaling instigated by PI3K activity and mediated by effector proteins such as Akt and Bad. Long term stimulation of PI3K can result in tumorigenesis as cells are unable to undergo apoptosis in response to DNA damage or other oncogenic stimuli. Our data show that NS5A-mediated stimulation of PI3K results in the up-regulation of cell survival cascades, including Akt activation and caspase 3 inhibition. Although these effects were seen in stably transformed cell lines, we do not believe that they result from clonal differences because both the replicon and NS5A-expressing cells were polyclonal stocks generated by pooling G418-resistant cells derived from either RNA or DNA transfections. In addition the observation that NS5A up-regulates PI3K and inhibits apoptosis in two different cell types (Huh7 and COS-7) supports the hypothesis that this attribute of NS5A is cell type-independent and argues against the possibility that these data can be explained by clonal variation.

What might be the effect of NS5A-mediated PI3K activation and up-regulation of cell survival in the HCV-infected liver? We hypothesize that such stimulation would result in an impaired apoptotic response to oncogenic events in HCV-infected hepatocytes and could therefore play a role in the association between HCV infection and the development of hepatocellular carcinoma. It is difficult to see why predisposing the host to tumorigenesis would be beneficial to HCV; however, stimulation of PI3K-mediated inhibition of apoptosis could potentially...
contribute to the persistence of HCV. One of the key features of the host response to viral infection is the induction of apoptosis of virus-infected cells after recognition of these cells by cytotoxic T-cells. The persistence of HCV in hepatocytes (or other cell types) could be assisted by inhibiting the induction of apoptosis by liver-infiltrating cytotoxic T-cells.

Aside from influencing cell survival, other signaling pathways downstream from PI3K regulate cell growth, cytoskeletal rearrangement, vesicular trafficking, and protein synthesis. It is conceivable that any or all of these might impinge on HCV replication. A complete discussion of the potential implications of these processes for HCV replication would seem overspeculative at this juncture; however, it is interesting to note that the PI3K pathway regulates the activity of the cell translation machinery via the activation of the mammalian target of rapamycin (mTOR) (32) and ribosomal S6 kinase (p70S6K) (10). Taken together with the recent observations that activity of the HCV internal ribosome entry site (IRES) is regulated during the cell cycle (33), and expression of NS5A stimulates IRES function (34), it is intriguing to speculate that up-regulation of

![Fig. 7](image_url)
NS5A activates PI3K

PI3K by NS5A might modify the cellular environment to favor IRES-mediated translation.

Activation of the PI3K pathway is a common theme among both RNA and DNA viruses that establish chronic infections. Both the latent membrane protein 1 of Epstein-Barr virus (13) and the middle-T antigen of polyoma virus (35) bind to the p85 subunit, in the latter case the interaction involves phosphotyrosines in middle-T and the two SH2 domains of p85. Both of these proteins activate PI3K, although the mechanisms of activation are unclear and may involve translocation of PI3K to the membrane rather than any conformational changes. The hepatitis B virus pX protein (12) up-regulates the PI3K pathway, although there is no evidence for direct interaction with PI3K. Lastly, the human immunodeficiency virus type 1 Nef protein has been shown to bind to p85 both in vitro (14) and in vivo (14, 15). Cells expressing Nef showed elevated PI3K activity; however, unlike NS5A, Nef did not stimulate the activity of PI3K in in vitro assays (14), consistent with our observations (Fig. 4a). In the majority of these cases viral protein-mediated up-regulation of PI3K has been shown to promote cell survival. We propose that our data demonstrate that HCV NS5A can now be added to the list of these viral anti-apoptotic factors that function via the PI3K pathway. The availability of replication cell lines and other systems for the delivery of full-length HCV into hepatocytes (36) will allow the future analysis of the role of NS5A-mediated PI3K up-regulation in viral replication and pathogenesis.

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REFERENCES

1. Kim, W. R. (2002) Hepatology 36, 30–34
2. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Science 244, 359–362
3. Bartsch, R., and Lohmann, V. (2000) J. Gen. Virol. 81, 1631–1648
4. Bartsch, R. (1999) J. Viral Hepatitis 6, 165–181
5. Tanji, Y., Kaneko, T., Satoh, S., and Shimotohno, K. (1995) J. Virol. 69, 3980–3986
6. Pawson, T. (1995) Nature 373, 573–580
7. Tan, S. L., Nakao, H., He, Y. P., Vijay, S., Neddermann, P., Jacobs, B. L., Mayer, B. J., and Katz, M. G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5533–5538
8. Macdonald, A., Crowder, K., Street, A., McCormick, C., and Harris, M. (2004) J. Gen. Virol. 85, 721–729
9. He, Y. P., Nakao, H. H., Tan, S. L., Polyak, P. J., Neddermann, P., Vijay, S., Jacobs, B. L., and Katz, M. G. (2002) J. Virol. 76, 9207–9217
10. Cantley, L. C. (2002) Science 296, 1655–1657
11. Daub, S., and Driscoll, P. C. (2002) J. Biol. Chem. 277, 426–432
12. Lee, Y. I., Kang-Park, S., Do, S. I., and Lee, Y. I. (2001) J. Biol. Chem. 276, 16969–16977
13. Dawson, C. W., Tramontin, G., Elia, P. G., and Young, L. S. (2003) J. Biol. Chem. 278, 3694–3704
14. Linnemann, T., Zheng, Y. H., Mandic, R., and Peterlin, B. M. (2002) Virology 294, 240–255
15. Wolf, D., Witte, V., Lafer, B., Blume, K., Stromer, E., Trapp, S., d’Alòja, P., Schurmann, A., and Baur, A. S. (2001) Nat. Med. 7, 1217–1224
16. Yanagi, M., Purrell, R. H., Emerson, S. U., and Bukh, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8738–8743
17. Yanagi, M., St. Claire, M., Shapiro, M., Emerson, S. U., Purrell, R. H., and Bukh, J. (1998) Virology 244, 161–172
18. Yanagi, M., Purrell, R. H., Emerson, S. U., and Bukh, J. (1999) Virology 262, 250–263
19. Green, S., Inam, I., and Sheer, E. (1988) Nucleic Acids Res. 16, 369
20. Macdonald, A., Crowder, K., Street, A., McCormick, C., Sakakula, K., and Harris, M. (2003) J. Biol. Chem. 278, 17775–17784
21. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 63, 31–40
22. Yeung, M. C. (2002) BioTechniques 33, 724, 736
23. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Cell 80, 237–248
24. Aouabala, M., Holt, J., Clegg, R. A., Rowlands, D. J., and Harris, M. (2001) J. Gen. Virol. 82, 1657–1664
25. Harris, M., and Coates, K. (1995) J. Gen. Virol. 74, 1581–1589
26. Rebec, N., Lohmann, V., and Bartenschlager, R. (2001) J. Virol. 75, 4614–4624
27. Chang, F., Lee, J. T., Navolanic, P. M., Steinman, L. S., Shelton, J. G., Bhalock, W. J., Franklin, R. A., and Coubey, J. A. (2003) Leukemia 17, 590–603
28. Mongiovì, A. M., Romano, P. R., Panini, S., Mendoza, M., Weng, W. T., Musacchio, A., Cesareni, G., and Di Fiore, P. P. (1999) EMBO J. 18, 500–509
29. Kang, H., Freund, C., Duke-Cohan, J. S., Musacchio, A., Wagner, G., and Rudd, C. E. (2000) EMBO J. 19, 2889–2899
30. Braas, V., Bieck, E., Montserrat, R., Wolk, B., Hellinge, J. A., Blum, H. E., Penin, F., and Moradpour, D. (2000) J. Gen. Virol. 81, 843–858
31. Cuenca, B. D., Liu, Y. L., Mao, M. L., Zhang, Y. J., Lu, P., Srinivastic, K., and Mills, G. B. (2001) J. Biol. Chem. 276, 24554–24561
32. Sokol, A., Hudson, C. C., Hompe, J. L., You, P., Otten, D. M., Karnitz, L. M., and Abraham, T. R. (2000) Cancer Res. 60, 3504–3513
33. Honda, M., Kaneko, S., Matsushita, E., Kobayashi, K., Abe, G. A., and Lemon, S. M. (2000) Gastroenterology 118, 152–162
34. He, Y. P., Yan, W., Coto, C., Li, Y., Gale, M., and Katz, M. G. (2003) J. Gen. Virol. 84, 535–543
35. Ichi, N., and Dilworth, S. M. (2001) Oncogene 20, 7908–7916
36. McCormick, C. J., Rowlands, D. J., and Harris, M. (2002) J. Gen. Virol. 83, 383–394