Hepatitis C Virus Stimulates the Phosphatidylinositol 4-Kinase III Alpha-Dependent Phosphatidylinositol 4-Phosphate Production That Is Essential for Its Replication[▽]

Kristi L. Berger, Sean M. Kelly, Tristan X. Jordan, Michael A. Tartell, and Glenn Randall*

Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

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Phosphatidylinositol 4-kinase III alpha (PI4KA) is an essential cofactor of hepatitis C virus (HCV) replication. We initiated this study to determine whether HCV directly engages PI4KA to establish its replication. PI4KA kinase activity was found to be absolutely required for HCV replication using a small interfering RNA transcomplementation assay. Moreover, HCV infection or subgenomic HCV replicons produced a dramatic increase in phosphatidylinositol 4-phosphate (PI4P) accumulation throughout the cytoplasm, which partially colocalized with the endoplasmic reticulum. In contrast, the majority of PI4P accumulated at the Golgi bodies in uninfected cells. The increase in PI4P was not observed after infection with UV-inactivated HCV and did not reflect changes in PI4KA protein or RNA abundance. In an analysis of U2OS cell lines with inducible expression of the HCV polyprotein or individual viral proteins, viral polyprotein expression resulted in enhanced cytoplasmic PI4P production. Increased PI4P accumulation following HCV protein expression was precluded by silencing the expression of PI4KA, but not the related PI4KB. Silencing PI4KA also resulted in aberrant agglomeration of viral replicase proteins, including NS5A, NS5B, and NS3. NS5A alone, but not other viral proteins, stimulated PI4P production *in vivo* and enhanced PI4KA kinase activity *in vitro*. Lastly, PI4KA coimmunoprecipitated with NS5A from infected Huh-7.5 cells and from dually transfected 293T cells. In sum, these results suggest that HCV NS5A modulation of PI4KA-dependent PI4P production influences replication complex formation.

Hepatitis C virus (HCV) is a major public health problem with limited therapeutic options. Approximately 3% of the world's population is chronically infected with HCV (46) and is at risk for progression to end-stage liver disease and hepatocellular carcinoma. The current standard of care is treatment with pegylated interferon-alpha plus ribavirin, which is successful in only half of treated patients (12). An intensive and prolonged effort to develop therapeutics that target HCV enzymes, including the nonstructural (NS) 3 protease and NS5B RNA-dependent RNA polymerase, is progressing to the clinic. More recently, a drug that appears to target another viral protein, NS5A, has been shown to have rather spectacular properties in terms of viral replication inhibition at picomolar concentrations (13). The first HCV-specific therapeutics targeting NS3 protease activity are likely to become widely available in the near future, with projected sustained virologic responses near 68% for telaprevir and 75% for boceprevir when either is combined with the current standard of care (18, 21, 27, 28). Despite this critical advance, concerns remain over a significant rate of nonresponse and emergence of antiviral resistance (38). For this reason, there is interest in identifying novel drug targets.

A number of groups have used small interfering RNA (siRNA) screens to identify cellular cofactors of HCV infection. This approach has a two-pronged benefit in identifying

cellular targets for anti-HCV therapeutics, while also uncovering important aspects of the virus-host interactions underlying HCV infection and pathogenesis. Although there has been notable divergence in the array of host cofactors of HCV infection identified in these screens, a remarkable constant has been the identification of phosphatidylinositol (PI) 4-kinase III alpha (PI4KA) as an essential cofactor of HCV RNA replication (5, 8, 22, 40, 42, 43). This finding is further supported by data showing that the kinase inhibitors wortmannin or LY294002, used at doses known to favor inhibition of type III PI4Ks, significantly decreased HCV replication (5, 40). PI kinases and the lipids they phosphorylate are essential regulators of membrane trafficking and protein sorting. PI4KA is one of four cellular PI 4-kinases that all function to phosphorylate phosphatidylinositol at the 4 position of an inositol head group, which then serves as a beacon to recruit proteins containing lipid-binding motifs with affinity for PI4P (reviewed in references 3, 10, and 45). The PI 4-kinases differ with respect to their subcellular localization, thus generating pools of PI4P at specific membrane compartments. PI4KA is the predominant endoplasmic reticulum (ER) resident PI 4-kinase (3). In HCV-infected cells, PI4KA colocalizes with HCV NS5A and viral double-stranded RNA, the HCV replication intermediate, suggesting a role in replication complex formation or function (5, 6).

All positive-stranded RNA viruses reorganize cellular membranes to create their sites of replication. In the case of HCV, infection produces an accumulation of heterogeneous, ER-associated vesicular structures that has been termed the membranous web (11, 15, 31). This is thought to be the site of HCV

* Corresponding author. Mailing address: Department of Microbiology, The University of Chicago, CLSC 707B, 920 E. 58th St., Chicago, IL 60637. Phone: (773) 702-5673. Fax: (773) 834-8150. E-mail: grandall@bsd.uchicago.edu.

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replication, since *de novo*-synthesized viral RNA colocalizes with these structures (15). The expression of NS4B in the absence of other viral proteins produces structures reminiscent of the membranous web (11), suggesting that it is a primary mediator of membrane reorganization. PI4KA is also implicated in HCV replication complex formation. siRNAs that reduce PI4KA accumulation appear to perturb membranous web formation. In cell lines expressing the HCV polyprotein, PI4KA siRNAs alter the localization of the HCV NS5A protein (40), while membranous webs are difficult to detect in HCV-infected cells pretreated with PI4KA siRNAs (5).

This led to the hypothesis that HCV may hijack PI4KA to generate PI4P, which would serve to nucleate viral and cellular proteins to ER membranes for HCV replication complex formation (6). To investigate whether HCV infection actively engages PI4KA, as opposed to PI4KA being a passive requirement for HCV replication, we examined the PI4KA enzymatic requirements for HCV replication and whether HCV infection perturbs cellular stores of PI4P. We present data that HCV replication requires PI4KA kinase activity, leading to the accumulation of PI4P that partially colocalizes with the viral NS5A protein. This increase in PI4P accumulation required PI4KA, but not the related PI4KB. Silencing PI4KA altered the subcellular localization of viral replicase proteins. PI4P accumulation and PI4KA kinase activity could be stimulated by NS5A *in vitro* and *in vivo*, and NS5A physically interacted with PI4KA from HCV-infected cells. These results demonstrate that PI4KA is actively engaged by HCV NS5A and that its kinase activity is required for HCV replication.

MATERIALS AND METHODS

Cells. The human hepatoma Huh-7.5 cell line, described previously (7), and HEK 293T cells were grown in Dulbecco modified Eagle medium (DMEM)-high glucose (+ glutamine, + sodium pyruvate) with 0.1 mM nonessential amino acids, 5% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Invitrogen). U2OS human osteosarcoma-derived cell lines with inducible genotype 1a HCV protein expression (32) (kindly provided by Darius Moradpour, University of Lausanne) were cultured in DMEM-high glucose with 10% FBS, 1% penicillin-streptomycin, 500 µg of G418 (Invitrogen)/ml, 1 µg of puromycin (Sigma)/ml, and 1 µg of tetracycline (Sigma)/ml to repress expression. U2OS clones included UHCvcon57.3 (39), Ucp7con-9.1 (11), UNS3-4A-24 (48), UNS4Bcon-4 (20), UNS5Acon-37.2 (9), and UNS5Bcon-5 (39). To induce expression, cells were washed four times in media without tetracycline. All cells were incubated at 37°C and 5% CO₂.

Reagents and primary antibodies. Wortmannin (Sigma) and phenylarsine oxide (Sigma) were resuspended in dimethyl sulfoxide (DMSO). The primary antibodies used included mouse anti-NS5A (9E10; a gift from Charles Rice, Rockefeller University), rabbit anti-NS5B (ab35586; Abcam), mouse anti-NS3 (catalog no. 1878; ViroStat, Inc.), rabbit anti-PI4KA (catalog no. 4902; Cell Signaling Technology), rabbit anti-PI4KB (catalog no. 06-578; Millipore), mouse anti-PI4P (Echelon Biosciences), rabbit anti-GM130 (ab40881; Abcam), rabbit anti-calnexin (SPA-860; Stressgen), mouse anti-HA (HA.11 clone 16B12; Covance), and rabbit anti-actin (A2066; Sigma). For use in combination with other monoclonal antibodies, mouse anti-NS5A-488 was derived by directly conjugating Alexa Fluor-488 to 9E10 antibody using the Alexa Fluor-488 monoclonal antibody labeling kit (Invitrogen) according to the manufacturer's instructions.

Viruses. Infectious genotype 2a virus stock was synthesized by electroporating Huh-7.5 cells with viral RNA transcribed from the intragenotypic clone pJF-HxJ6-CNS2C3 as previously described in detail (26, 34). Infectious HCV was quantified via limiting dilution analysis and immunohistochemical staining of naive cells for NS5A as described previously (26). HCV was UV-inactivated by exposure to four pulses of 120 mJ/cm² positioned 7 cm from the source using a UV cross-linker (Spectronics Corp.). Subgenomic replicon RNA was transcribed from the previously constructed pSG-JFH1-RLuc (5) and electroporated into cells in the same manner as pJF-HxJ6-CNS2C3.

To generate pseudotyped lentivirus for transgene expression, HEK 293T cells

were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with three plasmids, one encoding the HIV *gag* and *pol* genes, one encoding vesicular stomatitis virus glycoprotein (VSV-G), and the transgene subcloned into the pTrip-CMV-GFP HIV-based vector (33, 49). Supernatants were collected 48 h posttransfection, centrifuged (1,200 × g, 5 min), and stored at 4°C. The cells were infected with pseudotypes in cell culture media with 8 µg of Polybrene (Sigma)/ml for 6 h total, including a spinoculation step (500 × g, 30 min).

Plasmids. PI4KA expression constructs were generated as follows. pEF1A-HA-PI4KA-WT was made by PCR amplification from the previously published pTrip-PI4KA-GFP (formerly called pTrip-PIK4CA-GFP) construct (5) with forward primer incorporating SpeI, start codon, and hemagglutinin (HA) tag (5'-AAA CTA GTC GCC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT TGT CCA GTG GAT TTC CAT-3') and reverse primer with a stop codon and SpeI (5'-AAA AAC TAG TTC AGT AGG GGA TGT CAT TCT GA-3'). High-fidelity Phusion DNA polymerase (Finnzymes) was used. pEF1A/V5-His vector (Invitrogen) and PCR products were digested with SpeI and ligated such that the vector's V5-His tag is excluded.

pTrip-PI4KA-WT was made by PCR amplification from pTrip-PI4KA-GFP (5) with forward primer with PmeI and start codon (5'-AAA GTT TAA ACC GCC ACC ATG TGT CCA GTG GAT TTC CAT G-3') and reverse primer with stop codons and XhoI (5'-AAA CTC GAG TCA TCA GTA GGG GAT GTC ATT CTG A-3'). PCR products and the pTrip-CMV-GFP vector (33, 49), which was redesigned with PmeI in the multicloning site, were digested with PmeI and XhoI and ligated such that the vector's green fluorescent protein (GFP) tag is removed.

For the kinase-deficient versions, a K1792L mutation was introduced into PI4KA. The cloning strategy was devised by using the wild-type pTrip-PI4KA-GFP construct in which the future mutation site is 13 bp away from a NaeI site and flanked by two unique XmaI sites. Segment 1 was PCR amplified using forward-XmaI primer (5'-GTT GGA CCC GGG AGC CGT TAG-3') and reverse mutation-NaeI primer (5'-CCT GGC GGC AGT CGT CTC CCA CCA AGA AGA TG-3'). Segment 2 was PCR amplified using forward mutation-NaeI primer (5'-CAT CTT CTT GGT GGG AGA CGA CTG CCG GCA GG-3'; the AA→TT mutation is underlined) and reverse-XmaI primer (5'-CTG GTC CCG GGA GGT GCA GT-3'). *Taq* polymerase (New England Biolabs) was used to generate "A" overhangs amenable to TOPO vector cloning. PCR segments were digested with NaeI, ligated, and inserted into pCR2.1-TOPO vector (Invitrogen). The segment was excised from pCR2.1-TOPO with XmaI and ligated back into the XmaI sites in pTrip-PI4KA-GFP, generating pTrip-PI4KA-KD-GFP. This plasmid served as a template for generating other kinase-deficient expression constructs using primers described earlier. All PI4KA open reading frames were fully sequenced to assure no incorporation of deleterious stop codons during PCR.

The pEF1A-NS5A construct for mammalian expression was constructed as follows. NS5A was PCR amplified from HCV genotype 2a by using a forward primer including a SpeI site (5'-AAA ACT AGT CGC CAC CAT GTC CGG ATC CTG GCT CCG CGA-3') and a reverse primer including an EcoRI site (5'-AAA GAA TTC TCA TCA GCA GCA CAC GGT GGT ATC GT-3'). pEF1A/V5-His vector (Invitrogen) and PCR products were digested with SpeI and EcoRI and ligated such that the vector's V5-His tag is excluded. The pET151-HisV5-NS5A construct for bacterial expression was generated using a pET151 Directional TOPO expression kit (Invitrogen). NS5A was PCR amplified from HCV genotype 2a by using the forward primer 5'-CACC TCC GGA TCC TGG CTC CGC G-3' and the reverse primer 5'-TTA TTA GCA GCA CAC GGT GGT ATC-3'. NS5A amplicons were inserted into pET151/D-TOPO vector according to TOPO expression kit guidelines (Invitrogen).

To express NS5BΔ21 in bacteria, which has a C-terminal truncation excluding the transmembrane domain, NS5BΔ21 was cloned into a modified pET22b(+) vector. pET22b(+) was redesigned with a 73-bp deletion following the NdeI site, which includes the *pelB* leader sequence to the BamHI site, placing BamHI in-frame with the C-terminal His₆ tag. NS5BΔ21 was PCR amplified from HCV genotype 2a by using a forward primer including BamHI (5'-AAA GGATCC TCC ATG TCA TAC TCC TGG AC-3') and a reverse primer including XhoI (5'-AAA CTCGAG GCG GGC TCG GGC GCG CGA CA-3'). Amplicons were ligated into the restriction sites in modified pET22b(+) to generate the pET22b-NS5BΔ21-His construct.

Purification of recombinant protein. N-terminally 6×His-tagged NS5A was expressed in *Escherichia coli* Rosetta strain [BL21(DE3)/(pLysS)] (Invitrogen) from the pET151-HisV5-NS5A construct by induction of a 1-liter culture at an optical density at 600 nm (OD₆₀₀) of 0.6 with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 25°C for 16 h. NS5A was then purified as described previously, with modifications (37). Pelleted bacteria were resuspended in 25 ml

of lysis buffer (100 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM MgCl₂, 2% Triton X-100, 2 mg of lysozyme [Sigma]/ml, 750 U of benzonase [Merck], Complete-Mini protease inhibitor cocktail [Roche]) and incubated on ice for 30 min. Lysates were repeatedly freeze-thawed, and cell membranes were pelleted by centrifugation at 10,000 rpm using a Sorvall SLA600TC rotor at 4°C for 15 min. Pelleted membranes were resuspended in 20 ml of resuspension buffer A (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 10% glycerol, 10 mM imidazole, 10 mM 2-mercaptoethanol, 0.1% β-octyl glucopyranoside, Complete-Mini protease inhibitor cocktail), sonicated on ice five times for 15 s each time, and rotated at 4°C for 90 min. Membranes were pelleted at 10,000 rpm and 4°C for 10 min, and the supernatants were incubated with 1 ml of Ni-NTA (Qiagen; 50% slurry) for 1 h. The beads were washed four times with 10 ml of resuspension buffer A plus 50 mM imidazole, followed by 1 wash with low-salt buffer (25 mM Tris-HCl [pH 7.5], 25 mM NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1% β-octyl glucopyranoside) containing 50 mM imidazole. His-tagged NS5A was eluted with 5 ml of low-salt buffer plus 250 mM imidazole. Protein was concentrated by using Amicon Ultra-4 filters (Millipore) and buffer exchanged with 25 mM Tris-HCl (pH 7.5)–25 mM NaCl, followed by the addition of 15% glycerol.

C-terminally His₆-tagged NS5BΔ21 was expressed in *E. coli* BL21(DE3) Star strain (Invitrogen) from the pET22b-NS5BΔ21-His construct by induction of a 1-liter culture at an OD₆₀₀ of 0.8 with 1 mM IPTG at 25°C for 4 h. Bacteria were resuspended in 30 ml of resuspension buffer B (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol) with 10 mM imidazole and French pressed twice at 14,000 lb/in². Lysate was clarified by centrifugation at 15,000 rpm for 15 min and flowed through a 1-ml Ni-NTA gravity column. The column was washed with 20 ml of buffer B plus 20 mM imidazole, followed by 10 ml of buffer B plus 50 mM imidazole. Protein was eluted in 5 ml of buffer B plus 250 mM imidazole. Since eluted protein was highly concentrated, NS5BΔ21-His was diluted in 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, and 15% glycerol. All purified proteins were quantitated by SDS-PAGE and Coomassie blue staining against bovine serum albumin standards.

Western blot analysis. Adherent cells were washed twice in 1× phosphate-buffered saline (PBS), lysed in 1× Laemmli buffer (250 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.0025% bromophenol blue), and sonicated. Proteins were separated on 4 to 20% SDS-PAGE gels (Lonza, Inc.) and transferred to nitrocellulose. After being blocked in 10% dry, nonfat milk (1× PBS, 0.1% Tween 20), primary antibodies were added overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were added for 30 min in 5% dry milk and included goat anti-rabbit (catalog no. 31462; Thermo Scientific) and rabbit anti-mouse (catalog no. 31452; Thermo Scientific), which were detected using SuperSignal-Femto chemiluminescent substrate (Pierce-Thermo Scientific) and exposure to film.

Immunoprecipitation. Plasmids were transfected into HEK 293T cells cultured on 10-cm dishes for 48 h using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Cells were washed twice in cold 1× PBS (pH 7.5) and lysed for 30 min on ice in NP-40 lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol) supplemented with Complete-Mini protease inhibitor cocktail tablets (Roche). Lysates were cleared by centrifugation (20,000 × g, 30 min) and quantified by using a Bradford assay (Bio-Rad). HA-PI4KA protein was immunoprecipitated from 500 μg of lysate by using 100 μl of prewashed M-280 sheep anti-mouse IgG magnetic Dynabeads (Invitrogen) and 5 μl of mouse anti-HA antibody (HA.11; Covance) after rotation overnight at 4°C. NS5A was immunoprecipitated similarly but using primary 9E10 antibody. Immune complexes were washed three times with NP-40 lysis buffer. For immunoblots, immune complexes were boiled in 1× Laemmli buffer. For kinase assays, HA-PI4KA immunoprecipitates were resuspended in kinase assay buffer (see below).

PI4KA *in vitro* kinase assay. To verify the kinase-deficient mutant, kinase activity was measured as previously described with slight modifications (14) using reagents resuspended in kinase assay buffer A (116 mM HEPES/KOH [pH 7.5], 27 mM MgCl₂, 116 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.4% Triton X-100 [pH 7.5], 1 mM dithioerythritol [DTE]). Each reaction (60 μl, final volume) contained 20 μl (1/40) of HA-PI4KA pulldown (beads included) and 2 mM PI. The L-α-phosphatidylinositol substrate in chloroform from bovine liver (Avanti-Polar Lipids) was prepared by first evaporating using a Speed-Vac centrifuge and then sonicating in kinase assay buffer. HA-PI4KA and PI were preincubated with drug inhibitors or DMSO vehicle for 15 min at room temperature prior to the addition of 5 μCi of [γ-³²P]ATP (Perkin-Elmer; 6,000 Ci/mmol, 150 mCi/ml) for 30 min at room temperature. Reactions were stopped by the addition of 180 μl of 1 N HCl and 480 μl of CHCl₃-methanol (1:1) and vortexed. After phase separation, the organic layer (50 μl) in 5 ml of scintillation fluid was assayed by using a liquid scintillation counter.

To assess the effects of recombinant protein on kinase activity, HA-PI4KA

FIG. 1. PI4KA kinase activity is required for HCV replication. Transcomplementation assays of Huh-7.5 cells were performed 86 h after siRNA electroporation, 72 h after pTrip-pseudoparticle infection with wild-type (WT) or kinase-deficient (KD) PI4KA, and 48 h after HCV infection. siRNAs included si-irrelevant (IRR) and si-PI4KA-endogenous (PI4KA-endg). (A) PI4KA protein expression was verified by immunoblot compared to a β-actin control. (B) HCV replication as measured by qRT-PCR and calculated relative to si-IRR-treated, vector-transduced cells. (C) Infectious virus production as measured by tissue culture infectious dose 50 (TCID₅₀)/ml from limiting dilution analysis. For panels B and C, the data are expressed based on triplicates + the SEM in a log scale. **, *P* < 0.001.

immunoprecipitates were washed four times in 1× PBS (pH 7.5) and resuspended in 2.5× concentrated kinase buffer B (1× concentrated kinase buffer B is composed of 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 0.5 mM EGTA, and 0.4% Triton X-100). PI4KA protein concentration was determined by SDS-PAGE and silver staining (SilverSNAP stain; Pierce). Small, 25-μl reactions containing 10 μl of immunoprecipitated HA-PI4KA (~25 ng diluted in 2.5× kinase buffer B) and 200 μM PI in water were preincubated for 15 min with recombinant proteins diluted in their respective buffers. Drug inhibitors (0.5 μl) or DMSO vehicle control were also preincubated during this 15 min. Reactions were started by addition of 5 μCi of [γ-³²P]ATP for 40 min. The reactions were stopped, lipids were extracted as described above, and 70 μl of organic layer in 5 ml of scintillation fluid was assayed.

siRNA transfection. siRNAs were electroporated as described previously (35, 36). Briefly, 20 million cells in 400 μl of cold 1× PBS (pH 7.4) were electroporated with 2 nmol of siRNA using 2 mM cuvettes and an ECM 830 electroporator (BTX Genetronics). Alternatively, siRNAs were reverse transfected using serum-free Opti-MEM (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. siRNA sequences included the follow-

FIG. 2. HCV infection increases PI4P production. (A) Confocal microscopy of PI4P and NS5A in Huh-7.5 cells at 48 h after treatment with infectious HCV (multiplicity of infection [MOI] = 0.2), UV-inactivated HCV, or electroporation with subgenomic (sg) HCV replicons (JFH-1 isolate). sgJFH-1 electroporated cells and null-electroporated cells were mixed 1:1 and seeded on coverslips for side-by-side comparison. PI4P (red) was detected by indirect immunofluorescence. NS5A (green) was directly detected using anti-NS5A-488-conjugated antibody staining. Nuclei were stained with DAPI. Scale bar, 20 μ m. (B) Quantitation of PI4P fluorescence per cell from multiple images from triplicate coverslips. Averages $\pm$ the SEM are shown. **, $P < 0.001$. (C) Detection of PI4P from lipid extracts of HCV- or mock-infected Huh-7.5 cells 48 h postinfection using PI4P mass strips, with findings representative of two experiments. Column a consists of three sets of extractions (1 to 3) from experimental samples. Column b consists of prespotted PI4P standards as follows (from top to bottom): 10, 5, 4, 2, 1, and 0.5 pmol.

ing (5'→3' sense strand): irrelevant siRNA (si-IRR), GGC GCU UGU GGA CAU UCU G-dT-dT (custom Thermo Scientific); full-length (230-kDa) PI4KA, GGA UAA AGC UAU UCA GAA AUU (custom Thermo Scientific); PI4KA-endogenous siRNA (si-PI4KA-endg), CCU CAA AGC UGU CCC ACA AUU; and PI4KB, GGA GGU GUU GGA GAA AGU CTT (Silencer Validated siRNA AM51331; Ambion).

Real-time RT-PCR. RNA was extracted from 96-well tissue culture cells by using an RNeasy 96 kit (Qiagen) and eluted in $\sim$ 130 μ l of RNase-free water. Extracts (2 μ l) were reverse transcribed and PCR amplified by using the SuperScript III Platinum One-Step qRT-PCR system with Platinum *Taq* (Invitrogen). HCV RNAs were amplified using 260 nM forward primer (5'-ACT TCA TTA GCG GCA TCC AAT AC-3'), 260 nM reverse primer (5'-CGG CAC TGA ATG CCA TCA T-3'), and 180 nM probe (5'-6FAM-CAG GAT TGT CAA CAC TGC CAG GGA ACC-IowaBlack-3') (Integrated DNA Technologies), which recognizes NS4B of HCV JFH-1. Reactions were multiplexed with a 0.5 $\times$ amount of 18S rRNA TaqMan gene expression assay (4319413E; Applied Biosystems) as an internal control. PI4KA RNAs were amplified by using a TaqMan gene expression assay (Hs01021073_m1; Applied Biosystems) multiplexed with a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene expression assay (#4326317E; Applied Biosystems) for normalization. Reverse transcription-PCR (RT-PCR) was programmed for 50°C for 30 min, 95°C for 6 min, and then 50 cycles of 95°C for 15 s and 60°C for 1 min using an ABI 7300 system (Applied Biosystems). The data were analyzed with SDS v1.4 software (Applied Biosystems) and normalized to internal controls. Relative quantitation was calculated by comparing the cycle threshold (C_T) values using $2^{-\Delta\Delta C_T}$.

Immunofluorescence microscopy. Glass coverslips in 24-well dishes were first coated with 100 μ g of poly-L-lysine/ml for 10 min, washed with sterile water, and air dried before seeding 50,000 cells. All washes and reagents were prepared in 1 $\times$ PBS (pH 7.5) and used at room temperature. The cells were fixed with 4% paraformaldehyde (15 min). To detect PI4P immunostaining, the cells were permeabilized for 5 min with 20 μ M digitonin (Sigma), and aldehydes were quenched for 15 min with 50 mM NH_4Cl . For NS5B and NS3 antibodies, cells were permeabilized with 0.1% Triton X-100 and 0.1% saponin. Coverslips were

then blocked for 30 min in 20% normal goat serum (Millipore). Primary antibodies in 5% goat serum were incubated overnight at 4°C. NS5B and NS3 antibodies were supplemented with 0.05% saponin. Alexa Fluor 488 or 594 secondary antibodies (Invitrogen) were used at 1:1,200 in 5% goat serum for 30 min. The mouse anti-NS5A-488 conjugated tertiary antibody in 5% goat serum was used for 1 h after several washes following secondary antibody incubations. Coverslips were mounted in ProLong Gold AntiFade with DAPI (4',6'-diamidino-2-phenylindole) nuclear stain (Invitrogen). The samples were imaged using an Olympus DSU spinning disc confocal microscope equipped with a Photometrics Evolve EMCCD camera. Digital images were taken using Slidebook v5.0 software and processed using ImageJ (National Institutes of Health). Quantification of fluorescence intensity was determined from multiple images taken from triplicate coverslips using ImageJ.

PI4P mass strips. Cells were seeded in duplicate six-well dishes, one for lipid extractions and one for cell counting with a hemacytometer. Equivalent numbers of cells (4 million) were collected in PIPES buffer (pH 6.8; 20 mM PIPES, 137 mM NaCl, 3 mM KCl, 2 mM MgCl_2 , 5.6 mM glucose) and PI4P extracted according to a detailed protocol from a PI4P Mass Strip kit (Echelon Biosciences). Lipids were spotted in triplicate onto the provided nitrocellulose membrane prespotted with PI standards and detected by using the kit's PI4P-specific detectors and SuperSignal Pico chemiluminescent reagent (Pierce).

Statistical analysis. Data are presented as means $\pm$ the standard errors of the mean (SEM). To assess statistical significance, two-tailed, paired Student *t* tests were performed.

RESULTS

PI4KA kinase activity is required for HCV replication and virus production. In order to assess the requirement of PI4KA kinase activity for HCV replication, we generated mammalian expression constructs encoding wild-type (WT) or kinase-defi-

cient (KD) PI4KA. A previously validated kinase-deficient mutant, K1792L, was based on a characterization of the ATP-binding site, which identified a well-conserved lysine residue in the C terminus that was required for catalytic activity (44). PI4KA-WT or -KD expression constructs were tested for their ability to support HCV replication using an siRNA transcomplementation assay. In this assay, siRNAs that target the 3'-untranslated region (3'UTR) of PI4KA mRNA (si-PI4KA-endg) are introduced, followed by transduction of PI4KA expression constructs, which lack the PI4KA 3'UTR and are thus resistant to siRNA treatment. This enables silencing of endogenous PI4KA, followed by complementation with WT or KD recombinant PI4KA. Huh-7.5 cells were electroporated with irrelevant siRNAs (si-IRR) or si-PI4KA-endg, maintained for 24 h, and then infected with pseudotyped pTrip-based lentiviruses expressing empty vector, PI4KA-WT, or PI4KA-KD. After lentiviral transduction for 6 h, cells were infected with J6/JFH-1 HCV for 24 h. Viral RNA and infectious virus was quantified 48 h postinfection.

Immunoblots verified that in vector-transduced cells, si-PI4KA-endg efficiently reduced endogenous PI4KA protein levels compared to si-IRR, whereas the expression of WT and KD PI4KA from plasmids in *trans* was indeed refractory to si-PI4KA-endg RNAs (Fig. 1A). Interestingly, overexpression of PI4KA-WT did not enhance the accumulation of HCV RNAs (Fig. 1B) or the release of infectious virus (Fig. 1C) relative to controls, suggesting that endogenous levels of PI4KA are adequate for optimal HCV replication and virus production in Huh-7.5 cells. When empty-vector-transduced cells were treated with si-PI4KA-endg, there was a 200-fold decrease in HCV RNA and a 141-fold decrease in titer compared to si-IRR/vector treatment. When endogenous PI4KA is silenced (si-PI4KA-endg), only transduction with the enzymatically active PI4KA-WT significantly rescued HCV replication and virus production ($P < 0.001$, Fig. 1B and C, respectively). Complementation with the siRNA-resistant PI4KA enhanced infectious HCV production 110-fold compared to the vector alone. Even though transduction with PI4KA-KD resulted in higher PI4KA protein expression than WT (Fig. 1A), PI4KA-KD was unable to transcomplement HCV replication and infectious virus production. These results further validate PI4KA as a critical cellular cofactor for viral replication and demonstrate that its lipid kinase activity is specifically required for HCV replication.

HCV infection enhances PI4P production but not PI4KA abundance. We next evaluated the effects of HCV replication on the accumulation of PI4P, the end product of PI4K activity. Huh-7.5 cells were infected for 48 h with untreated or UV-inactivated J6/JFH-1 HCV, fixed and immunostained for HCV NS5A and PI4P. We observed elevated PI4P fluorescence in cells infected with HCV, as identified by positive staining for the viral NS5A protein (Fig. 2A). PI4P partially colocalized with or was in close proximity to NS5A foci under these fixation and permeabilization conditions. Quantification of PI4P fluorescence shows that HCV infection significantly induced PI4P by at least ~2.5-fold per cell compared to uninfected cells ($P < 0.001$) (Fig. 2B). In parallel, lipid extracts were prepared from mock- or HCV-infected cells and probed for PI4P content using PI4P mass strips that contain known loading standards (Fig. 2C, column b). Analysis of PI4P from triplicate sets

FIG. 3. PI4KA abundance is unaltered by HCV infection. Huh-7.5 cells were infected with UV-inactivated (UV-HCV) or infectious HCV (MOI = 3) and analyzed at indicated hours postinfection (HPI). (A) Total PI4KA mRNA was quantified by real-time RT-PCR from triplicate infected samples for each time point and calculated relative to a 2-h sample. The SEM is shown. ns, not significant. Results are representative of three experiments. (B) HCV RNA transcripts from the same samples as in panel A, similarly quantified. (C) Proteins analyzed at the indicated time points (in hours) postinfection by immunoblotting for PI4KA, NS5A, and β -actin.

of lipid extracts showed an ~8-fold stimulation of PI4P production in HCV-infected cells compared to mock-infected cells as quantified using ImageJ (Fig. 2C, column a).

UV-inactivated HCV did not alter PI4P fluorescence, suggesting that viral entry events cannot account for the enhanced PI4P production (Fig. 2A). Furthermore, cells electroporated with subgenomic JFH-1 replicons (seeded with null-electroporated cells for comparison) also displayed enhanced PI4P fluorescence, suggesting this phenotype does not require expression of the viral structural proteins but rather establishment of replication complexes by nonstructural NS3-NS5B proteins (Fig. 2A).

We next examined whether the increase in PI4P reflected a general increase in PI4KA RNA or protein accumulation. PI4KA and HCV RNA levels were measured over a time course of HCV infection or UV-inactivated HCV treatment (Fig. 3A and B). PI4KA RNA levels did not significantly differ between HCV infection or UV-HCV treatment. Similarly, PI4KA protein levels remained unchanged over time in HCV-infected cells and were similar to cells treated with UV-inactivated HCV (Fig. 3C). These data suggest that the observed increased production of PI4P in HCV-infected cells is likely due to stimulation of resident PI4KA by HCV and does not reflect elevated PI4KA transcript or protein levels.

FIG. 4. Redistribution of PI4P by HCV infection. PI4P (red), GM130 (green), and calnexin (green) were detected by indirect immunofluorescence in Huh-7.5 cells either mock or HCV infected for 48 h (MOI = 3). Viral infection was detected by direct immunostaining for NS5A using anti-NS5A-488-conjugated antibody. PI4P fluorescence is normalized between images of mock-infected and infected cells for comparison (red = 166-ms exposure). Nuclei were stained with DAPI. *, Cell magnified in the bottom panel. Scale bar, 10 μ m.

Localization of PI4P during HCV infection. PI4P localization has a distinct pattern in cells infected with HCV (extensive cytoplasmic distribution) compared to uninfected cells (Fig. 2). To further study the localization of PI4P, Huh-7.5 cells were either mock or HCV infected and probed with antibodies to PI4P and markers of the ER (calnexin) or Golgi bodies (GM130). In mock-infected cells, PI4P localized similarly to GM130, but not calnexin (Fig. 4), as expected since the majority of PI4P normally resides at the Golgi apparatus and not the ER. HCV-infected cells displayed enhanced PI4P production throughout the cytoplasm. Although a fraction of PI4P remained localized with the Golgi apparatus, the majority of PI4P accumulated throughout the cytoplasm and partially colocalized with the ER marker calnexin (Fig. 4).

HCV polyprotein or NS5A expression enhances PI4P accumulation. To investigate whether a viral protein was responsible for PI4P induction, we utilized U2OS cell lines that inducibly express individual genotype 1a HCV proteins or the entire polyprotein upon removal of tetracycline from cell culture media. These cell lines have been previously used to describe HCV-induced membranous web formation by the HCV polyprotein and NS4B (11, 32). This approach had two benefits: (i) it allowed us to investigate the contribution of individual viral proteins to PI4P accumulation, and (ii) it enabled us to examine the roles of cellular PI 4-kinases for PI4P production independent of their effects on viral replication. For each cell line examined, the confocal imaging parameters were kept constant between uninduced and induced conditions

for comparative analysis of fluorescence intensity. Induction of viral proteins was verified using HCV-specific antibodies.

In U2OS cell lines that were uninduced, the majority of PI4P accumulated at the perinuclear Golgi region (Fig. 5A), as was the case for uninfected Huh-7.5 cells (Fig. 4). The induction of HCV polyprotein expression in UHCV cells resulted in increased PI4P accumulation with a broad cytosolic distribution in which a portion of PI4P foci colocalized with NS5A (arrowheads, Fig. 5B), indicating that HCV genotype 1a is also capable of stimulating PI4P accumulation. Some PI4P foci also were observed without NS5A colocalization (arrows, Fig. 5B). For induced UHCV cells, 99% displayed redistributed cytosolic PI4P (Fig. 5C). We next examined cells expressing the individual proteins to pinpoint the viral protein responsible for PI4P induction. Induction of UNS5A cells resulted in the increased accumulation of PI4P foci in the cytoplasm, many of which colocalized with NS5A (arrowheads, Fig. 5B). PI4P foci, which were redistributed throughout the cytoplasm, were observed in 65% of induced UNS5A cells and only 11% of uninduced UNS5A cells (Fig. 5C). Induction of NS5B had no effect on PI4P accumulation in UNS5B cells (Fig. 5B and C), and this was also true for UCp7 (core through p7), UNS34-A, and UNS4B cell lines (data not shown). In sum, HCV polyprotein and NS5A expression stimulated accumulation of PI4P in the cytosol, suggesting NS5A is involved in modulating PI4P levels during infection. We cannot rule out, however, possible accessory contributions of other viral proteins in modulating PI4P production.

FIG. 6. PI4KA coimmunoprecipitates with NS5A. (A) Huh-7.5 cells were electroporated with full-length HCV RNAs, and lysate was collected 48 h later from a 150-mm dish. Lysates were divided equally between immunoprecipitation reactions with anti-mouse IgG magnetic beads containing NS5A primary antibody (+Ab) or without (–Ab). NS5A and endogenous PI4KA (endg.PI4KA) were detected by immunoblotting (IB). The input represents 1% extract, and IP is 25% of the total pull-down; the results are representative of three independent experiments. (B) HEK 293T cells were cotransfected with HA-tagged PI4KA and untagged NS5A expression constructs, and lysates were collected at 48 h posttransfection, immunoprecipitated, and immunoblotted as in panel A. The PI4KA immunodetection in panel B represents both HA-tagged and endogenous proteins.

NS5A interacts with PI4KA in HCV-infected Huh-7.5 cells or cotransfected 293T cells. Relocalization of PI4P production in the cytoplasm upon induction of NS5A expression could result from a physical interaction between NS5A with PI4KA. Analysis of detergent-resistant membranes showed NS5A and PI4KA cofractionate (5), and a previous yeast two-hybrid analysis reported the interaction of NS5A with PI4KA (1). We report here that this interaction occurs in HCV-infected Huh-7.5 cells, showing that endogenous PI4KA immunoprecipitates with NS5A (Fig. 6A). Since NS5A interacts with multiple cellular proteins, it is reasonable that only a fraction of input PI4KA was capable of associating during immunoprecipitation. In addition, 293Ts were cotransfected with NS5A and HA-PI4KA expression constructs. Exogenous PI4KA coimmunoprecipitated with NS5A immune complexes (Fig. 6B), demonstrating their interaction can occur independent of other HCV proteins.

Development of an *in vitro* PI4KA kinase assay. PI4KA was N terminally tagged with an HA epitope to facilitate immunoprecipitation with a commercially available HA antibody, as has been done in PI4K type II enzyme assays (4). Empty pEF1A vector control, HA-tagged PI4KA-WT, or HA-tagged PI4KA-KD constructs were transfected into HEK 293T cells and then immunoprecipitated using anti-HA antibody and

magnetic beads conjugated to anti-mouse IgG (Fig. 7A, lanes 1 to 3), compared to IgG-coated beads alone (no HA antibody) (lanes 4 and 5). As shown, equivalent amounts of HA-PI4KA-WT and HA-PI4KA-KD were immunoprecipitated and measured for enzyme activity using an *in vitro* kinase assay (Fig. 7B) based on previous studies (14, 44). The data show HA-PI4KA-WT, but not HA-PI4KA-KD, was catalytically active in our kinase assay. We verified that the kinase activity reflected the properties of PI4KA by demonstrating that it could be inhibited by known PI4KA inhibitors (3), including 500 nM wortmannin and 5 μ M phenylarsine oxide (PAO).

NS5A enhances PI4KA kinase activity. Given the physical interaction between NS5A and PI4KA and NS5A's ability to stimulate PI4P production *in vivo*, we next tested whether NS5A stimulates PI4KA kinase activity *in vitro*. Increasing amounts of purified recombinant HCV NS5A or HCV NS5B protein (Fig. 7C) were added to kinase assay reactions containing immunoprecipitates of HA-PI4KA (Fig. 7C). We observed significant dose-dependent increases in PI4KA activity with increasing amounts of NS5A, but not NS5B (Fig. 7D, $P < 0.005$). This activity was inhibited by 5 μ M PAO, demonstrating that the stimulation was PI kinase specific. No PI kinase activity was associated with purified NS5A alone, as expected. These results demonstrate that NS5A directly stimulates the activity of PI4KA.

PI4KA is required for PI4P induction in cells expressing the HCV polyprotein. There is a consensus in the literature regarding a requirement for PI4KA in HCV replication (5, 8, 22, 40, 42, 43). The case for PI4KB is less convincing across HCV genotypes, since the majority of studies have failed to agreeably identify it as a cofactor of HCV replication. We have previously shown that PI4KA, but not PI4KB or type II PI 4-kinases, is required for efficient HCV genotype 2a replication (5). To address which type III PI 4-kinase is responsible for enhanced PI4P synthesis following HCV polyprotein expression, the U2OS UHCV cell line was treated with siRNAs—either si-IRR, si-PI4KA, or si-PI4KB—and then uninduced or induced for viral polyprotein expression. Immunoblots and immunofluorescence were examined in samples at 54 h after siRNA treatment and at 32 h postinduction.

Immunoblots show specific and efficient knockdown of PI4KA or PI4KB by their corresponding siRNAs compared to si-IRR (Fig. 8A). Delivery of siRNAs by transfection targeted nearly 100% of cells, as evidenced by the loss of PI4KB immunofluorescence staining in si-PI4KB-treated cells compared to si-IRR (Fig. 8B). We first examined the effect of HCV polyprotein expression on PI4KB localization and whether PI4KB localization overlapped with that of the induced PI4P accumulation. The majority of PI4KB in uninduced cells was localized to the Golgi region in si-IRR-treated cells, as ex-

FIG. 5. HCV NS5A enhances PI4P accumulation in stable U2OS cell lines with inducible HCV protein expression. U2OS osteosarcoma UHCV (polyprotein), UNS5A, and UNS5B cell lines were grown in either uninduced conditions (A) or induced expression conditions (B) and imaged 48 h postinduction. In both panels A and B, PI4P (red) and NS5B (green) were detected using indirect immunofluorescence. NS5A was directly detected using anti-NS5A-488-conjugated antibody. PI4P fluorescence imaging parameters were kept constant for all images in A and B (red = 100-ms exposure). *, Cell magnified in the bottom image in panels A and B. Arrowheads denote the foci of PI4P colocalized with NS5A. Arrows denote PI4P foci without NS5A. Nuclei were stained with DAPI. Scale bar, 20 μ m. (C) Quantitation of images represented in panels A and B as the percent cells with PI4P foci redistributed throughout the cytosol from uninduced or induced cells, as verified by viral protein immunostaining. n, the total number of cells. The data were from two independent experiments for UHCV and UNS5A and one for UNS5B.

FIG. 7. NS5A stimulates PI4KA activity. (A) HEK 293T cells were transfected with empty vector or plasmids encoding HA-tagged wild-type (WT) or a kinase-deficient (KD) mutant (K1792L) of PI4KA. PI4KA was immunoprecipitated (IP) using anti-HA antibody [IP (+)Ab; lanes 1 to 3] and verified by PI4KA immunoblotting (IB) compared to lysates incubated with beads alone without HA antibody [IP (-)Ab; lanes 4 to 5]. IP is 8% of total pull-down. (B) Immunoprecipitates of beads only or HEK 293T cells transfected with vector, HA-PI4KA-WT, or HA-PI4KA-KD were added to an *in vitro* kinase reaction in triplicate (see Materials and Methods). The concentrations of drugs used were as follows: 500 nM wortmannin (WORT) or 5 μ M phenylarsine oxide (PAO) diluted in DMSO. The SEM is shown for all data points but is indiscernible for some samples. (C) Silver-stained 4 to 20% SDS-PAGE gel showing the purity of recombinant HisV5-NS5A and NS5BΔ21-His proteins. HA-PI4KA (~230 kDa) was specifically immunoprecipitated by HA antibody, (+)Ab. The negative control, (-)Ab, consisted of immunoprecipitates from HA-PI4KA-transfected lysate lacking HA antibody. (D) The kinase assay activity of ~25 ng of immunoprecipitated HA-PI4KA, or equivalent (-)Ab control, was measured after the addition of purified NS5A or NS5B (see Materials and Methods). 1 $\times$, 5 $\times$, and 25 $\times$ represent the molar excesses of viral proteins compared to HA-PI4KA. For PI4KA inhibition, 5 μ M PAO diluted in DMSO was used. Counts per minute (CPM) + the SEM represent results for triplicate reactions. *, $P < 0.005$ compared to empty buffer (none) from HA-PI4KA reactions. The results are representative of four experiments.

pected, since the role of PI4KB in generating Golgi apparatus-resident PI4P is well documented (47). However, PI4KB localization was unaffected by the induction of HCV polyprotein expression (Fig. 8B, si-IRR panels). Also, PI4KB did not co-localize with HCV-induced PI4P accumulation, and silencing PI4KB had no effect on the enhanced PI4P accumulation following HCV protein induction (Fig. 8B, si-PI4KB panels). These results indicate that PI4KB is not targeted by HCV for increased PI4P production.

We next examined the role of PI4KA in HCV-induced PI4P accumulation. Induction of HCV polyprotein expression was first verified by NS5A immunofluorescence for all siRNAs tested (Fig. 9A). si-PI4KA treatment resulted in the generation of aberrant, large globules of NS5A immunostaining, as previously reported (40), compared to smaller NS5A foci observed in si-IRR- and si-PI4KB-treated cells (Fig. 9A). These globular NS5A structures are thought to represent abnormalities in membranous web formation (40).

We then assessed the contribution of each type III PI 4-kinase to changes in PI4P localization with respect to the Golgi complex following HCV expression (Fig. 9B). Critical for analysis of PI4P localization, images were captured at a time (described earlier) when PI4P lipid pools were still detectable in si-PI4KB-treated cells, despite the loss of PI4KB protein ex-

pression (Fig. 8). Although GM130 fluorescence was less intense in si-PI4KB-treated cells than for other siRNAs, the Golgi apparatus did not appear to be completely disrupted at this time point (Fig. 9B). Longer periods of PI4KB silencing resulted in the eventual loss of PI4P signal and Golgi integrity, as previously suggested (41, 47), which would preclude analysis. Under all uninduced conditions, PI4P resided at the perinuclear Golgi region, which was positive for GM130 (Fig. 9B). Again, HCV polyprotein induction in si-IRR-treated cells enhanced PI4P accumulation throughout the cytoplasm (Fig. 9) that extended beyond the Golgi complex (Fig. 9B). However, treatment with PI4KA siRNAs prevented widespread PI4P accumulation in the cytosol upon HCV induction (Fig. 9), since PI4P fluorescence was restricted to only the Golgi bodies (Fig. 9B). Analysis of PI4P in si-PI4KB-treated cells that were NS5A positive revealed a broad, cytoplasmic accumulation of PI4P that was similar to si-IRR-treated induced cells (Fig. 9A). This PI4P accumulation in si-PI4KB-treated cells corresponded to enhanced cytosolic PI4P staining that extended beyond PI4P detected at the Golgi apparatus (Fig. 9B).

We assessed the changes in PI4P distribution by quantifying the number UHCV cells with PI4P immunostaining that was broadly extended beyond association with the Golgi marker GM130, termed PI4P ER/cytosolic, from the microscopy im-

FIG. 8. RNA interference analysis of PI4P production in UHCV cells. The U2OS cell line with inducible HCV polyprotein expression (UHCV) was treated with siRNAs targeting PI4KA, PI4KB, or irrelevant (IRR), followed by growth in either uninduced or induced media conditions. The data were collected 54 h after siRNA and at 32 h postinduction. (A) Immunoblot of PI4KA, PI4KB, and β -actin control of UHCV cells treated with siRNAs and grown under induction conditions. (B) PI4P (red) and PI4KB (green) were detected by indirect immunofluorescence for siRNAs and growth conditions indicated. For red and green channels, imaging parameters were constant between all images for comparison (exposure: red = 42 ms; green = 295 ms). A DAPI nuclear stain was performed. Scale bar, 20 μ m.

ages represented in Fig. 9B. For induced HCV expression, the number of NS5A-positive cells in each siRNA-treated population was also counted from images represented in Fig. 9A. Under uninduced conditions, NS5A expression was repressed, and there was no appreciable cytosolic accumulation of PI4P in regions outside of the Golgi apparatus for all siRNA treatments (Fig. 9, uninduced). After induction, ~80% of the UHCV cells treated with si-IRR or si-PI4KA expressed NS5A, whereas only ~50% of cells treated with si-PI4KB had detectable NS5A foci (Fig. 10). The quantitation confirmed that HCV protein induction resulted in increased widespread cytosolic PI4P in both si-IRR- or si-PI4KB-treated cells, which corresponded to the number of NS5A-expressing cells (Fig. 10). In contrast, si-PI4KA prevented the cytosolic accumulation of PI4P following HCV polyprotein induction (Fig. 10). The results of our siRNA analysis confirm that HCV targets PI4KA, not PI4KB, to enhance PI4P production in the cytoplasm. Taken together, our overall analysis of U2OS inducible cell lines shows that HCV NS5A is involved in upregulating cytosolic PI4P production and that this upregulation requires PI4KA.

PI4KA impacts the subcellular localization of HCV replicase proteins. Since silencing PI4KA leads to aberrant aggregation of NS5A immunostaining, we next examined the impact on other viral proteins known to associate with viral replication complexes, such as the viral RNA-dependent RNA polymerase NS5B and viral protease NS3. We transfected the U2OS UHCV cell line with irrelevant (si-IRR) or PI4KA siRNAs, followed by HCV polyprotein induction for 48 h. NS5A, NS5B, and NS3 immunostaining were not detected above background in uninduced cells (data not shown). Similar to our observations at 32 h postinduction (Fig. 9), si-PI4KA prevented the enhanced cytosolic accumulation of PI4P and led to large ag-

gregates of mislocalized NS5A at 48 h postinduction (Fig. 11). For si-IRR treatment, NS5B had a widespread, reticular pattern of staining with foci that colocalized with NS5A. NS3 similarly colocalized with NS5A foci. However, when PI4KA was silenced, NS5B's overall reticular localization was unchanged, but larger foci appeared that overlapped with the aberrant NS5A globular immunostaining. Likewise, NS3 immunostaining accumulated into larger masses which entirely colocalized with NS5A. Although si-PI4KA did not disrupt the ability of NS5A to localize with other replicase proteins, it did cause an aberrant agglomeration of replicase proteins and thus further highlights PI4KA's role in organizing HCV replication complexes.

DISCUSSION

In this study, we investigated the requirements of PI4KA enzymatic activity for HCV replication and the effect of HCV on this lipid kinase activity for PI4P generation. An siRNA transcomplementation assay was developed, wherein ectopic expression of siRNA-resistant, wild-type PI4KA, but not a kinase-deficient PI4KA containing a K1792L mutation, could restore HCV replication in cells silenced for endogenous PI4KA expression. Although PI4P was predominant at the Golgi apparatus in uninfected Huh-7.5 cells, PI4P accumulated in a broader cytosolic distribution that partially overlapped with NS5A and an ER marker in HCV-infected cells. These results implicate the generation of PI4P as an essential step during HCV replication.

Enhanced PI4P accumulation was demonstrated in three systems, including HCV 2a infection, subgenomic HCV 2a replication, and inducible HCV 1a polyprotein expression in the established U2OS cell line. Inducible expression of NS5A

FIG. 9. PI4P induction by HCV polyprotein expression is dependent on PI4KA. U2OS UHCV cells were examined under the same conditions as in Fig. 8. (A and B) Indirect immunofluorescence of PI4P (red) in uninduced cells or cells induced for HCV polyprotein expression treated with irrelevant (IRR), PI4KA, or PI4KB siRNAs with direct detection of NS5A (green) using anti-NS5A-488 antibody (A) or with indirect detection of Golgi-marker GM130 (B). The labels for panel B are as described for panel A. Representative images are shown. Scale bar, 10 μ m. Red and green fluorescence imaging parameters were kept constant among all images within each panel (exposure, panel A: red = 102 ms, green = 73 ms; exposure, panel B: red = 85 ms, green = 2,208 ms).

FIG. 10. ER/cytosolic PI4P accumulation following HCV polyprotein expression is dependent on PI4KA. Quantitation is based on the experimental setup and microscopy shown in Fig. 8. Comparing uninduced versus induced growth conditions for each siRNA treatment, the number of UHCV cells with broad cytosolic PI4P distribution beyond GM130 (PI4P-ER/cytosolic) was quantified from multiple images represented in panel B. The number of NS5A-expressing cells in each siRNA-treated population was quantified from multiple images represented in panel A. The total number of cells counted (n) is indicated.

alone generated foci of PI4P throughout the cytoplasm, which colocalized with NS5A. NS5A has been reported to physically interact with PI4KA (1), and we demonstrated this interaction in infected cells and cells dually transfected with plasmids expressing each protein. Thus, NS5A interacted with PI4KA in the absence of other viral proteins and its induced expression resulted in the increased accumulation of the PI4KA product, PI4P. These results correspond to an ability of NS5A to directly stimulate PI4KA activity. While the present report was in review, results were published that are consistent with a role for HCV NS5A in the PI4KA interaction and its role in stimulating PI4KA activity (23, 37).

Although two publications reported an increased accumulation of PI4P in cells with replicating HCV, they differ in their identification of the kinase responsible, with one identifying PI4KB as the dominant kinase (19), while another study implicated PI4KA (37). We have previously shown that silencing PI4KA, but not PI4KB, inhibits HCV 2a replication (5). In the present study, we evaluated the contribution of the two type III PI 4-kinases to cytosolic PI4P accumulation by HCV using the inducible U2OS cell lines. PI4KB is required for sustained PI4P synthesis at the Golgi body, a process that was unchanged by HCV infection (Fig. 4) or HCV polyprotein expression (Fig. 9B). In contrast, PI4KA silencing had a minimal effect on PI4P accumulation in uninduced cells but completely precluded the enhanced accumulation of PI4P in the cytoplasm that is associated with HCV polyprotein expression. These results implicate PI4KA as the major contributor to stimulated PI4P production by HCV. Furthermore, PI4KA was required for proper localization of viral replicase proteins, NS5A, NS5B, and NS3. In sum, our data suggest that HCV hijacks and stimulates PI4KA to generate PI4P, and possibly other phosphatidylinositol phosphates (PIPs), to promote the proper organization of HCV replication complexes.

The purpose of PI4P production and PI4KA activity for HCV replication remains poorly defined. Interestingly, NS5A expression in the absence of other viral proteins resulted in PI4P accumulation that colocalized with NS5A, while full HCV polyprotein expression leads to more widespread PI4P accumulation with a lower degree of NS5A colocalization (Fig. 5). This difference suggests that NS5A stimulation of PI4P production may precede the further membrane alterations associated with the formation of HCV replication complexes. It is possible that another nonstructural viral protein, e.g., NS4B,

FIG. 11. PI4KA is required for proper localization of HCV replicase proteins. The U2OS UHCV cell line was examined at 72 h after siRNA treatment, either si-irrelevant (si-IRR) or si-PI4KA, and at 48 h postinduction of HCV polyprotein expression. For (red) indirect immunofluorescence detection of PI4P, the cells were permeabilized with digitonin, whereas for the detection of NS5B and NS3, Triton X-100 and saponin were used. NS5A was directly detected using anti-NS5A-488-conjugated antibody (green). NS5A, NS5B, and NS3 were not detected under uninduced conditions (data not shown). For red channels, imaging parameters were constant between si-IRR and si-PI4KA within each antibody sample set. DAPI nuclear stain was included. Scale bar, 10 μ m.

or the full repertoire of nonstructural proteins could contribute to the widespread distribution of PI4P-laden membranes.

A number of cellular proteins with PI4P-binding domains are required for HCV replication, including OSBP (2), PIP5K1A (8), and PIK3C2G (5), suggesting PI4P may be required to recruit these or other cellular cofactors to replication complexes. PI4P may also act as a substrate for other cellular kinase cofactors to generate other PIPs, such as PI(4,5)P₂ or PI(3,4)P₂. Alternatively, viral proteins may bind PIPs. Although HCV proteins do not contain canonical PIP-binding motifs, a number of nonpredicted PIP-binding proteins have been identified experimentally. For example, in the case of poliovirus, the RNA-dependent RNA polymerase preferentially binds PI4P, and this may influence replicase activity (19). This leads to a model wherein HCV hijacks PI4KA to generate PI4P, and perhaps other PIPs, to nucleate cellular vesicles trafficking with the viral replicase to establish replication complexes (6). More work is required to test the validity of this model and function(s) of PI4P in HCV replication.

The identification of PI4Ks as replication cofactors for HCV and, recently, for enteroviruses (19) suggests that they may belong to a common class of viruses that rely on PI4K modulation of vesicular trafficking to establish replication complexes. Neither PI4KA nor PI4KB are required for replication of dengue virus (16). Instead, flaviviruses such as West Nile virus and dengue virus hijack lipid synthetic enzymes to generate excess lipids at sites of viral replication that are contiguous with the ER (18, 25, 60). West Nile virus drives cholesterol synthesis at sites of viral replication (25), while dengue virus NS3 recruits fatty acid synthase to sites of viral replication and stimulates its activity (16). These excess ER membranes are presumably bent by proteins that induce membrane curvature (25, 29, 30, 50). Thus, there appear to be at least two major models for viral replication complex formation: one that targets PI4Ks to modify vesicular trafficking and a second mechanism that stimulates *de novo* lipid synthesis coupled with membrane curvature (17).

Cellular cofactors have attractive properties as antiviral targets. They are likely to have different drug resistance profiles than viral targets. In addition, host factors are distinct targets from the viral enzymes and, as such, are attractive to include in combination therapies to limit resistance. PI 3-kinases have been successfully targeted for cancer therapeutics (24). Although PI4K inhibitors are not yet in the clinic, they have potential in the development of HCV therapeutics. The present study demonstrates that the PI4KA kinase activity is essential for HCV replication and also describes an assay that can be used to identify PI4KA inhibitors. This may aid in the development of PI4KA inhibitors to include in the therapeutic arsenal against HCV.

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