Phosphatidylinositol 4,5-Bisphosphate Is an HCV NS5A Ligand and Mediates Replication of the Viral Genome

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BACKGROUND & AIMS: Phosphoinositides (PIs) bind and regulate localization of proteins via a variety of structural motifs. PI 4,5-bisphosphate ($PI[4,5]P_2$) interacts with and modulates the function of several proteins involved in intracellular vesicular membrane trafficking. We investigated interactions between $PI(4,5)P_2$ and hepatitis C virus (HCV) nonstructural protein 5A (NS5A) and effects on the viral life cycle. **METHODS:** We used a combination of quartz crystal microbalance, circular dichroism, molecular genetics, and immunofluorescence to study specific binding of PI(4,5)P2 by the HCV NS5A protein. We evaluated the effects of PI(4,5)P2 on the function of NS5A by expressing wild-type or mutant forms of Bart79I or FL-J6/JFH-5'C19Rluc2AUbi21 RNA in Huh7 cells. We also studied the effects of strategies designed to inhibit $PI(4,5)P_2$ on HCV replication in these cells. **RESULTS:** The N-terminal amphipathic helix of NS5A bound specifically to $PI(4,5)P_2$, inducing a conformational change that stabilized the interaction between NS5A and TBC1D20, which is required for HCV replication. A pair of positively charged residues within the amphipathic helix (the basic amino acid PI(4,5)P2 pincer domain) was required for $PI(4,5)P_2$ binding and replication of the HCV-RNA genome. A similar motif was found to be conserved across all HCV isolates, as well as amphipathic helices of many pathogens and apolipoproteins. **CONCLUSIONS:** $PI(4,5)P_2$ binds to HCV NS5A to promote replication of the viral RNA genome in hepatocytes. Strategies to disrupt this interaction might be developed to inhibit replication of HCV and other viruses.

Keywords: QCM; Antiviral Strategies; Signaling Molecule; Phospholipid.

Hepatitis C virus (HCV) is an important cause of chronic liver disease. Many key details of the viral life cycle remain unknown. The HCV nonstructural protein 5A (NS5A) harbors an N-terminal amphipathic helix (AH) that is necessary and sufficient for mediating the association of NS5A with cellular-derived and model membranes.[1–3] This membrane association appears to involve a host cell membrane protein partner[2] and is essential for membrane-associated viral RNA replication.[1,3] NS5A also has been found to interact with regulators of host cell vesicular membrane trafficking machinery.[4,5] Phosphoinositides (PIs) have long been known to mediate key intracellular signaling pathways[6–8] and, more recently, also have been recognized as playing important roles in the subcellular localization of PI-interacting proteins that bind PIs via a variety of structural motifs.[9,10] In particular, PIs such as phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) are recognized by, and can modulate the function of, several proteins involved in intracellular vesicular membrane trafficking.[11,12] We therefore hypothesized that the NS5A AH also might bind PIs and that this interaction is essential for viral replication. Indeed, we found that although the amphipathic nature of the AH is the primary determinant of the association of NS5A with lipid membranes, the NS5A AH also harbors a motif that specifically binds $PI(4,5)P_2$, that such binding induces a key conformational change within the AH, and that $PI(4,5)P_2$ binding is essential for viral genome replication. Moreover, this novel $PI(4,5)P_2$-binding motif is found in a variety of pathogens. These results provide a molecular explanation for recent reports[13–16] of PI-kinase involvement in the HCV life cycle, and suggest novel potential antiviral therapeutic strategies.

Materials and Methods

Vesicles Containing Polymerized PIPs

The molar ratios of PIPs, -diyne, PC-diyne, and PE-diyne (5:65:30 molar ratios), were combined in an 8-mL glass vial, to which 1 mL chloroform was added, followed by drying on a SpeedVac (Thermo Fischer Scientific Inc, Waltham, MA) overnight. (Multiple vials of dried lipid mixture were prepared in advance and stored at -20°C until needed.) For each μmol of total lipid, phosphate-buffered saline buffer (150 mmol/L NaCl, pH 7.5) made with Milli-Q water (EMD Millipore Corporation, Billerica, MA) was added to make a 1-mmol/L final solution. Probe sonication (~2–3 W) at 60°C for approximately 60 minutes was followed by extrusion through 400 nm polycarbonate track-etched membrane using the conventional vesicle extrusion method. The solution was transferred to a

beaker and placed in a slightly larger beaker in an ice bath, followed by irradiation for 60–90 minutes with a hand-held UV lamp (254 nm) placed directly on top of the larger beaker, with the solution turning faintly red. Resulting PIP-containing vesicles were characterized by their average size distribution and ζ potential with dynamic light scattering and ζ potential measurements, respectively.

Peptides

The peptides used in this study were synthesized by Anaspec Corporation (San Jose, CA). The sequences of the NS5A protein–derived amphipathic α-helical peptide and nonamphipathic nonhelical control peptide (NH) were H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Ile-Cys-Thr-Val-Leu-Thr-Asp-Phe-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-Asp-Tyr-Lys-Asp-NH2 and H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-**Asp**-Trp-Asp-Trp-**Glu**-Cys-Thr-Val-Leu-Thr-Asp-**Asp**-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-Asp-Tyr-Lys-Asp-NH2, respectively (the introduced charged amino acids differentiating NH from AH are indicated in bold underline). Synthesized basic amino acid PI(4,5)P₂ pincer (BAAPP) domain mutant AH peptides have the same sequence as AH except for substitution of alanine for lysine at position 20 (AH-K20A), position 26 (AH-K26A), or both (AH-K20AK26A).

Dynamic Light Scattering

Dynamic light scattering was performed on a 90Plus Particle Size Analyzer and the results were analyzed by digital autocorrelation software (Brookhaven Instruments Corporation, Holtsville, NY). All measurements were taken at a scattering angle of 90° so that the reflection effect was minimized. All autocorrelation functions obtained also were analyzed by CONTIN algorithm and non-negatively constrained least squares algorithms to check for multimodal distributions.

E4 Quartz Crystal Microbalance-Dissipation

Quartz crystal microbalance-dissipation (QCM-D) measurements were performed essentially as previously described,[2] and as detailed in the Supplementary Methods and Materials. We used the Sauerbrey equation[17] to convert frequency to a real mass of bound peptide. All experiments were repeated at least 3 times, with a SD of less than 5%. For QCM-D experiments involving endoplasmic reticulum membranes, the latter were purchased from Celsis In Vitro Technologies (Chicago, IL) and enriched by incubation with solutions of either PI, PI(3,5)P2, or PI(4,5)P2 before loading into the QCM chambers.

Circular Dichroism Measurements

Circular dichroism measurements were performed as described in the Supplementary Methods and Materials. The scans obtained with ellipticity (Θ) were converted to mean molar residue ellipticity ([Θ]) as previously described.[18] Spectra were processed with CD6 software, baseline corrected, and smoothed using a third-order, least-square polynomial fit. The fraction helicity of the peptide in solution was determined as previously described.[19]

Plasmids

Bart79I, a high-efficiency subgenomic replicon of HCV,[20] harbors the neomycin resistance gene and the HCV nonstructural proteins. The Bart–Luciferase plasmid, Bart79I-luc, was cloned from the Bart79I parent[3] and the pGL3-basic parent (Promega, Madison, WI), as described in the Supplementary Methods and Materials. FL-J6/JFH-5'C19Rluc2AUbi—a monocistronic, full-length HCV genome that expresses Rluc and was derived from the previously described infectious genotype 2a HCV genome J6/JFH1[21]—was a gift from Dr Charles Rice (Rockefeller University).[22] The nucleotide sequence AAG that encodes for lysine at amino acid positions 20 and 26 of NS5A was changed to GCG (encoding for alanines) through the use of the Quick-Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described by the manufacturer and confirmed by sequencing.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described in the Supplementary Methods and Materials.

Purification of Recombinant HCV NS5A and TBC1D20

Full-length NS5A and TBC1D20 proteins were purified from bacteria as described elsewhere.[23,24]

Colony Formation Assays

Colony formation assays were performed using 5 μg of in vitro–transcribed wild-type and mutant Bart79I RNAs as previously described[3] and detailed in the Supplementary Methods and Materials.

Viral Sequencing Analysis

TRIzol reagent (Gibco BRL, Grand Island, NY) was used to extract total RNA from the pool containing the Huh7 cells that survived the colony formation assays after electroporation with in vitro–transcribed wild-type or mutant Bart79I RNAs, and the HCV-RNA sequences were determined as described in the Supplementary Methods and Materials.

Transient Replication Assays

Wild-type or BAAPP domain mutant versions of Bart79I-luciferase or FL-J6/JFH-5'C19Rluc2AUbi[22] RNAs were electroporated into Huh7 cells followed by determination of luciferase activity at 8, 48, 96, and 144 hours after electroporation as described in the Supplementary Methods and Materials.

Cell Viability Assays

Cells were incubated with cell culture media containing 10% alamarBlue (Biosource International, Inc, Camarillo, CA) for 2 hours. Relative cell viabilities were compared by measuring the absorbance of cell culture media at 544 nm.

Identification of Putative BAAPP Domain-Containing Proteins

Putative BAAPP domains were identified by analyzing protein sequences using Jpred3[25] to predict their secondary structure. Regions found to form α helices then were plotted in a helix wheel format (http://www.tcdb.org/progs/pepwheel. php) to determine if the helix contained a hydrophobic face and positive charges (basic amino acids lysine [K], arginine [R], or histidine [H]) that flanked the hydrophobic face.

Results

## The NS5A Amphipathic Helix Specifically Binds PI(4,5)P₂

To test the hypothesis that the NS5A AH binds PIs, we determined the ability of a synthetic peptide corresponding to the NS5A AH to bind to polymerized lipid vesicles containing different PIs, using the QCM-D technique[2] (Supplementary Figure 1). QCM-D provides for very sensitive and reproducible real-time measurements of mass binding to a target oscillating quartz crystal. The greater the mass bound, the greater the recorded decrease in resonating frequency of the quartz crystal nanosensor.[26,27] For our QCM-D assay, target polymerized lipid vesicles are first deposited on an oscillating SiO₂ quartz crystal nanosensor (Q-sense, Gothenbug, Sweden). The binding mass of peptide to this vesicle platform then is determined as a function of the change in resonance frequency of the piezoelectric AT-cut crystal.[26,27] As shown in Figure 1A, significant binding of the NS5A AH to PI(4,5)P₂-containing vesicles was observed. To determine the specificity of the observed binding, lipid vesicles containing PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂, or PI(3,4,5)P₃, were used in parallel assays. As shown in Figure 1A, the NS5A AH shows a high degree of binding specificity for PI(4,5)P₂. Related PI bisphosphates such as PI(3,4)P₂ and PI(3,5)P₂, which have a similar number of negative charges to PI(4,5)P₂, showed only minimal binding to the NS5A AH. This shows that the interaction of the NS5A AH with PI(4,5)P₂ was not derived simply from a positive electrostatic attraction for multivalent, negatively charged PI bisphosphates. Furthermore, no specific binding to the NS5A AH was observed with other PIs containing 1 or 3 phosphate-modified inositol headgroups, such as PI(3)P, PI(4)P, PI(5)P, or PI(3,4,5)P (Figure 1A). Readily available control proteins reported to have binding specificity for some of these PI substrates, however, performed as expected (Figure 1B).

QCM-D monitoring allows for determination of binding kinetics, and the K_d for the NS5A AH association with PI(4,5)P₂ vesicles was found to be 4.5 μmol/L (Supplementary Figure 2). By comparison, the K_d for the approximately 120 amino acid pleckstrin homology domain—one of the best characterized PI(4,5)P₂ binding domains to date—has been reported to be quite similar (2–30 μmol/L).[28,29]

In contrast, a negative control peptide (termed NH), derived from the NS5A AH in which 3 charged point mutations were introduced into the hydrophobic face, showed minimal binding to any of the PI-containing substrates (Figure 1C).

To provide additional evidence supporting the specific binding of the NS5A AH to PI(4,5)P₂, and to confirm that the results obtained with the isolated NS5A AH peptide appropriately reflect the behavior of this AH segment when present in the context of the full-length NS5A protein, we assessed the binding of full-length NS5A to PI(4,5)P₂ and related phosphoinositides (PI[3,4]P₂ and PI[3,5]P₂,). As shown in Supplementary Figure 3, the full-length NS5A protein shows substantial binding to PI(4,5)P₂. Again, as in the experiments with peptides, preferential binding to PI(4,5)P₂ compared with the other PI-bisphosphate isoforms was observed.
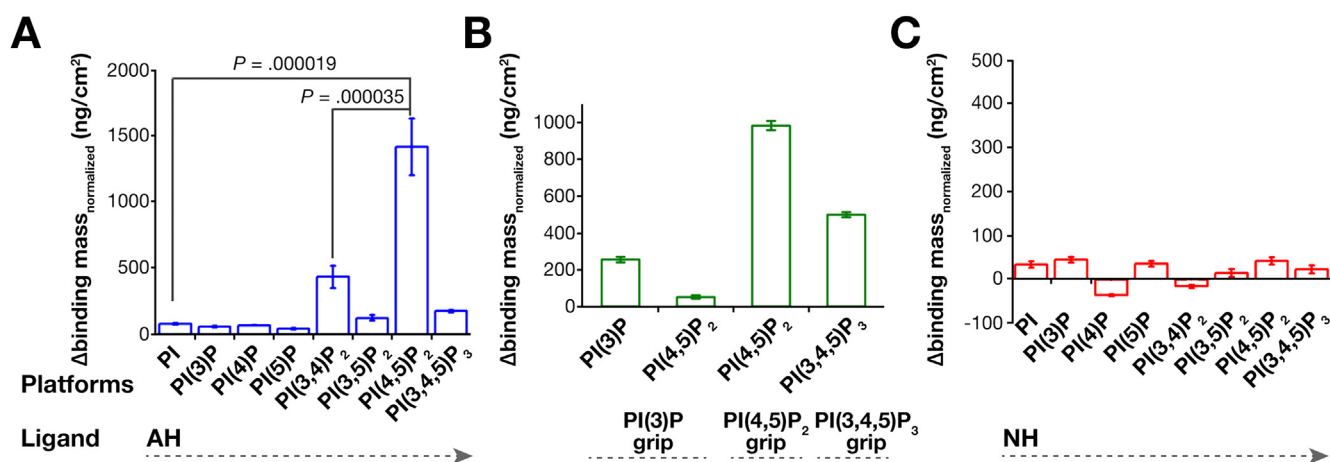
Figure 1. The HCV-NS5A AH specifically binds lipid vesicles containing PI(4,5)P₂. QCM-D measurements quantify mass changes caused by the binding of the NS5A-AH peptide to vesicles containing various PIs. The system consists of polymerized vesicles containing the PIs on a SiO₂ solid substrate. We used the Sauerbrey[17] equation to convert frequency to a real mass of bound peptide. (A) The NS5A AH peptide (~8 μmol/L) binds significantly more to vesicles containing PI(4,5)P₂ than those containing PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, or PI(3,4,5)P₃, where the lower amount of binding is consistent with prior work using model lipid vesicles alone.[2] (B) As positive controls, we used 3 different Grip (Echelon Biosciences, Inc, Salt Lake City, UT) proteins that are known to bind PI(3)P, PI(4,5)P₂, and PI(3,4,5)P₃. PI(3)P Grip contains recombinant p40PX domain GST-tagged protein, PI(4,5)P₂ Grip contains recombinant PLC-δ1 pleckstrin homology domain GST-tagged protein, and PI(3,4,5)P₃ Grip contains recombinant GRP1-PH domain GST-tagged protein.[42] (C) As a negative control, we used the NH peptide, in which 3 point mutations were introduced into the NS5A AH to disrupt amino acids on the hydrophobic face.[3] Notably, the NH peptide showed no significant binding to any of the target lipid vesicles. Error bars represent standard deviations, P values from the Student t test. Note the scale differences between the figures.
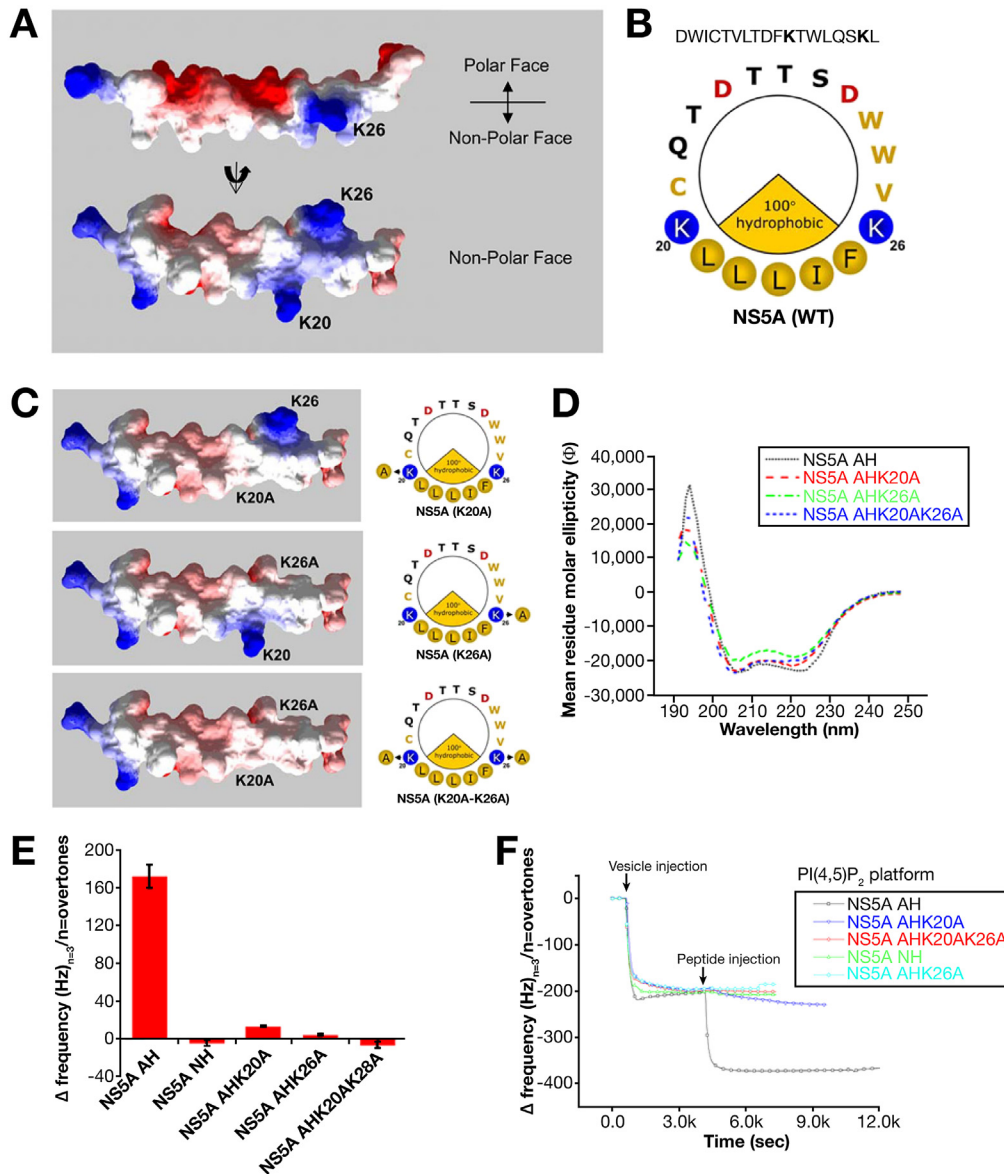
Figure 2. PI(4,5)P$_2$ binding to the NS5A AH is mediated by a pair of conserved positively charged amino acids, or Basic Amino Acid PI(4,5)P$_2$ Pincer (BAAPP) domain. (*A*) A molecular surface model of the BAAPP domain of the NS5A AH (genotype 1b) was created using the program DeepView/Swiss-PdbViewer (http://spdbv.vital-it.ch/refs.html) (V3.7),[43] based on the nuclear magnetic resonance structure 1R7C. The surface was colored by electrostatic potential, which was calculated using default parameters and included only charged residues. *Blue* denotes positive electrostatic potentials, *white* denotes neutral potentials, and *red* denotes negative electrostatic potentials. (*B*) Helix wheel plot of the BAAPP domain in the NS5A AH, with the hydrophobic face denoted by the *yellow pie slice* and *filled yellow circles*. Positively charged residues that flank the hydrophobic face are indicated by the *filled blue circles*. (*C*) *Left*: Molecular surface models of BAAPP mutants in the NS5A AH. Mutations (as indicated, K20A, K26A, and K20AK26A) in the molecular structure were modeled using DeepView. *Right*: Helix wheel plots of BAAPP mutants in the NS5A AH. (*D*) Far-UV circular dichroism analyses of the NS5A AH and the single mutant (K20A and K26A) and double-mutant (K20AK26A) peptides. The circular dichroism spectra were recorded in 10 mmol/L phosphate-buffered saline buffer, pH 7.5. (*E*) The binding kinetics of the NS5A AH peptide and mutant variants (~19 μmol/L) to PI(4,5)P$_2$-containing vesicles, using the same technique used in Figure 1. (*F*) A comparison of the total frequency changes (Δf) caused by binding of NS5A AH peptide and mutant variants, as shown in *panel E*.

NS5A Binds PI(4,5)P$_2$ via a Novel Structural Motif: A BAAPP Domain

Although a variety of structural motifs are known to bind PI(4,5)P$_2$,[9] the NS5A AH does not appear to conform to any of these. As shown in Figure 2*A* and *B*, inspection of the NS5A AH showed a pair of basic amino acids (Lys 20 and Lys 26) that flank the hydrophobic face of the AH. As such,

they are oriented toward the lipid bilayer, with which the AH likely interacts in a monotypic fashion.[30] We hypothesized that these positively charged lysines ideally might be suited to interact with the negatively charged phosphates of the PI(4,5)P$_2$ lipid headgroups. To test this hypothesis, we synthesized mutant versions of the NS5A AH peptide in which one or both of these lysines were mutated to alanines

(Figure 2C). Circular dichroism measurements confirmed that these mutations did not alter the helical nature of the AH (Figure 2D). These mutations, however, dramatically impaired the ability of the corresponding peptides to bind $PI(4,5)P_2$-containing vesicles (Figure 2E and F). Taken together, these results suggest that the $PI(4,5)P_2$ binding domain in the NS5A AH represents a novel structural motif for $PI(4,5)P_2$ binding that we term the Basic Amino Acid PI(4,5)P2 Pincer (BAAPP) domain.

HCV Replication Sites Are Localized to Sites Enriched in $PI(4,5)P_2$

Because lysines 20 and 26 are highly conserved across HCV isolates, despite a fair amount of sequence variation within the AH, this suggested that $PI(4,5)P_2$ binding by the NS5A AH is important for the HCV life cycle. We hypothesized that NS5A and $PI(4,5)P_2$ therefore might be co-localized at the site of viral replication. As shown in Figure 3, similar to previous work,[31] a monoclonal antibody against $PI(4,5)P_2$ shows both a nuclear staining pattern and distinct small speckles distributed in the cytoplasm. Because HCV replication is known to be localized to similar-appearing cytoplasmic speckle-like structures, we hypothesized the latter now might be found to be enriched in $PI(4,5)P_2$. Indeed, in cells harboring HCV replicons, significant co-localization of NS5A with $PI(4,5)P_2$ was observed (Figure 3A). To eliminate the possibility of antibody-specific effects, we performed similar experiments with a yellow fluorescent protein-tagged version of NS5A (Figure 3B), which showed identical results, with more than 90% of the NS5A-staining speckles found to co-stain with the anti-$PI(4,5)P_2$ antibody. Expression of just an isolated NS5A failed to co-localize with the $PI(4,5)P_2$ speckles (Figure 3C). Moreover, the intracellular distribution of NS5A was unaffected by mutation of the BAAPP domain (Supplementary Figure 4A). Therefore, NS5A with an intact BAAPP domain appears to co-localize with $PI(4,5)P_2$ only in the context of a
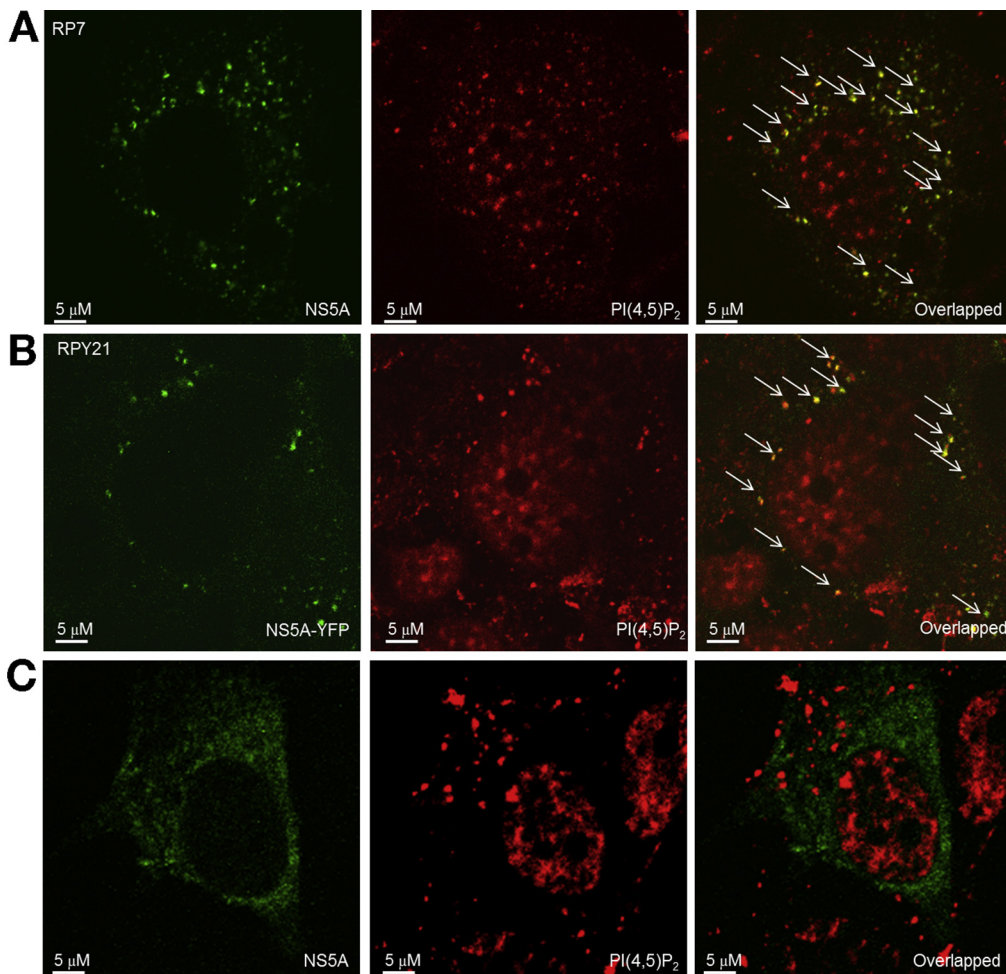
Figure 3. NS5A co-localizes with $PI(4,5)P_2$ at sites of HCV replication. (A) Subcellular localization of $PI(4,5)P_2$ was examined by immunofluorescence using a monoclonal antibody specific for $PI(4,5)P_2$ and Alexa 594–conjugated secondary antibody in the RP7 cells harboring Bart79I replicating HCV subgenomic replicons, where NS5A was detected with a mouse monoclonal antibody specific for NS5A and Alexa 488–conjugated secondary antibody. (B) RPY21 cells harboring replicating HCV replicons with a yellow fluorescent protein-tagged version of NS5A. White arrows indicate sites of colocalization of NS5A and $PI(4,5)P_2$, shown in yellow. Quantitation of confocal images showed that 91% of NS5A-staining speckles co-stain with the anti-$PI(4,5)P_2$ antibody. (C) When expressed alone off of a plasmid vector, NS5A fails to co-localize with the $PI(4,5)P_2$ speckles. Scale bars: 5 micron. Magnification, ×100.

replicating genome where NS5A is localized to HCV replication complex sites. This suggests that HCV replication complexes are established at $PI(4,5)P_2$ sites or the HCV replication complex promotes formation of $PI(4,5)P_2$ sites. Either way, $PI(4,5)P_2$ represents a new marker for HCV replication complexes. These results also suggest that $PI(4,5)P_2$ binding by NS5A might mediate an interaction important for viral replication. We hypothesized that such an interaction might involve a $PI(4,5)P_2$-induced conformational change in the NS5A AH.

PI(4,5)P2 Binding Induces an Important Conformational Change in NS5A

To test the earlier-described hypothesis, circular dichroism measurements of the NS5A AH peptide were performed in the presence or absence of $PI(4,5)P_2$-containing lipid vesicles. As shown in Figure 4A, a dramatic alteration of the helical structure of the NS5A AH was observed upon interaction with $PI(4,5)P_2$. No such changes were noted with vesicles only or vesicles containing $PI(3,4)P_2$. Therefore, $PI(4,5)P_2$ binding appears to mediate a conformational change in the NS5A AH.

NS5A has been reported to interact with a variety of host proteins. Among these is TBC1D20, a guanosine triphosphatase activating protein for Rab1.[5,24] TBC1D20 is required for efficient HCV replication and the interaction between TBC1D20 and NS5A is mediated by the latter's N-terminal AH.[5] To test the hypothesis that the $PI(4,5)P_2$-induced AH conformational change might regulate the interaction of NS5A with TBC1D20, we performed QCM-D binding experiments of TBC1D20 to NS5A bound to human liver–derived microsomal membranes, with or without prior $PI(4,5)P_2$ enrichment. As shown in Figure 4B, the kinetics of the TBC1D20:NS5A interaction showed a striking dependence on $PI(4,5)P_2$. In particular, the rate of TBC1D20

absorption to membrane-bound NS5A was increased specifically when the membranes were enriched in $PI(4,5)P_2$. Moreover, the rate of TBC1D20 desorption from NS5A was affected even more dramatically when the membranes were enriched in $PI(4,5)P_2$, as opposed to the control membranes enriched with $PI(3,5)P_2$. This is reflected in the corresponding calculated values for the respective k_{on} and k_{off} rate constants, which together indicate a prolonged and stable interaction between bound TBC1D20 and the membrane-bound NS5A in the presence of $PI(4,5)P_2$ (Figure 4B and Supplementary Figure 5). That no such effect was observed upon substitution of $PI(3,5)P_2$ for $PI(4,5)P_2$, indicates the selectivity of the effect of $PI(4,5)P_2$ on the TBC1D20:NS5A interaction. The binding mass result showed that $PI(4,5)P_2$ promotes the binding of TBC1D20 to NS5A up to 57% more on $PI(4,5)P_2$-enriched membranes than on $PI(3,5)P_2$-enriched membranes (Figure 4C). There were no differences in TBC1D20 binding to $PI(4,5)P_2$-enriched vs $PI(4,5)P_2$-unenriched membranes in the absence of prior NS5A addition (Supplementary Figure 6).

The NS5A BAAPP Domain Mediates a Critical Role in HCV Genome Replication

To test the hypothesis that the ability of NS5A to engage in the interaction with $PI(4,5)P_2$ is essential for genome replication, we first performed standard HCV colony formation assays using high-efficiency, second-generation replicons encoding wild-type or mutant (K20AK26A) NS5A.[20] As shown in Figure 5A, although the wild-type replicon yielded numerous colonies and the negative control replicon containing a lethal mutation in the polymerase gene yielded none, approximately 75% fewer colonies were obtained with the K20AK26A mutant replicon compared with the wild-type replicon. When the replicons in the colonies surviving on the mutant plate were sequenced, they were
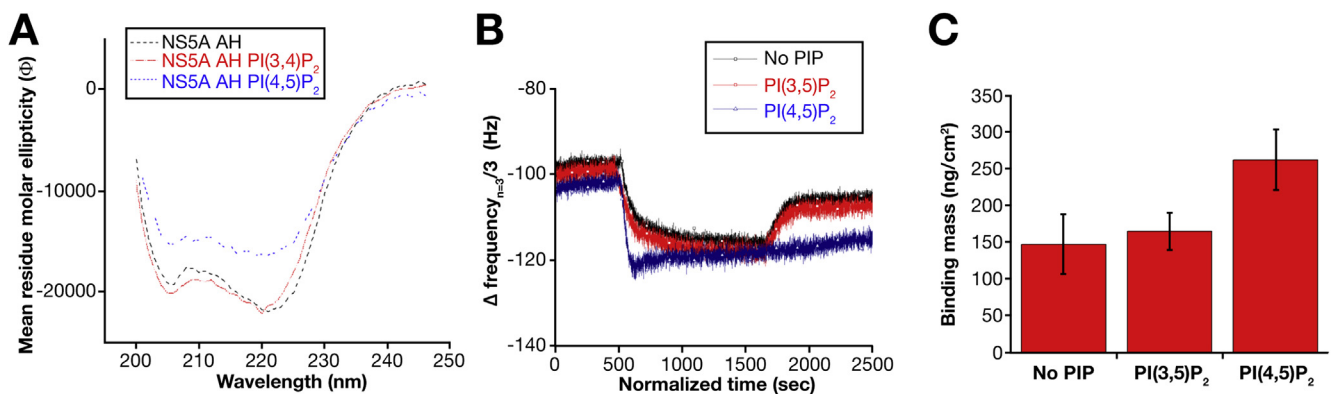

Figure 4. $PI(4,5)P_2$ induces a conformational change in the NS5A AH and stabilizes the NS5A-AH–mediated interaction with TBC1D20. (*A*) Far-UV circular dichroism analyses of the NS5A-AH peptide in the presence or absence of $PI(4,5)P_2$ containing polymerized vesicles, showing that a conformational change occurs upon addition of $PI(4,5)P_2$-containing vesicles, but not $PI(3,4)P_2$-containing vesicles. (*B*) The normalized frequency change (Δf) upon addition of TBC1D20 to 3 different platforms containing human liver–derived microsomal membranes, as is, or after enrichment with either $PI(3,5)P_2$ or $PI(4,5)P_2$, followed by preloading of NS5A before addition of TBC1D20 (each flowed into the QCM-D chambers at ~1 μg/mL). The kinetics of the frequency change (Δf) show that human liver–derived membranes alone, or after $PI(3,5)P_2$ enrichment, fail to promote stable association of TBC1D20 with NS5A full-length protein. The $PI(4,5)P_2$-enriched membrane, however, promoted a strong interaction of NS5A with TBC1D20, so that there was no such dissociation. (*C*) Calculated bound mass density from binding of TBC1D20 to NS5A on the 3 different platforms after equilibration (2500 sec).
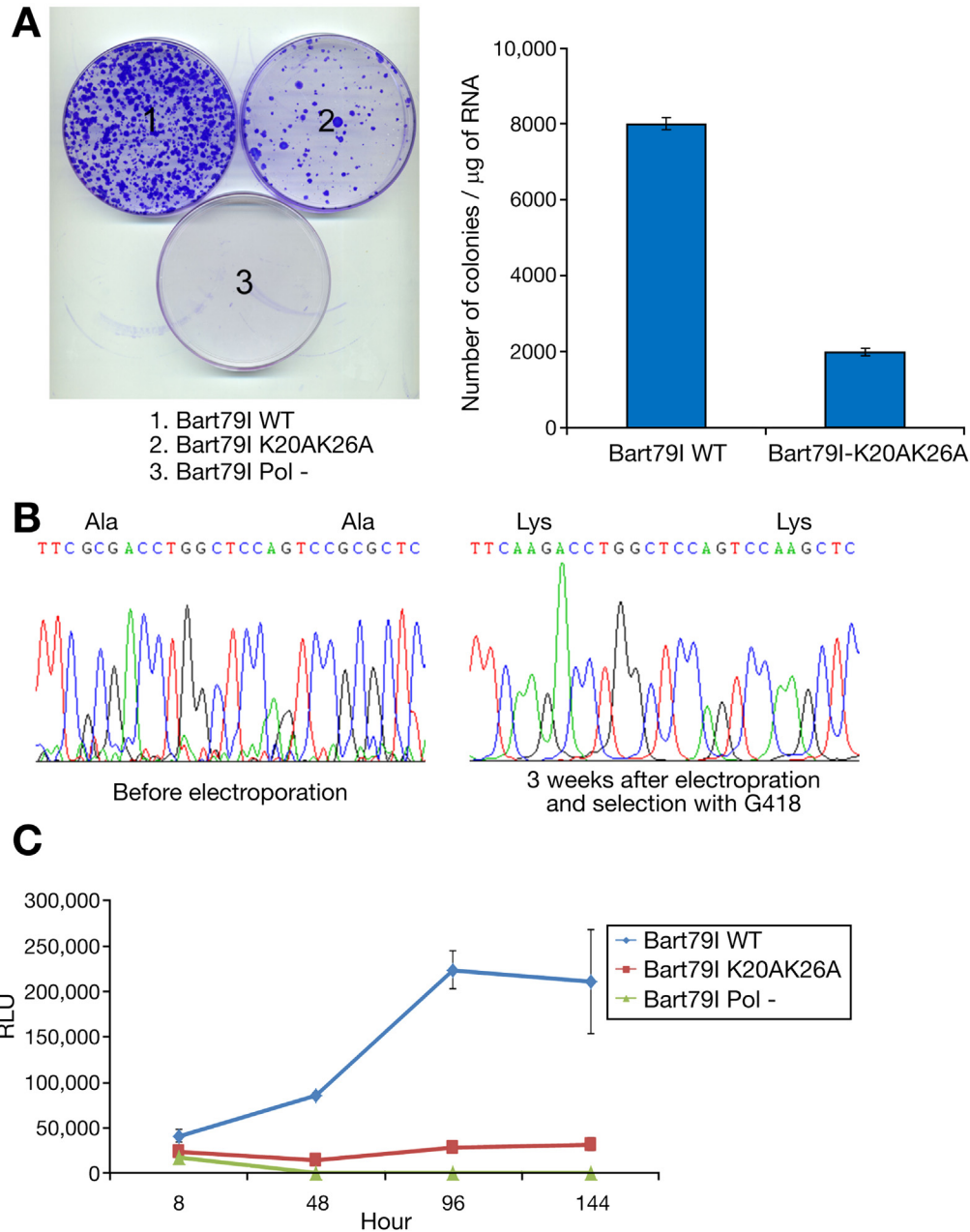
Figure 5. PI(4,5)P$_2$ mediates HCV-RNA genome replication. (*A*) Colony formation assay. Huh7 cells were electroporated with 5 µg of in vitro–transcribed wild-type Bart79I, mutant Bart79I encoding NS5A (K20AK26A), or Bart79I (Pol-) RNAs followed by selection with 750 µg/mL of G418 for 3 weeks. Surviving colonies were stained with crystal violet and the number of colonies was counted from 3 different plates to calculate the average number of colonies and SD. (*B*) Reversion to wild-type sequence. *Left*: Sequence of input mutant (K20AK26A) HCV replicon RNA. *Right*: Sequence analysis of replicon RNA isolated from colonies growing on mutant plate from colony formation assay. Note that the mutant GCG alanine-encoding codons at positions 20 and 26 of NS5A have reverted back to the wild-type AAG sequences encoding lysine residues. (*C*) Luciferase reporter-linked transient HCV replication assay. Huh7 cells were electroporated with 10 µg of in vitro–transcribed wild-type Bart79I-luc, mutant Bart79I-luc (K20AK26A), or Bart79I-luc (Pol-) RNAs. Firefly luciferase activities were measured at 8, 48, 96, and 144 hours after electroporation. RLU, relative light unit.

found to have reverted to wild-type during the approximate 3-week selection process (Figure 5*B*). This suggests the following: such reversion was essential for viral fitness, and that the ability of NS5A to bind PI(4,5)P$_2$ is important for efficient genome replication. To directly test this hypothesis, we performed transient HCV replication assays with luciferase reporter-linked, wild-type or K20AK26A mutant replicons. As shown in Figure 5*C*, mutation of the PI(4,5)P$_2$ interaction domain of the AH NS5A indeed severely impaired HCV genome replication. Similar results were observed upon introduction of these BAAPP domain mutations into an infectious clone of HCV (Supplementary Figure 7). Although the PI(4,5)P$_2$ interaction with the human immunodeficiency virus Gag matrix protein has been implicated in virus
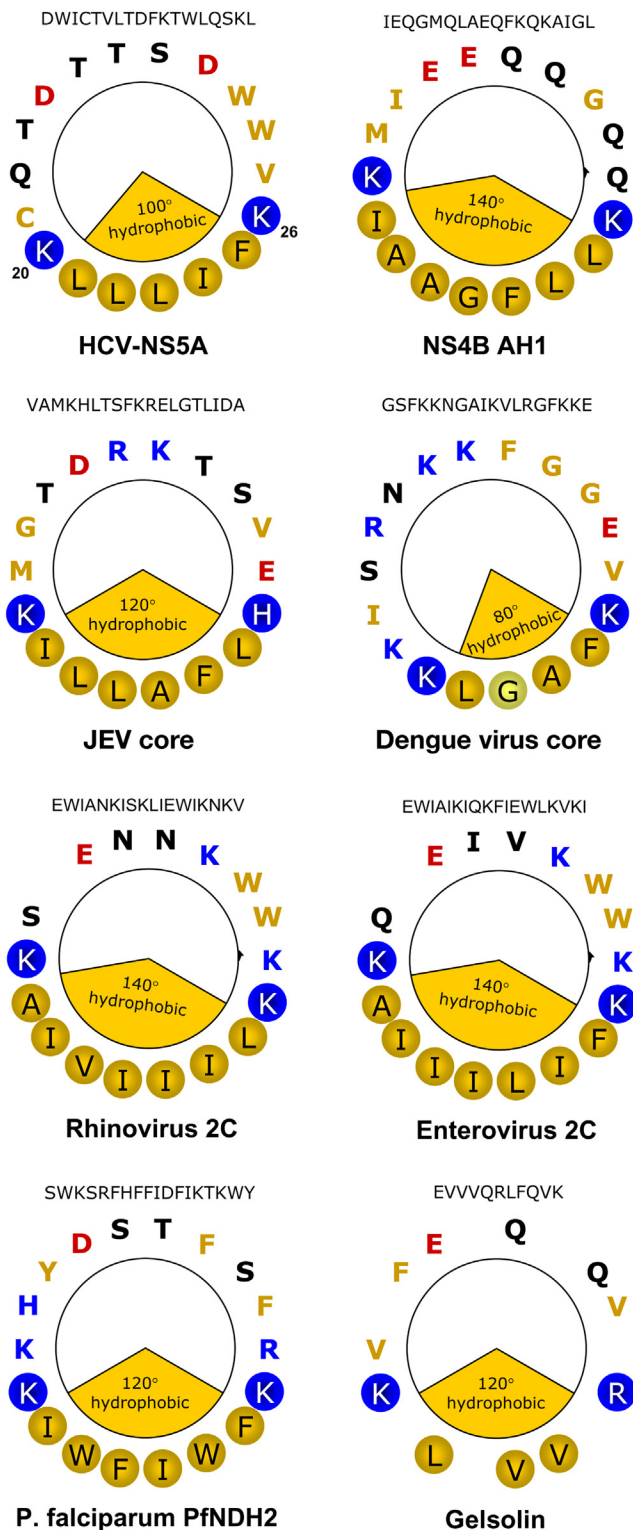
Figure 6. A BAAPP domain is found in the AHs of other pathogens and host cell proteins. Putative BAAPP domain in the AHs of other proteins. Hydrophobic face denoted by the *yellow pie slice* and *filled yellow circles*. Positively charged residues that flank the hydrophobic face are indicated by the *filled blue circles*, with *blue* denoting that they are positively charged residues. JEV, Japanese encephalitis virus; Apo, apolipoprotein.

particle assembly,[32] we now show an example of PI(4,5)P$_2$ mediating viral genome replication.

Finally, an analysis of sequences from a variety of organisms showed that putative BAAPP domains are present in other viral proteins (Figure 6). BAAPP domains also are found in a variety of host proteins, including apolipoproteins (Figure 6). Analysis of several of these BAAPP domains using methods analogous to those described here (Figures 1 and 2) shows that they also mediate binding to PI(4,5)P$_2$ (Supplementary Figure 8).

Discussion

The earlier-described experiments show an exciting role for PI(4,5)P$_2$ in the HCV life cycle and provide intriguing insights into the relevant molecular mechanism of action. In particular, we discovered that the HCV-NS5A amphipathic helix specifically binds PI(4,5)P$_2$ (Figure 1) and that this binding is mediated by a novel structural motif, the BAAPP domain (Figure 2A and B). PI(4,5)P$_2$ binding induces an important conformational change in NS5A (Figure 4A), affecting the latter's interaction with a key regulator of HCV replication (Figure 4B). Importantly, disrupting the ability of the BAAPP domain to bind PI(4,5)P$_2$ profoundly impairs HCV genome replication (Figure 5). Moreover, the apparent widespread presence of BAAPP domains in pathogen proteins (Figure 6) suggests that BAAPP domain-mediated PI(4,5)P$_2$ binding plays equally important roles in other pathogen life cycles, and that our findings might be translated into novel anti-infective therapies.

Previous work has implicated the involvement of a host cell membrane protein(s) (the identity of which remains unknown) in the binding of NS5A to intracellular membranes, as well as an inherent membrane-associating activity of the NS5A AH with model membranes.[2] Consistent with this previous work,[2] we observed significant binding of the NS5A AH to model lipid vesicles alone (Figure 1), which reflects the strong membrane-associating activity provided by this amphipathic segment of NS5A. The current findings, however, also extend our understanding of the interaction of NS5A with membranes. In particular, we discovered that the NS5A AH can specifically bind PI(4,5)P2—both in the context of an isolated AH peptide (Figure 1) and when contained within full-length NS5A (Supplementary Figure 3). The role of this PI(4,5)P2 binding does not appear to be a primary determinant for either NS5A membrane association or intracellular localization (Figure 3 and Supplementary Figure 4). Rather, PI(4,5)P2 binding induces a conformational change within the AH (Figure 4A) that alters the interaction with a key NS5A binding partner (Figure 4B and C).

Our results also provide new insights into, and a molecular mechanism to account for, the relevance of recent data showing the sensitivity of HCV replication to small interfering RNA-mediated knockdown of PI4K-IIIα and PI4K-IIIβ, the reported interaction with, and activation of, these PI(4)P-generating kinases by viral proteins,[13,14,16,33,34] as well as the role for the reported presence of PI(4)P at sites of HCV replication.[35] In particular, in addition to PI4

kinase involvement in recruiting PI4P-binding proteins such as OSBP,[36] and modulation of NS5A phosphorylation,[37] our results suggest that the source of the PI(4,5)P2 relied on by HCV likely is derived via the PI(4)P produced by one or both of these members of the PI4-kinase family.

Together, this suggests a model whereby the HCV polyproteins initially become membrane associated via PI(4,5)P2-independent mechanisms, and subsequently HCV replication complexes are established at sites that become locally enriched in PI(4,5)P2 (likely via hijacking of the requisite PI kinases to the future replication sites by NS5A [eg, Ahn et al[13] and Reiss et al[16]] and possibly other proteins). This latter PI(4,5)P2 is sensed by NS5A, triggering a key regulatory switch. This model is supported by data showing that NS5A is associated with PI(4,5)P2 only in the context of active replication (Figure 3), PI(4,5)P2 binding by NS5A induces a conformational change in the NS5A AH (Figure 4A), and that interaction of PI(4,5)P2 with NS5A regulates the association of NS5A with TBC1D20 (Figure 4B and C)—a host cell partner required for viral genome replication.[5]

Importantly, NS5A is not the only protein containing a BAAPP domain. Residues 150–169 of the host protein gelsolin have been identified previously as comprising a structurally unclassified type of PI(4,5)P2 binding domain.[38] nuclear magnetic resonance studies have shown that this region forms an AH that is capable of binding PI(4,5)P2.[39] This information, along with a helix wheel representation of this region, shows that this region now can be classified as a BAAPP domain (Figure 6).

Moreover, we speculate that the BAAPP domains we have identified in apolipoproteins (Figure 6) may mediate interaction with PI(4,5)P2 and that this interaction plays an important role in the genesis of certain lipoprotein particles, with localized PI(4,5)P2 domains representing a common platform for the initial stages of very low density lipoprotein and HCV particle assembly. In particular, HCV either may compete for or hijack limiting components of host cell PI(4,5)P2-associated machinery to help effectuate viral assembly. This could account for the reciprocal relationship observed between serum levels of very low density lipoprotein and HCV titer before and after successful treatment of HCV (Siagris et al[40] and personal observations).

Finally, these results suggest a variety of new potential antiviral strategies. For example, neomycin is known to be a ligand of PI(4,5)P2.[41] As such, either neomycin or analogues thereof could be considered as inhibitors of PI(4,5)P2–BAAPP interactions. A complementary approach would be to deplete PI(4,5)P2 pharmacologically at HCV replication sites, such as by stimulating a phosphatase involved in PI(4,5)P2 degradation, or inhibiting a kinase on which HCV depends for its source of PI(4,5)P2. Moreover, because there appear to be back-up mechanisms to such contemplated disruption of PI(4,5)P2 metabolism that are available to the host but not the virus, relevant inhibitors with sufficient therapeutic indices can be contemplated readily. Such inhibitors might represent a valuable new class of antiviral agents to be included in future therapeutic cocktails designed to maximize pan-genotypic efficiency of, and minimize resistance to, therapies for treating hepatitis C or other BAAPP domain–harboring pathogens.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.11.043.

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