Phosphatidylserine-Specific Phospholipase A1 Involved in Hepatitis C Virus Assembly through NS2 Complex Formation

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ABSTRACT

Several members of the phospholipase family have been reported to be involved in hepatitis C virus (HCV) replication. Here, we identified another phospholipase, phosphatidylserine-specific phospholipase A1 (PLA1A), as a host factor involved in HCV assembly. PLA1A was upregulated by HCV infection, and PLA1A knockdown significantly reduced J399EM (genotype 2a) HCV propagation at the assembly step but not the entry, RNA replication, and protein translation steps of the life cycle. Protein localization and interaction analysis further revealed a role of PLA1A in the interaction of NS2-E2 and NS2-NS5A, as the formation of the NS2-E2 and NS2-NS5A complexes was weakened in the absence of PLA1A. In addition, PLA1A stabilized the NS2/NS5A dotted structure during infection. These data suggest that PLA1A plays an important role in bridging the membrane-associated NS2-E2 complex and the NS5A-associated replication complex via its interaction with E2, NS2, and NS5A, which leads to a coordinating interaction between the structural and nonstructural proteins and facilitates viral assembly.

IMPORTANCE

Hepatitis C virus (HCV) genomic replication is driven by the replication complex and occurs at the membranous web, while the lipid droplet is the organelle in which virion assembly is initiated. In this study, we identified phosphatidylserine-specific phospholipase A1 (PLA1A), a member of phospholipase A 1 family, as a novel host factor involved in the assembly process of HCV. PLA1A, which is induced by HCV infection at a late infection stage, interacts with HCV E2, NS2, and NS5A proteins and enhances and stabilizes the NS2-E2 and NS2-NS5A complex formation, which is essential for viral assembly. Thus, PLA1A is an important host factor which is involved in the initiation of the viral assembly in close proximity to Core-decorated lipid droplets through bringing together the HCV replication complex and envelope complex.

Hepatitis C virus (HCV) is a major cause of chronic liver disease, affecting approximately 185 million people worldwide (1). HCV is a positive single-stranded RNA virus belonging to the *Flaviviridae* family. The HCV 9.6-kb genome contains a large open reading frame encoding a single polyprotein that is processed into its structural proteins (Core, E1, and E2) and nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by host and viral proteinases (2). The structural proteins are components of the virion, while nonstructural proteins NS3 to NS5B compose the minimal viral replicase governing RNA replication (3, 4).

The overall HCV life cycle has been well defined since the development of an infectious HCV cell culture system (5–7). HCV genomic replication is driven by the replication complex (RC) and occurs at the membranous web, a rearranged membrane structure induced by virus infection (8–10). Recent progress regarding the study of the assembly process demonstrates that the lipid droplet (LD) is the organelle in which virion assembly is initiated (11) and that, in addition to the structural proteins, almost all the nonstructural proteins and many host factors are involved in this process (12). The roles of NS2 and P7 in virion assembly attract attention because these molecules are not components of the virion or the replicase. NS2 is a polytopic transmembrane protein containing 3 putative transmembrane segments (13) and is suggested to serve as the scaffold for virus assembly by interacting with both structural and nonstructural proteins such as E1/E2, NS3/4A, and NS5A (14–16). P7 is a small protein with 63 amino acids (aa)

harboring ion channel activity. Its role in HCV morphogenesis has been studied by mutation analysis and has been shown to be important for capsid assembly and envelopment (17–19). NS5A is another critical factor in the assembly process and is recruited to the Core-decorated LD through the interaction between its domain II and Core, bringing HCV RNA to the assembly site (20).

In addition to viral proteins, several host factors participate in the HCV assembly process by influencing the localization of HCV proteins or by mediating the interactions between HCV proteins. Triglyceride-synthesizing enzyme diacylglycerol acyltransferase-1 (DGAT1) was first found to recruit the Core protein to LD (21). Recently, the interaction between DGAT1 and NS5A was confirmed and was shown to be the bridge between Core and NS5A, facilitating HCV assembly (21, 22). Two additional proteins,

Rab18 and TIP47, were shown to interact with NS5A and promote the interaction between viral replication sites and LD (23–25). In addition, signal peptidase complex subunit 1 (SPCS1) facilitates the interaction between NS2 and E2 and is involved in the early step of the assembly of infectious particles (26).

Several members of the phospholipase A2 family such as PLA2G4A, PLA2G4C, and PLA2GXIIB are involved in HCV replication via various mechanisms (27, 28, 30), implying a role for the phospholipase family in HCV replication. Here, we identified another member of the phospholipase family, phosphatidylserine (PS)-specific phospholipase A1 (PLA1A), as a host factor involved in HCV assembly. PLA1A was first identified in rat pellets (29), and PLA1A mRNA is present at high levels in human liver tissue (31). PLA1A specifically acts on phosphatidylserine (PS) and 1-acyl-2-lysophosphatidylserine (lyso-PS) to hydrolyze fatty acids at the sn-1 position of these phospholipids (32). PLA1A is a secreted protein, and its substrate, PS, typically localizes to the inner leaflet of the lipid bilayer, and when PS is exposed on the surface of the cell membrane, PLA1A exerts its lipase functions. Although the lipase activity of PLA1A has been verified, its functions *in vivo* remain unknown. However, PLAIA was shown to act with its substrate PS and product lyso-PS (32), which is a lipid mediator that plays a role in the inflammation response (33).

In the present study, we revealed a role for PLA1A in HCV assembly. PLA1A interacts with the HCV E2, NS2, and NS5A proteins, facilitating NS2-E2 and NS2-NS5A complex formation during virus assembly.

MATERIALS AND METHODS

Cell culture. Huh7.5.1 cells (kindly provided by Frank Chisari) and Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 2 mM L-glutamine, nonessential amino acids, and 10% fetal bovine serum (FBS) (Invitrogen). The subgenomic HCV replicon cell lines (Huh7.5.1-SGR [30] and Con1 [34] containing subgenomic genotype 2a and 1b HCV, respectively) were grown in the same medium supplemented with 500 μg ml^{-1} G418.

Virus production. The JFH-1 virus used in this study was based on the pJFH-1 plasmid kindly provided by T. Wakita (6). The J399EM strain was derived from the JFH-1 virus by insertion of enhanced green fluorescent protein (eGFP) into the HCV NS5A region (35). The JFH1-HA virus with a hemagglutinin (HA) epitope at the N terminus of NS2 was constructed as previously described (15). The viral titer in the culture supernatants and cell lysates was determined by a modified endpoint dilution assay as previously described (30, 34).

Plasmid construction. The PLA1A (GenBank accession no. NM_001206960) coding sequence was amplified by PCR and inserted into the pXJ40-HA and pXJ40-Flag plasmids. To generate the HA- or Flag-tagged HCV protein expression plasmids, the coding sequences of HCV Core, E1, E2, NS2, NS3, NS34A, NS4B, NS5A, and NS5B of genotype 2a (JFH1; GenBank accession no. AB047639) were amplified and inserted into the pXJ40-HA and pXJ40-Flag vectors. The bicistronic reporter, pHCV-IRES, was previously described (30).

RNA interference. The following small interfering RNAs (siRNAs) were used: siRNAs specific to PLA1A (siPLA1A-2 [D-008411-02-0002], siPLA1A-3 [D-008411-03-0003], and siPLA1A-4 [D-008411-04-0004]; Thermo Scientific Dharmacon), AllStars negative-control siRNA (siControl; catalog no. 1027281, Qiagen), and siRNA specific to HCV (siHCV; target sequence 5-GGUCUCGUAGACCGUGCAC-3). The siRNAs were transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 20 nM according to the manufacturer's instructions. To maintain the gene-silencing effect from the beginning of infection to the last time point analyzed, the cells were split at 24 h posttrans-

fection (hpt) and transfected with the same siRNA again. HCV infection was performed 6 h after the second transfection.

Stable cell line construction. The coding sequences for short hairpin RNA (shRNA; shPLA1A#4 [5′-GGTTTCCTTTGCCGATCTTAT-3′]) targeting the PLA1A gene and a negative-control shRNA (5′-GGTGCAG CAGATTGTGAATACCCATAGTAA-3′) were cloned into shRNA expression vector pSUPER.retro.neo (OligoEngine, Inc.) following the manufacturer's instructions. The retroviruses were produced in 293T cells by cotransfection of the shPLA1A or shNC plasmid, pVPack-GP, and pVPack-VSV-G (Stratagene). The retrovirus-containing supernatants were harvested at 72 hpt. To generate the PLA1A knockdown cell line, the Huh7.5.1 cells were transduced with the retrovirus in the presence of 8 μg ml^{-1} Polybrene (Sigma), and stable knockdown pools were isolated by G418 selection and named Huh7.5.1-shNC (or shNC) and Huh7.5.1-shPLA1A#4 (or shPLA1A#4).

Western blot and coimmunoprecipitation analysis and indirect immunofluorescence staining. Whole-cell lysates were prepared and quantified using the Bradford method (Bio-Rad catalog no. 500-0006). Equal amounts of protein samples (30 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose filter membrane (Millipore). After blocking with 5% nonfat milk–Tris-buffered saline with Tween 20 (TBST), the membranes were incubated with specific primary antibodies and corresponding peroxidase-conjugated secondary antibodies. The following antibodies were used for Western blot analysis: anti-NS3 (catalog no. ab65407; Abcam), anti-Core (catalog no. ab2740; Abcam), anti-Flag (catalog no. F1084; Sigma), anti-HA (catalog no. H9658; Sigma), anti-PLA1A (produced by Abmart), and anti-beta-actin (catalog no. sc-47778; Santa Cruz). The proteins were visualized using suitable horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research) and SuperSignal-Femto chemiluminescent substrate (Pierce).

Coimmunoprecipitation and indirect immunofluorescence staining were performed as previously described (30). The Alexa Fluor 561/488/633-conjugated secondary antibodies used for indirect immunofluorescence staining were obtained from Invitrogen. LDs were stained with HCS LipidTOX Red (Invitrogen), and the nuclei were stained using Hoechst 33258 (Invitrogen).

Quantitative real-time RT-PCR. Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen) and digested with RNase-free DNase (Promega) according to the manufacturer's protocols. HCV RNA in the supernatant was prepared using TRIzol LS reagent (Invitrogen). Specific mRNAs and HCV RNAs were quantified by one-step real-time reverse transcription-PCR (RT-PCR) using a QuantiFast SYBR green RT-PCR kit (Qiagen). The mRNA and HCV RNA levels were normalized against the copy number of human beta-actin mRNAs. The forward and reverse primers used to amplify PLA1A were 5′-GGAACTGAGAAACAA GGACACC-3′ and 5′-AAACTCGGTTGGAAGACTGAAA-3′, and the primers for HCV and actin were previously described (36).

Membrane flotation fractionation. For cell fractionation, 9×10^7 cells were resuspended in 1 ml of cold hypotonic buffer (10 mM Tris-HCl [pH 7.8], 10 mM NaCl, EDTA-free protease inhibitor cocktail [Roche]) and mechanically disintegrated by forcing the cell suspension through a 25-gauge injection needle 30 times. The homogenate was centrifuged at $900 \times g$ for 5 min to pellet the nuclear fraction, followed by centrifugation at $15,000 \times g$ for 20 min to pellet the cell membrane fraction. The deposit contained the cytoplasmic fraction, and the cell membrane fraction was resuspended in 950 μl hypotonic buffer, NP-40 was added to reach a final concentration of 1%, and the solution was incubated for 30 min on ice.

Regarding sucrose density gradient centrifugation, 1-ml aliquots treated as described above were mixed with 1 ml 72% (wt/vol) sucrose in Laemmli sample buffer (LSB; 50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM MgCl$_2$) in an ultracentrifuge tube and overlaid with 4 ml 55% sucrose and 1.5 ml of 10% sucrose. The aliquots were then subjected to ultracentrifugation ($38,000 \times g$ for 16 h), and every 1-ml fraction was collected from the top of the gradient, mixed with 4 ml cold methyl alcohol, and centrifuged at $10,000 \times g$ for 10 min to pellet the membrane proteins. The
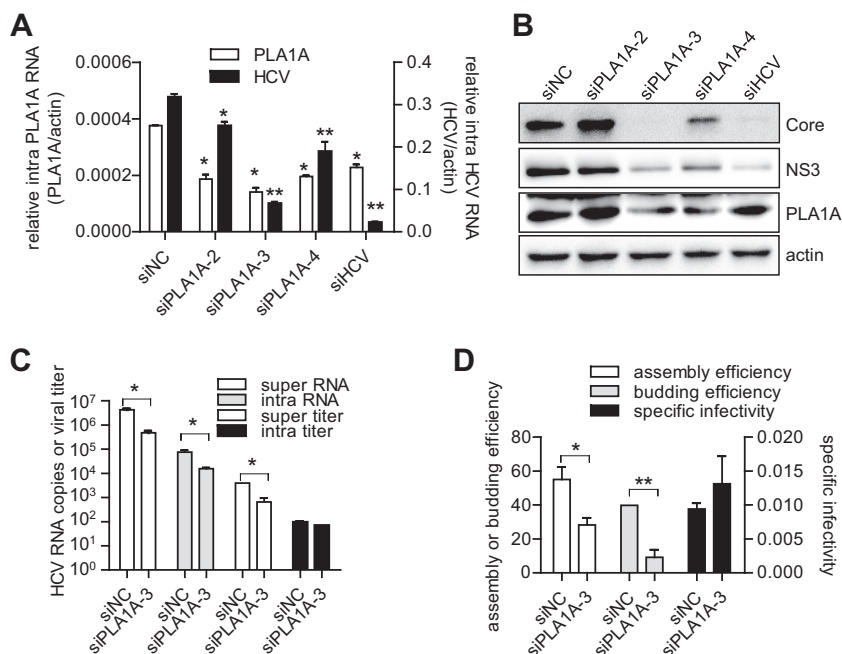
FIG 1 PLA1A participates in HCV propagation. (A and B) The Huh7 cells were transfected with different siRNAs as indicated and then infected with J399EM at a multiplicity of infection (MOI) of 0.1 for 96 h. RNA and protein samples were collected to determine the relative intracellular (intra) PLA1A mRNA levels and HCV RNA levels (A) and the HCV Core, NS3, and endogenous PLA1A protein levels (B). (C and D) The Huh7 cells were transfected with siNC or siPLA1A-3 and then infected with J399EM at an MOI of 0.1 for 96 h. (C) The HCV level in supernatant (super RNA), intracellular HCV RNA level (intra RNA), viral titer in supernatant (super titer), and intracellular viral titer (intra titer) were determined. The intracellular HCV RNA levels are shown as numbers of copies per 10^6 copies of actin mRNA. (D) The assembly efficiency (super RNA/intra RNA), the budding efficiency (super titer/intra titer), and the specific infectivity (super titer/super RNA) were calculated.

protein samples were characterized using SDS-PAGE and immunoblotting.

Proteolytic digestion protection assay. HCV-infected cells seeded in 6-well dishes were collected in 170 μl proteinase K buffer (50 mM Tris-HCl [pH 8.0], 10 mM CaCl$_2$, 1 mM dithiothreitol [DTT]) and subjected to five freeze-thaw cycles. Next, 50 μl of the crude lysate was left untreated, 50 μl was treated with 50 μg ml^{-1} proteinase K (Qiagen) for 20 min on ice, and another 50 μl was lysed with 5% (vol/vol) Triton X-100 prior to proteinase K treatment. Proteinase K digestion was terminated by the addition of 5 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 10 min. Subsequently, 13 μl of 5× SDS sample buffer was added, and the sample was heated to 95°C for 10 min. The amount of residual core protein was determined by SDS-PAGE and immunoblotting.

Statistical analysis. Data were analyzed using a two-tailed unpaired t test. P values were calculated, and statistical significance was reported as highly significant with $P = <0.05$ (*) or $P = <0.01$ (**). Data are presented as means ± standard deviations.

RESULTS

PLA1A participates in HCV propagation. Previously, we identified PLA2G4C, which hydrolyzes phospholipase at the sn-2 and sn-1 positions, as a host factor involved in HCV replication (30). PLA1A, a member of the phospholipase family that hydrolyzes phosphatidylserine at the sn-1 position, was further analyzed for its role in the HCV life cycle. Three siRNAs targeting PLA1A (siPLA1A-2, siPLA1A-3, and siPLA1A-4) that have no cytotoxicity effect on Huh7 cells (data not shown), the small interfering negative control (siNC), and the siHCV-positive control targeting the internal ribosome entry site (IRES) region of HCV were introduced into the Huh7 cells. Among the three PLA1A siRNAs, siPLA1A-3 and siPLA1A-4 efficiently reduced the mRNA and pro-

tein expression of PLA1A, while siPLA1A-2 reduced only the mRNA but not the protein expression level. Interestingly, the reduced PLA1A expression was accompanied by and correlated with the decline of HCV RNA and viral protein expression in the cells (Fig. 1A and B). siPLA1A-3, which was the most efficient PLA1A-silencing siRNA, had the strongest effect on HCV replication, causing a 79% reduction in the intracellular HCV RNA level and a significant decrease in the expression of HCV proteins such as Core and NS3 compared with the siNC-transfected cell results (Fig. 1A and B).

siPLA1A-3 was then used in the following analysis. The indexes for viral assembly and release were further calculated by testing and comparing the viral titers and the HCV RNA copy numbers in cells as well as in the cell supernatant. The assembly efficiency was defined as the ratio of the number of supernatant HCV RNA copies to the number of intracellular HCV RNA copies, the budding efficiency was defined as the ratio of the HCV titer in cell supernatant to the HCV titer in cells, and the specific infectivity was defined as the ratio of the number of HCV RNA copies to the HCV titer in the cell supernatant (35). Silencing PLA1A expression using siPLA1A-3 reduced the HCV RNA levels in cells and in the supernatant, and the viral titer in the supernatant significantly declined (Fig. 1C). The (approximately 8.8-fold) decrease in the HCV RNA level in the cell supernatant caused by PLA1A silencing was more pronounced than the (approximately 4.9-fold) HCV RNA level decrease in the cells, and the infectious titer in the supernatant also dropped (approximately 6.1-fold) more dramatically than that in the cells (approximately 1.3-fold). As a result, the assembly efficiency and the budding efficiency in siPLA1A-3-
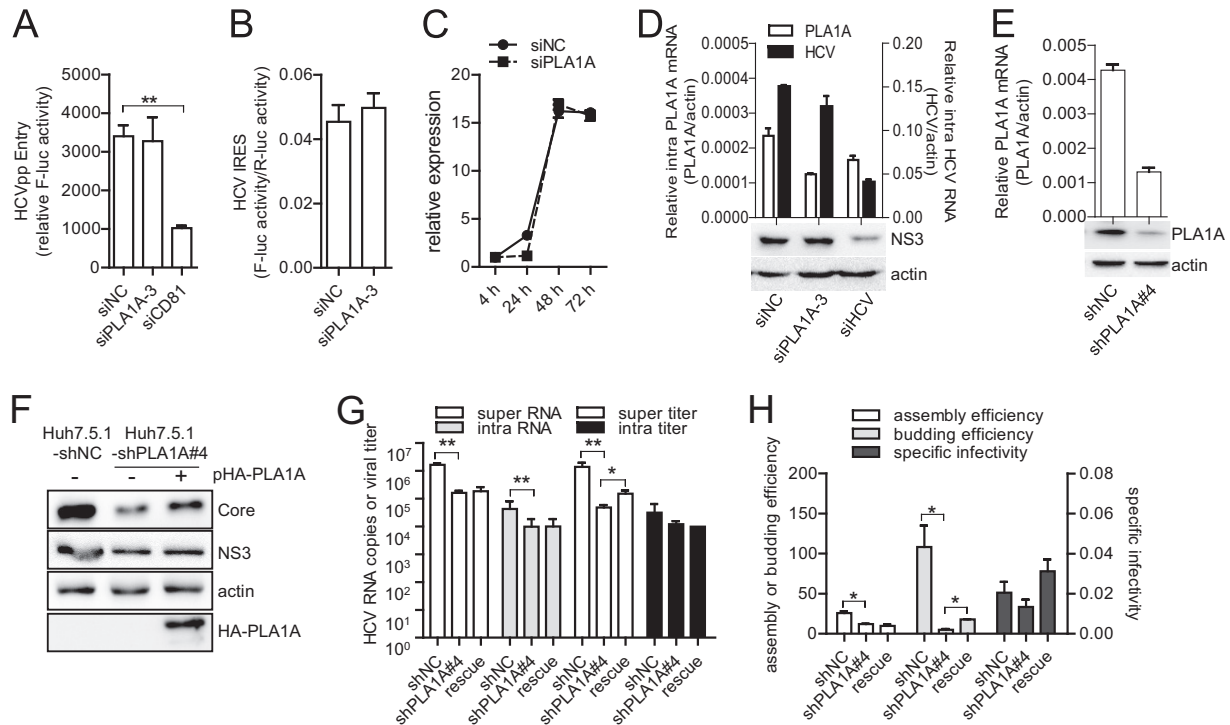
FIG 2 Knockdown of the expression of PLA1A primarily impairs HCV assembly and release. (A) Huh7 cells were transfected with individual siRNAs as indicated for 48 h and then transduced with HCVpp. The luciferase activity was measured 48 h postransduction. (B) Huh7 cells transfected with siNC or siPLA1A-3 were transfected with pHCV-IRES, and dual-luciferase assay was performed at 48 hpt with a dual-luciferase reporter assay system (Promega). The IRES translation efficiency was determined by the ratio of firefly luciferase (F-Luc) activity to Renilla luciferase (R-Luc) activity. (C) Huh7.5.1 cells were electroporated with JFH1-SGR-luc RNA (10 μg) together with siNC or siPLA1A (20 nM). Luciferase activity was measured at the indicated time point and normalized to the value at 4 h. (D) Huh7.5.1-SGR cells were transfected with the indicated siRNAs. The HCV RNA and PLA1A mRNA as well as the NS3 protein expression levels were detected at 72 hpt. (E) PLA1A mRNA and protein expression in Huh7.5.1-shNC and Huh7.5.1-shPLA1A#4 cells were tested by real-time RT-PCR and Western blot analysis, respectively. (F to H) Huh7.5.1-shNC and Huh7.5.1-shPLA1A#4 cells transfected with vector or pHA-PLA1A were infected with J399EM at an MOI of 0.1 for 72 h. HCV NS3, Core, and overexpression of HA-PLA1A were detected (E), HCV RNA copy numbers and viral titers in cells as well as in the supernatant were measured (F), and the assembly efficiency, the budding efficiency, and the specific infectivity were calculated (G).

transfected cells were reduced to 54.3% and 23.2%, respectively, compared with the siNC-transfected cells; however, the specific infectivity did not change significantly, implying that virus assembly and release were impaired by PLA1A silencing (Fig. 1D).

PLA1A is primarily involved in HCV virion assembly and release. To further reveal if PLA1A is involved in other steps of the HCV life cycle, the effect of PLA1A on viral entry, translation, and RNA replication was studied using HCVpp, the HCV IRES reporter plasmid and subgenomic replicon in Huh7 and Huh7.5.1 cells. Huh7 cells transfected with individual siRNAs were transduced with HCVpp, and the luciferase activity was measured at 48 h postransduction and used as an indicator of HCVpp entry efficiency. As shown in Fig. 2A, no difference in the levels of HCVpp entry efficiency between siNC- and siPLA1A-transfected cells was observed. As a positive control, siRNA targeting CD81 efficiently reduced HCVpp entry. Similar results were seen with Huh7.5.1 cells (data not shown); therefore, the knockdown of PLA1A expression did not influence the entry of HCV. pHCV-IRES, a bicistronic reporter plasmid that indicates the translation activity of the HCV IRES, was used to measure the effect of PLA1A on the translation potency of the HCV genome. The results shown in Fig. 2B indicate that the knockdown of PLA1A endogenous expression had no effect on HCV IRES-mediated translation. Furthermore, the role of PLA1A in HCV genomic replication was analyzed in a

transient-transfection assay. No significant difference in the levels of replication of JFH1-SGR-luc was observed between the siNC- and siPLA1A-transfected Huh7.5.1 cells (Fig. 2C). This result was further confirmed in Huh7.5.1-SGR cells containing the JFH1 subgenomic replicon of genotype 2a because the HCV RNA and protein expression levels did not change when PLA1A was knocked down (Fig. 2D). Thus, PLA1A is not involved in the entry, translation, or RNA replication processes of HCV in Huh7.5.1 cells.

To further confirm the role of PLA1A in assembly and release, HCV propagation was further analyzed in Huh7.5.1 cells with PLA1A stably knocked down. For this purpose, cell lines with stable shPLA1A expression were constructed via the lentiviral transduction of Huh7.5.1 cells as described in Materials and Methods. The expression of PLA1A was efficiently reduced in the stable knockdown cells (Huh7.5.1-shPLA1A#4, or shPLA1A#4) compared with the negative-control cells (Huh7.5.1-shNC, or shNC) (Fig. 2E). Consistent with the previous results, no significant difference in the levels of HCVpp entry efficiency, HCV IRES activity, or JFH1-SGR-luc replication were observed in the shNC and shPLA1A#4 cells (data not shown). Cell culture-grown HCV (HCVcc) propagation was then monitored in the shNC and shPLA1A#4 cells at 72 h postinfection (hpi). HCV protein (Core and NS3) expression in the shPLA1A#4 cells was significantly re-
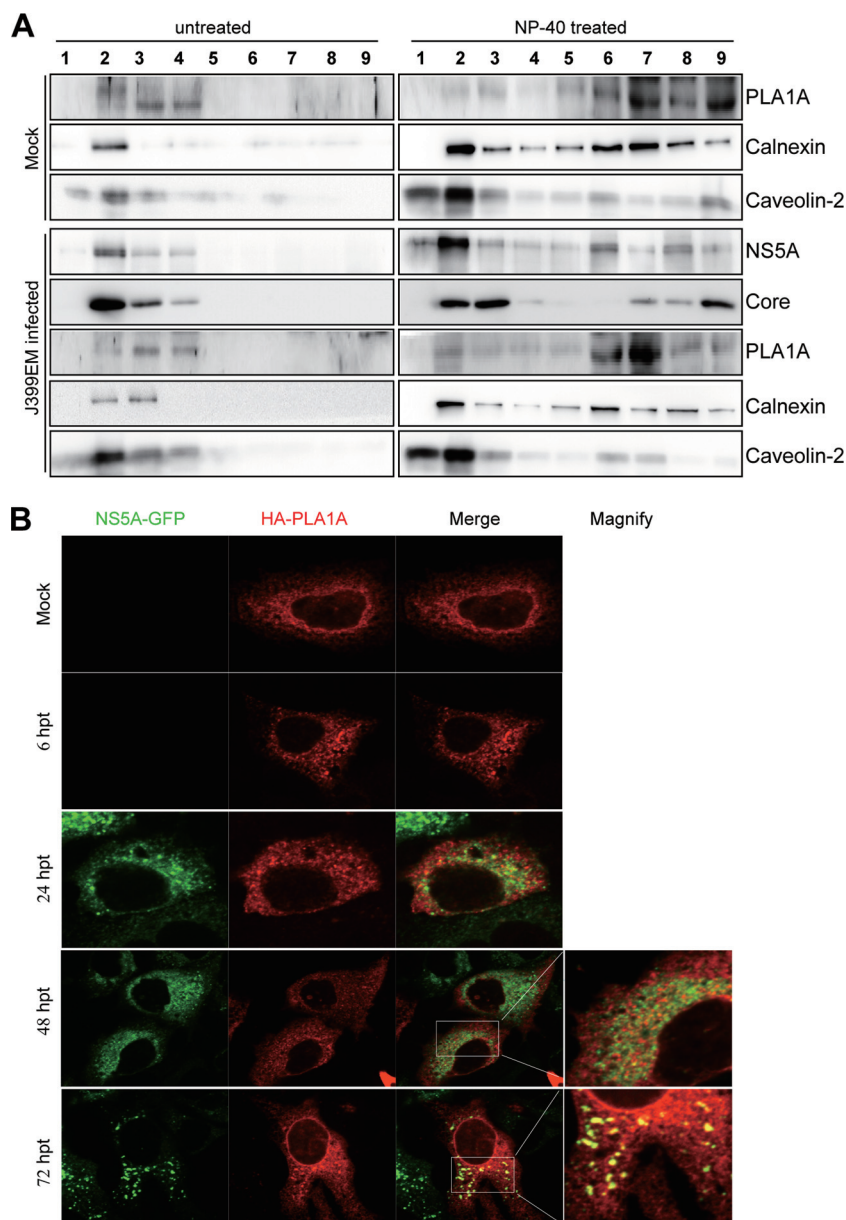
FIG 3 HCV infection has no influence on the localization and distribution of PLA1A. (A) Lysates from mock- or J399EM-infected Huh7 cells were submitted to a membrane flotation assay in a sucrose gradient (see details in Materials and Methods). NS5A-GFP, core, NS3, PLA1A, calnexin, and caveolin-2 were detected in each fraction by Western blot analysis. (B) Huh7 cells were transfected with pHA-PLA1A and then infected with J399EM at an MOI of 0.1. The intracellular PLA1A was immunostained with anti-HA antibody and Alexa Fluor 561-conjugated secondary antibodies at indicated time points and was observed under a fluorescence microscope. The NS5A expression was indicated by the eGFP expression seen under a fluorescence microscope.

duced (Fig. 2F), and the viral RNA and infectious virus levels released into the shPLA1A#4 cell supernatant were reduced to 6.7% and 7.8%, respectively, of the levels observed in the shNC cells (Fig. 2G). The assembly efficiency and budding efficiency in the shPLA1A#4 cells were reduced to 46.7% and 4.5%, respectively, compared with the shNC cell levels (Fig. 2H). Exogenous expression of PLA1A in the shPLA1A#4 cells partially rescued HCV protein expression in the cells, the infectious virus production in the supernatant, and the calculated budding efficiency (Fig. 2F to H). Together with the results shown in Fig. 1, these results indicate that PLA1A primarily participates in the assembly and

release steps but not in the entry and genomic replication steps of the HCV life cycle.

The localization of PLA1A during infection. To explore the potential mechanism underlying the role of PLA1A in HCV replication, the subcellular localization of PLA1A was examined by both biochemical fractionation and epitope-tagged expression. The cell lysates from mock- or J399EM-infected Huh7.5.1 cells were subjected to membrane flotation fractionation. Caveolin-2 was used as a detergent-resistant membrane (DRM)-positive control, and calnexin was used as a detergent-sensitive control (37). As previously reported, a large amount of HCV Core and NS5A
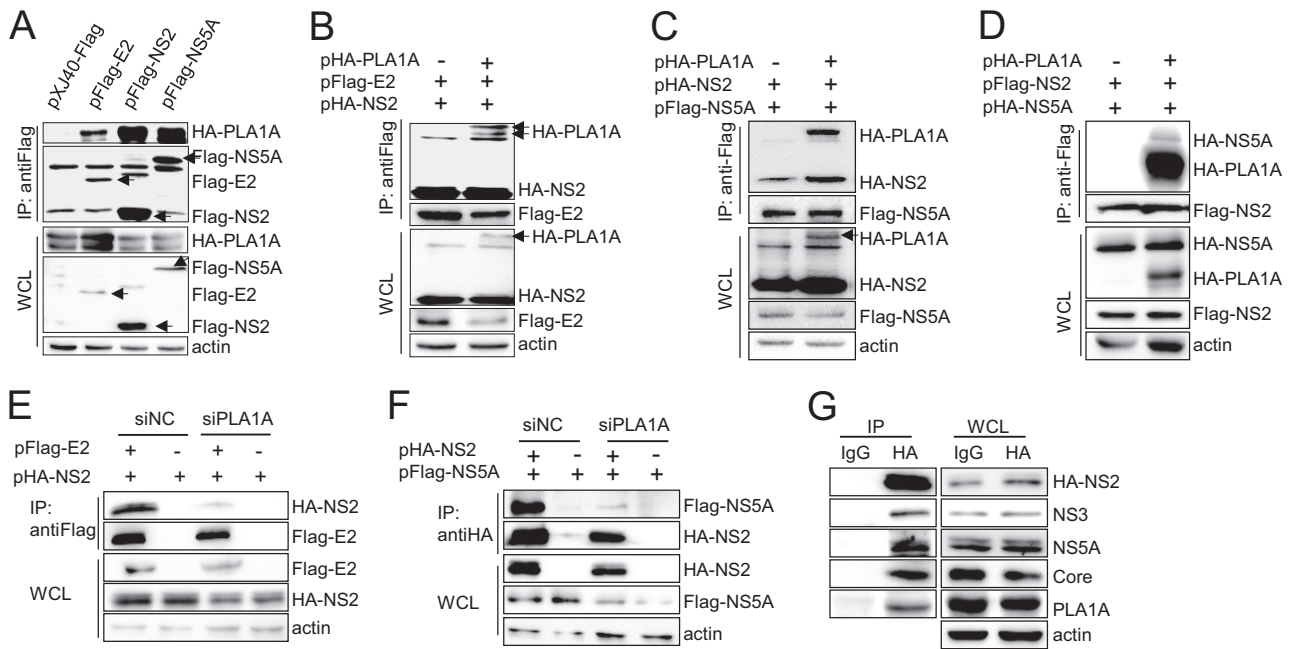
FIG 4 PLA1A participates in the NS2-E2 and NS2-NS5A interaction. (A) 293T cells were transfected with pHA-PLA1A together with pFlag-E2, pFlag-NS2, or pFlag-NS5A. Whole-cell lysates (WCL) were then submitted to coimmunoprecipitation (IP) and Western blot analysis performed with the indicated antibodies. (B) Lysates of 293T cells, which were cotransfected with pHA-NS2 and pFlag-E2 in the presence or absence of HA-PLA1A expression, were submitted to coimmunoprecipitation and Western blot analysis performed with the indicated antibodies. (C and D) Lysates of 293T cells, which were cotransfected with pHA-NS2 and pFlag-NS5A (C) or with pFlag-NS2 and pHA-NS5A (D) in the presence or absence of HA-PLA1A expression, were submitted to coimmunoprecipitation and Western blot analysis performed with the indicated antibodies. (E and F) Huh7 cells were transfected with siNC or siPLA1A and then transfected with pFlag-E2 and pHA-NS2 (E) or with pHA-NS2 and pFlag-NS5A (F). The cell lysates were submitted to coimmunoprecipitation and Western blot analysis performed with the indicated antibodies. (G) Huh7 cells were electroporated with RNA of JFH1-HA and incubated for 96 h. The cells were lysed for immunoprecipitation assay with IgG or anti-HA antibody. The resultant precipitates and whole-cell lysates were examined by Western blot analysis performed with antibody to HA, NS3, NS5A, Core, PLA1A, or actin.

proteins was fractionated in the DRMs (fractions 1 to 3); however, only a small subset of PLA1A was found in the DRMs (fractions 1 to 3) in both the mock- and J399EM-infected Huh7 cells. This result suggests that PLA1A primarily exists in the detergent-sensitive fraction or associates with membranes only weakly and that HCV infection does not change the distribution of PLA1A (Fig. 3A). Due to the lack of a suitable antibody that recognizes PLA1A for immunofluorescence, an expression plasmid with HA-tagged PLA1A (pXJ40-HA-PLA1A) was constructed and transfected into Huh7 cells. HA-PLA1A showed a reticular distribution in the cytoplasm (Fig. 3B). Interestingly, although the distribution pattern of PLA1A remained unchanged during HCV infection, the accumulated NS5A dot-like structures were highly colocalized with PLA1A at 72 hpi (Fig. 3B). This phenotype appeared at the late stage but not the early stage (at 24 and 48 hpi) of HCV infection (Fig. 3B).

PLA1A interacts with the HCV E2, NS2, and NS5A proteins. The marked colocalization of NS5A with PLA1A at the late stage of infection implied that an interaction occurs between the two proteins. Indeed, HA-PLA1A coprecipitated with Flag-tagged NS5A (Fig. 4A). Interestingly, HA-PLA1A also coprecipitated with Flag-tagged structural protein E2 and nonstructural protein NS2 using an anti-Flag antibody (Fig. 4A) but not with other proteins such as E1, NS34A, and NS4B (data not shown), indicating that PLA1A interacts with E2, NS2, and NS5A. A previous report suggested a role for NS2 as a scaffold that interacts with both structural and nonstructural proteins (14). Thus, the role of PLA1A in the

NS2-E2 and NS2-NS5A interactions was examined using PLA1A overexpression or PLA1A silencing.

The interactions between NS2 and E2 and between NS2 and NS5A were first examined in 293T cells in the presence or absence of the PLA1A expression plasmid. As shown in Fig. 4B, NS2 coimmunoprecipitated with E2 independently of PLA1A overexpression. NS2 coimmunoprecipitation with NS5A in 293T cells was observed and enhanced by PLA1A overexpression (Fig. 4C). The coimmunoprecipitation experiments were then performed in the reverse direction, and a small fraction of NS5A was coimmunoprecipitated using the HA-NS2 antibody in 293T cells during PLA1A overexpression but not in the mock-transfected cells (Fig. 4D). These results indicate that the NS2-NS5A interaction was enhanced by PLA1A.

Subsequently, the NS2-E2 and NS2-NS5A interactions were examined in Huh7 cells transfected with siNC or siPLA1A. The results showed that the precipitation of HA-NS2 together with Flag-E2 using the Flag antibody significantly decreased in the siPLA1A-transfected cells (Fig. 4E). Similar results were obtained when the NS2 and NS5A interaction was examined, and the precipitation of NS5A using the NS2-HA antibody was dramatically reduced by PLA1A silencing (Fig. 4F). Thus, endogenous PLA1A in Huh7 cells plays a role in promoting the NS2-E2 and NS2-NS5A interactions.

Finally, we determined the involvement of endogenous PLA1A in NS2 complex formation during HCVcc infection. To this end, the Huh7 cells were electroporated with an RNA genome from the
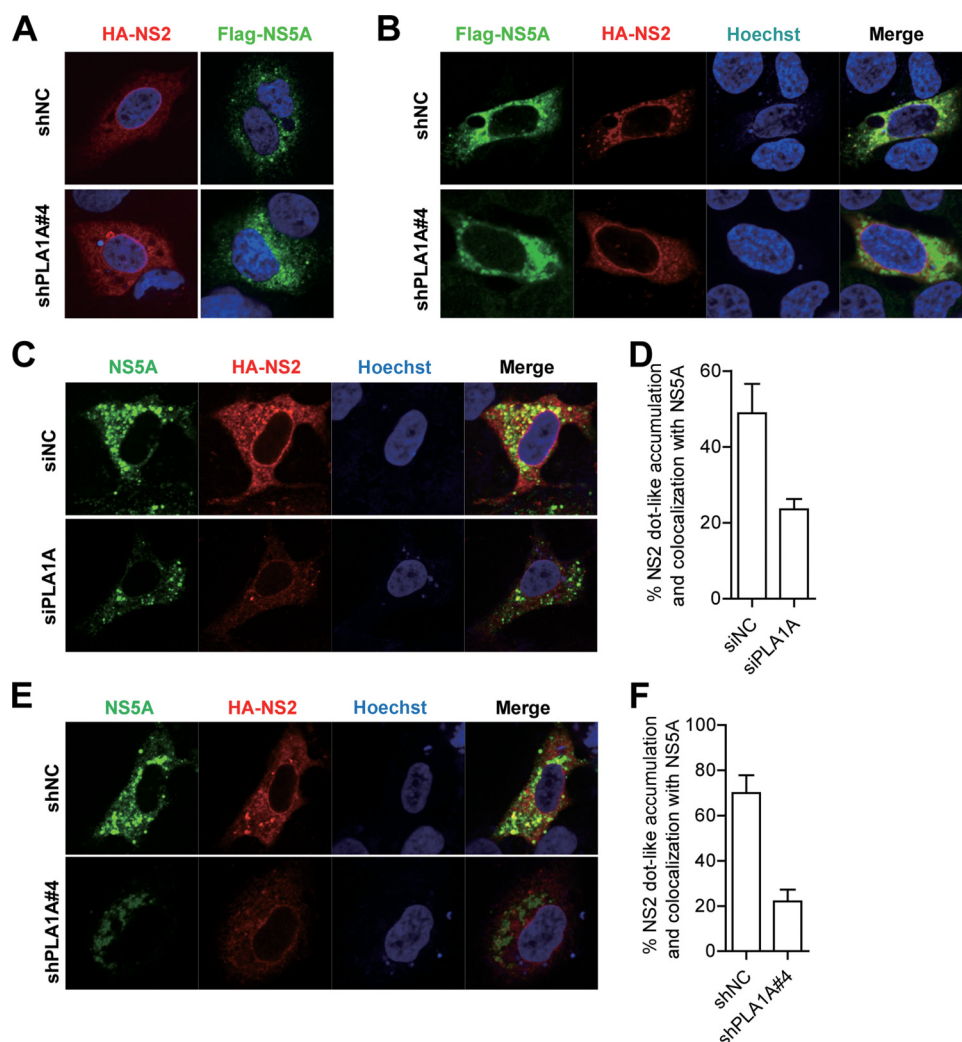
FIG 5 Knockdown of the expression of PLA1A influences the formation of NS2/NS5A dotted structures. (A) Huh7.5.1-shNC or shPLA1A#4 cells were transfected with pHA-NS2 or pFlag-NS5A and stained by indirect immunofluorescence with anti-HA or anti-Flag primary antibodies. The nuclei were stained with Hoechst 33258. (B) Huh7.5.1-shNC and shPLA1A#4 cells were cotransfected with pHA-NS2 and pFlag-NS5A and stained by indirect immunofluorescence with anti-HA and anti-Flag primary antibodies, respectively. The nuclei were stained with Hoechst 33258. (C) Huh7 cells were electroporated with JFH1-HA RNA together with siNC or siPLA1A and incubated for 96 h. Cells were then fixed and probed with antibodies against the HA tag and NS5A (9E10) for indirect immunofluorescence analysis. (D) The percentage of cells with NS2/NS5A dotted structures was counted and is shown. (E) JFH1-HA RNA was electroporated into Huh7.5.1-shNC or shPLA1A#4 cells and incubated for 96 h. Cells were then fixed and probed with antibodies against the HA tag and NS5A (9E10) for indirect immunofluorescence analysis. (F) The percentage of cells with NS2/NS5A dotted structures was counted and is shown.

JFH1-HA virus in which the NS2 protein was fused with an HA tag. The whole-cell lysate was extracted at 72 h postelectroporation and subjected to immunoprecipitation analysis using the anti-HA antibody. As expected, Core, NS3, NS5A, and PLA1A were precipitated by anti-HA, indicating that endogenous PLA1A is a part of the NS2 complex during infection (Fig. 4G).

Overall, these results suggest that PLA1A interacts with HCV E2, NS2, and NS5A proteins, plays an essential role in the NS2-E2 and NS2-NS5A interactions, and is a component of the NS2 complex.

PLA1A is required for the formation of NS2/NS5A dotted structures during HCV infection. The NS2 protein forms dotted structures that colocalize with NS5A gradually during HCV infection as virus production occurs, and mutations in E2 and NS5A influence NS2 dotted-structure formation (15). As in previous

reports, NS5A and NS2 presented an endoplasmic reticulum (ER)-like reticulated pattern in the cytoplasm when expressed alone in shNC and shPLA1A#4 cells (Fig. 5A). When NS2 and NS5A were coexpressed, a dotted NS2 structure colocalized with NS5A was observed in 34.35% ± 6.15% of doubly positive shNC cells; however, only 5.95% ± 1.34% of NS2- and NS5A-transfected shPLA1A#4 cells showed the accumulation of NS2 in dot-like structures (Fig. 5B). The localization of NS2 and NS5A was then analyzed in the context of an infectious virus, JFH1-HA. As previously reported, NS2 primarily distributed in an ER-like reticulated pattern at 24 and 48 hpi but gradually formed dotted structures which colocalized with NS5A at 96 hpi (Fig. 5C and data not shown). However, the percentage of cells with NS2/NS5A dotted structures was reduced in siPLA1A-transfected cells (23.53% ± 2.69%) compared with siNC-transfected cells (48.86% ± 7.72%) (Fig. 5D). The
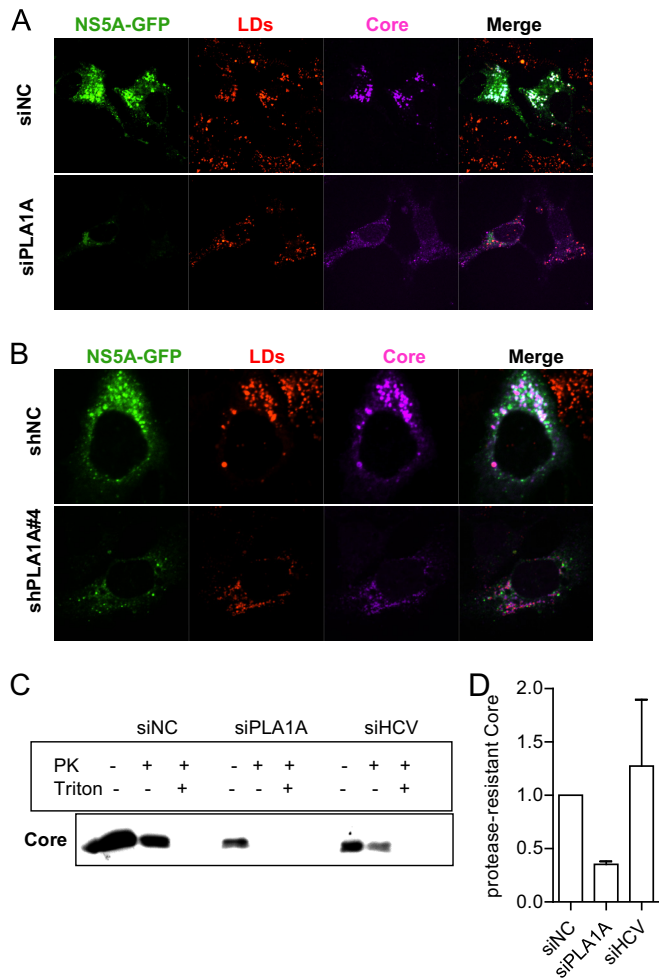
FIG 6 PLA1A influences the envelopment of the HCV virion. (A) Huh7 cells were transfected with siNC or siPLA1A and then infected with J399EM at an MOI of 0.1 for 96 h. Core was stained by indirect immunofluorescence with anti-Core. The LDs were stained with HCS LipidTOX Red. (B) Huh7.5.1-shNC and shPLA1A#4 cells were infected with J399EM at an MOI of 0.1 for 96 h. Cells were fixed and stained by indirect immunofluorescence with anti-Core and HCS LipidTOX Red (LD). (C) Huh7 cells were transfected with siNC, siPLA1A, or siHCV and infected with J399EM at an MOI of 0.1. Cells were treated using a proteolytic digestion protection assay (see Materials and Methods) at 96 hpi. Representative results are shown. (D) The percentage of protease-resistant Core was calculated and normalized to the level seen with an untreated control.

same results were obtained in JFH1-HA-electroporated shNC and shPLA1A#4 cell lines, as the dotted NS2 structure was found in 69.94% ± 7.94% of shNC cells compared with 22.02% ± 5.30% of shPLA1A#4 cells (Fig. 5E and F). In summary, these results indicate the requirement of PLA1A in the formation of NS2/NS5A dotted structures.

PLA1A influences the envelopment of the HCV virion. Next, we analyzed the influence of PLA1A in the substeps of assembly. Regarding the JFH1 virus, the localization of Core protein on the LD and the recruitment of NS5A are important for initial virus assembly (20). As anticipated, Core and NS5A accumulation around the LD was found in cells at 96 hpi. No significant difference was found between siPLA1A- and siNC-transfected cells or between shPLA1A#4 and shNC cells (Fig. 6A and B), indicating

that the accumulation of the Core protein around the LD and the recruitment of NS5A were not influenced by PLA1A silencing. The envelopment of the HCV virion was then evaluated using a proteolytic digestion protection assay (38). In this assay, the resistance of Core protein to digestion reflected the degree of envelopment. A representative result is shown in Fig. 6C, and a significant reduction in proteinase-resistant Core was observed in siPLA1A-transfected cells compared with siNC-transfected cells. This reduction was not due to a significant reduction of total Core protein levels because siHCV successfully reduced the total Core protein level but did not change its resistance to proteinase. Thus, we speculated that the knockdown of the expression of PLA1A influences the envelopment of HCV.

Upregulation of PLA1A expression by HCV infection. After confirming the involvement of PLA1A in the HCV life cycle, the modulation of PLA1A expression by HCV infection was analyzed because HCV replication knockdown in J399EM-infected Huh7 cells also reduced PLA1A mRNA levels (Fig. 1A). Quantitative RT-PCR (qRT-PCR) assay results indicated that PLA1A mRNA levels were upregulated significantly at 72 hpi in an HCV dose-dependent manner (Fig. 7A and B). Western blot analysis performed with PLA1A-specific antibody showed that PLA1A protein levels were also increased after HCV infection (Fig. 7C). To determine which viral component is involved in the upregulation of PLA1A, Huh7 cells were transfected with expression plasmids containing the individual HCV structural and nonstructural genes. None of the proteins induced PLA1A expression in Huh7 cells (Fig. 7D); however, poly(I·C), a synthetic double-stranded RNA (dsRNA) polymer, enhanced PLA1A expression by 4.5-fold at 72 h (Fig. 7E). These results imply that HCV genome replication is essential for the upregulation of PLA1A.

DISCUSSION

In the present study, we identified PLA1A as a host factor that participates in the assembly process of HCV through interactions with NS2, E2, and NS5A. PLA1A is required for the interaction between NS2 and E2 and for the interaction between NS2 and NS5A and determines the NS2/NS5A dotted structures, which are thought to be a transient state in the assembly process.

The details of HCV assembly are not fully understood; however, this process requires complex protein-protein interactions between HCV proteins and host factors. It has been generally accepted that the Core-decorated LD and the replication complex (RC) are brought together through the interaction of Core and NS5A, which is very important at an early step of HCV assembly (11, 20, 39). NS2 protein is required for virus assembly as a scaffold recruiting viral envelope proteins to the assembly sites in close proximity to LD (14–16, 40–42). It has been proposed that the E1E2NS2p7 complex migrates to a position close to the RC through the interaction between NS2 and NS5A. The argument is that the interaction between NS2 and NS5A was thought to be a transient or unstable interaction (14, 15, 41, 43). Our results provide evidence that PLA1A has an important role in the formation of the NS2-NS5A complex mediated by its interaction with both NS2 and NS5A. Upregulation of PLA1A expression induced by HCV infection in the late stage could enhance and/or stabilize the formation of the NS2-NS5A complex, while knockdown of PLA1A reduced the interaction of NS2 and NS5A (Fig. 4B) and the NS2/NS5A dotted structure (Fig. 5B, C, and E), which is thought to represent a transient phase during assembly. Therefore, PLA1A
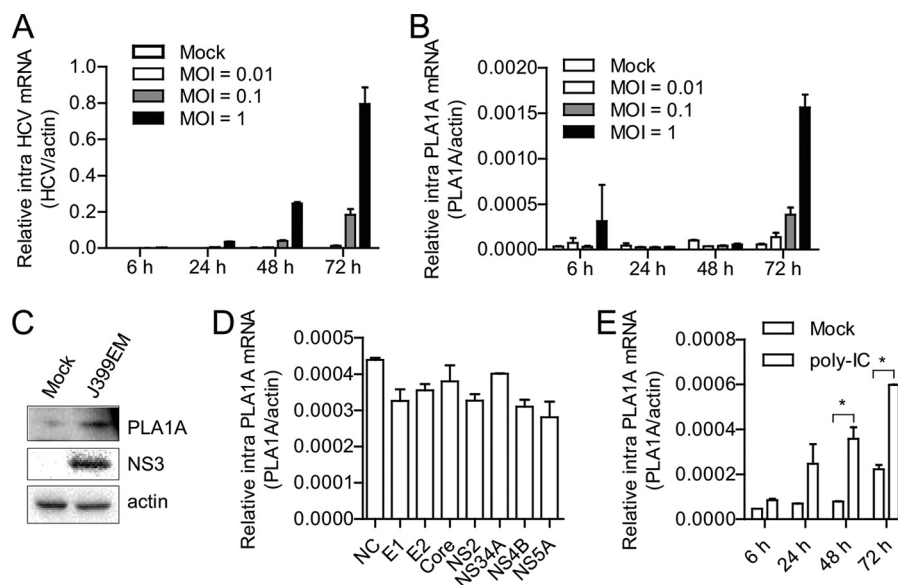
FIG 7 PLA1A expression is upregulated by HCV infection. Huh7 cells left uninfected or infected with J399EM at different MOIs (0.01, 0.1, and 1) were harvested at the indicated time points. (A and B) Intercellular HCV RNA levels (A) and PLA1A mRNA levels (B) were quantified by specific real-time RT-PCR. (C) Protein samples of HCVcc-infected (MOI of 0.1) and uninfected Huh7 cells were collected at 72 hpi. PLA1A expression and HCV NS3 expression were detected. (D) Huh7.5.1 cells were transfected with empty vector, E1, E2, Core, NS2, NS34A, NS4B, or NS5A expression plasmid. The PLA1A mRNA levels were determined at 72 hpt. (E) Huh7 cells transfected with 1 μg poly(I·C) were harvested at the indicated time points. The PLA1A mRNA levels were quantified by specific real-time RT-PCR.

may play an essential role in HCV assembly by recruiting the NS2 complex together with E1-E2 glycoprotein to Core-containing LDs via the interaction with NS2 and NS5A. HCV assembly also requires phosphorylation of NS5A by casein kinase IIα (44). Thus, it will be of interest to analyze whether PLA1A also influences the phosphorylation status of NS5A, which may contribute to the switch between replication and assembly (45).

Prior work showed that NS2 is not essential for RNA replication (9); however, NS2 brings together the viral E1-E2 glycoprotein complex, p7, and the NS3-4A enzyme complex (14–16, 40–42). The interaction between NS2 and E1-E2 envelope protein has been shown using biochemistry and genetic data (16, 42); however, whether these two proteins interact directly remains unclear. Recently, SPCS1 was identified as an NS2 interaction protein and was reported to mediate NS2-E2 complex formation, contributing to the HCV assembly process (26). A similar function for PLA1A in the NS2-E2 interaction was found in our research because PLA1A interacts with both E2 and NS2 (Fig. 4A). As PLA1A silencing reduced the interaction of NS2 and E2, as shown in Fig. 4E, PLA1A may contribute to NS2-E2 complex formation or stabilize the complex. Proteolytic analysis showed impaired HCV envelopment in PLA1A knockdown cells, demonstrating the functional importance for PLA1A in HCV assembly.

Besides PLA1A, several members of the phospholipase family such as PLA2G4C, PLA2G4A, and PLA2GXIIB are involved in HCV assembly (27, 28, 30). The exact mechanisms for the regulation of HCV assembly by PLA2G4C have not yet been revealed. However, PLA2G4C may function at the very early stage of HCV assembly, as knockdown of PLA2G4C does not impair HCV release (30). The hypothesis is further strengthened by evidence showing that PLA2G4C is also involved in HCV RNA replication by participation in membranous web formation and causes the relocalization of NS4B to LD, where virion assembly occurs.

PLA2G4A is involved in the early stage of HCV assembly, as inhibition of PLA2G4A activity by specific chemical inhibitor Py-2 reduced core protein abundance at lipid droplets, which may in turn limit core protein envelopment and particle release (28). PLA2GXIIB may function in the late stage of the HCV life cycle and is required for HCV secretion through the regulation of VLDL secretion (27). Thus, these phospholipases function at different stages of HCV assembly with diverse mechanisms. It will be valuable to perform further studies to explore how these phospholipases are involved together in HCV assembly and release.

The common feature for these phospholipases is the production of fatty acids by enzyme digestion. It has been reported that exogenous addition of arachidonic acid, but not of other related polyunsaturated fatty acids, is crucial for production of infectious HCV progeny but not for viral entry and RNA replication (28, 30). PLA1A specifically acts on phosphatidylserine (PS) and 1-acyl-2-lysophosphatidylserine (lyso-PS) to hydrolyze fatty acids at the sn-1 position of these phospholipids (29). Thus, PLA1A may also contribute to HCV particle production through the cleavage products of fatty acids such as arachidonic acid. As a secreted enzyme, PLA1A may also hydrolyze phosphatidylserine on the cell surface and produce lyso-PS, as HCV was previously shown to induce apoptosis of infected cells (46). Interestingly, exogenous addition of lyso-PS can partially rescue HCV replication reduced by PLA1A knockdown (data not shown). lyso-PS has been described as a potent activator of histamine release from mast cells, as a growth inhibitor of T cells, and as a chemotactic substance for fibroblasts and tumor cells (47–50). Further work will be conducted to disclose how lyso-PS regulates HCV replication.

PLA1A is expressed at a high level in the liver and prostate gland and at lower levels in other organs (32). Consistent with these findings, we found that expression of PLA1A was higher in hepatoma cell lines, including Huh7.5.1, Huh7, and Lunet cells,

than in non-hepatoma cell lines (293T and HeLa cells) (data not shown). Notably, the PLA1A mRNA levels in Huh7.5.1 cells, which support HCV replication most efficiently, were approximately 7-fold higher than in Huh7 and Lunet cells. HCV tropism is restricted to liver cells; therefore, the high expression level of PLA1A may be one of the factors explaining the support of Huh7.5.1 cells for HCV replication. Furthermore, the expression of PLA1A in hepatoma cells was enhanced by HCV at a late stage of infection, which is consistent with its role in the assembly process. Our results indicated that the dsRNA formed during virus propagation may promote HCV-induced PLA1A upregulation in Huh7 cells because the transfection of the synthetic analog of dsRNA, poly(I·C), induces PLA1A expression (51). Although none of the HCV proteins is solely responsible for PLA1A upregulation, we cannot rule out the possibility that a combination of HCV proteins may alter PLA1A expression. A previous report showed that PLA1A mRNA expression was enhanced at 12 h post-lipopolysaccharide (LPS) stimulation in THP-1-derived macrophages (52). It would be very interesting to analyze the cross talk between the innate immunity pathway and the induction of PLA1A expression during HCV infection.

In summary, a novel host factor, PLA1A, was identified as a participant in the HCV assembly process. Our data demonstrate that PLA1A plays a key role in the NS2-NS5 and E2-NS2 interaction. These results provide clues for understanding the details of the molecular mechanism of assembly and formation of infectious HCV particles.

ACKNOWLEDGMENTS

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