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A class II phosphoinositide 3-kinase plays an indispensable role in hepatitis C virus replication

Tomohiko Maehama [a,*], Masayoshi Fukasawa [a], Tomoko Date [b], Takaji Wakita [b], Kentaro Hanada [a]

[a] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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ABSTRACT

Phosphoinositides function as fundamental signaling molecules and play roles in diverse cellular processes. Certain types of viruses may employ host cell phosphoinositide signaling systems to facilitate their replication cycles. Here we demonstrate that the β isoform of class II PI3K (PI3K-C2β) plays an indispensable role in hepatitis C virus (HCV) propagation in human hepatocellular carcinoma cells. Knockdown of PI3K-C2β abrogated HCV propagation in the cell. Using an HCV replicon system, we found that knockdown of PI3K-C2β substantially repressed the full-genome replication, while showing relatively small reductions in sub-genome replication, in which structural proteins including core protein were deleted. We also found that HCV core protein showed the binding activity towards D4-phosphorylated phosphoinositides and overlapped localization with phosphatidylinositol 3,4-bisphosphate in the cell. These results suggest that the phosphoinositide generated by PI3K-C2β plays an indispensable role in the HCV replication cycle through the binding to HCV core protein.

1. Introduction

Phosphoinositides (PIs) are lipid molecules that play crucial roles in diverse cellular functions [1]. In most cases, PIs generated in response to specific stimuli recruit their binding proteins to certain intracellular sites where these proteins exert their functions [2]. Accumulating evidence has identified a number of PI-binding proteins and their functions in fundamental cellular activities, such as cell proliferation, cell growth, vesicle trafficking, and cytoskeletal reorganization [1,2]. PI species are interconverted through phosphorylation and dephosphorylation by a set of specific PI kinases and PI phosphatases. For example, phosphatidylinositol 4-phosphate (PtdIns(4)*P*) is phosphorylated by phosphatidylinositol phosphate 5-kinases to produce phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), which is further phosphorylated by class I PI 3-kinases (PI3Ks) to produce phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). This PtdIns(4)*P*—PtdIns(4,5)P_2—PtdIns(3,4,5)P_3 axis is well characterized in its regulation and physiological functions. PtdIns(4)*P* binds to the pleckstrin homology (PH) domain of certain proteins to regulate the intracellular trafficking of proteins and lipids. PtdIns(4,5)P_2 plays a role in regulating cytoskeletal reorganization through the binding to several actin-binding proteins, while PtdIns(3,4,5)P_3 governs cell proliferation and survival through the binding to a set of PH-domain proteins, such as the protein kinase AKT.

Hepatitis C virus (HCV) is an RNA virus that propagates in human hepatic cells and becomes a causative agent for several hepatic disorders, such as steatosis, fibrosis, and hepatocellular carcinomas [3]. HCV is an enveloped virus, of which the viral nucleocapsid is comprised of one type of protein, the core protein. An accumulating body of evidence suggests that the core protein affects diverse host cell function, including proliferation, apoptosis, and metabolism [4], although underlying molecular mechanism of how the core protein acts on these cellular processes remains largely undefined. HCV enters into cells via clathrin-mediated endocytosis, replicates on certain endomembrane structures, and exits from cells using the lipoprotein secretion system [5–7]. These observations clearly show that HCV employs fundamental host cell functions, including cytoskeletal reorganization and intracellular trafficking systems, to facilitate its replication, thereby suggesting that multiple PIs may participate in the HCV replication cycle. Indeed, several studies have demonstrated that type III phosphatidylinositol 4-kinases (PI4K-IIIα and PI4K-IIIβ), which produce PtdIns(4)*P*, are required for HCV replication in HuH-7 hepatocellular carcinoma cells [8–12]. It has been also reported that PI4K-IIIα binds to the HCV nonstructural NS5A protein and participates in the replication complex formation [13–15]. In addition, NS5A

* Corresponding author. Address: Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1157.
E-mail address: tmaehama@nih.go.jp (T. Maehama).

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protein is known to activate class I PI3K and increase survival signals [16,17]. However, participation of other PIs and their metabolizing enzymes in the HCV replication cycle remains largely unknown.

In this study, we show that the β isoform of class II PI3K (PI3K-C2β), a potential phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2)- and phosphatidylinositol 3-phosphate (PtdIns(3)P)-producing enzyme, is required for the HCV genome replication process. We also demonstrate that PtdIns(3,4)P_2 directly binds to HCV core protein *in vitro* and, in HCV-replicating cells, localizes at endomembrane structures with the core protein. Our study is the first report to demonstrate the requirement of PI3K-C2β for the HCV replication cycle.

2. Materials and methods

2.1. HCV replication assay

Replicon RNAs (SGR, SGR-GND, FGR, and FGR-GND) were prepared from pSGR-JFH1/luc, pSGR-JFH1(GND)/luc, pFGR-JFH1/luc, and pFGR-JFH1(GND)/luc as described previously [18,19]. HuH-7.5.1-8 cells on 48-well plates were transfected with one of the replicon RNAs as described above and then cultured for 22–72 h. Luciferase activity was determined using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

2.2. Lipid binding assay

In order to prepare liposomes, egg phosphatidylcholine (500 μg, Avanti Polar Lipids) and *sn*-1-palmitoy-2-oleoylphosphatidylethanolamine (125 μg, Sigma–Aldrich) along with 3 μg each of PtdIns(3)P, PtdIns(4)P, phosphatidylinositol 5-phosphate, PtdIns(3,4)P_2, PtdIns(4,5)P_2, or PtdIns(3,4,5)P_3 were dried under vacuum. All PIs used in this study were in the dipalmitoylated form and obtained from Cayman. The dried lipids were resuspended in 100 μl of resuspension buffer (50 mM 3-morpholinopropanesulfonic acid–NaOH [pH 7.2], 100 mM NaCl, and 1 mM DTT) and incubated at 65 °C for 1 h. The liposomes were then frozen in liquid nitrogen and thawed at 37 °C for three cycles. The liposome binding assay was performed in a solution containing 100 μl of liposome solution and 2 μl of fraction-17 (of Sephacryl S-300 column chromatography) containing recombinant core protein. After incubation at 4 °C for 1 h, liposomes were collected by centrifugation at 20,000*g* for 20 min, and core protein precipitated with liposomes

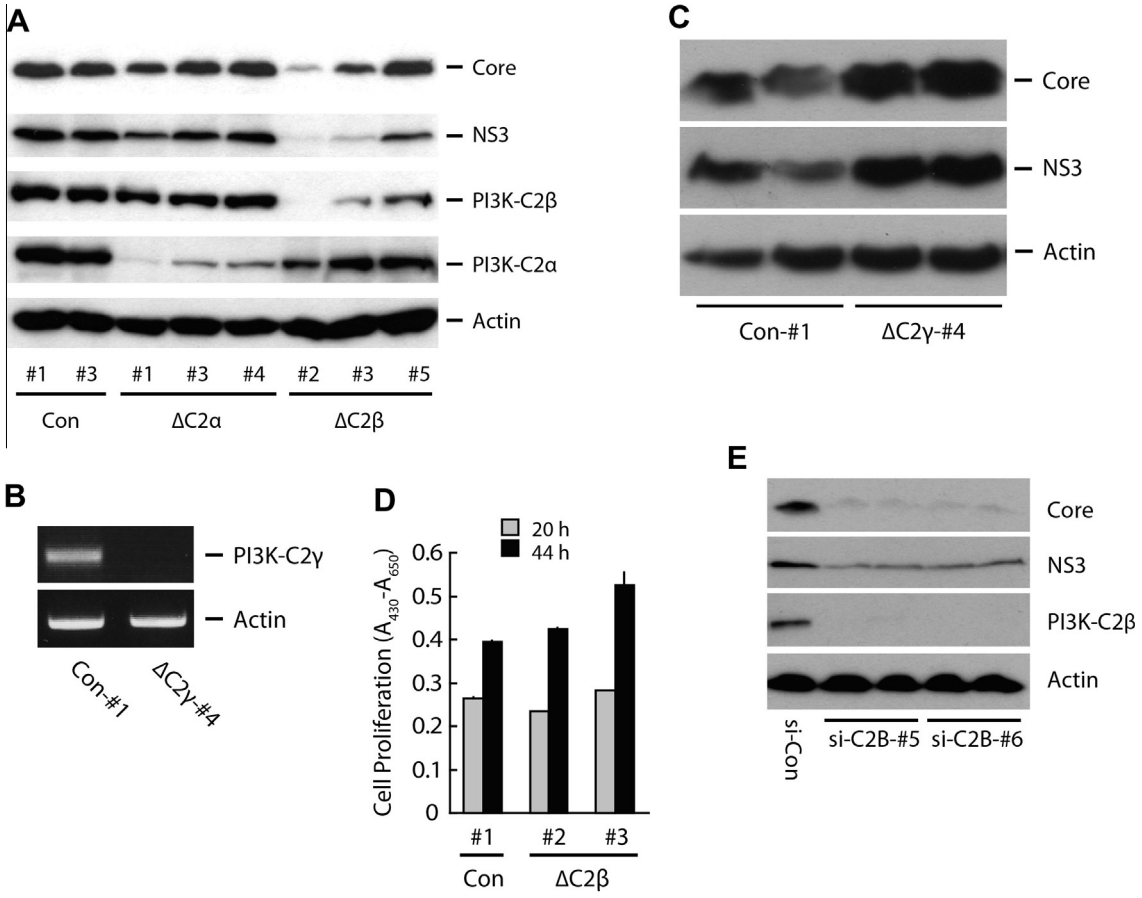

Fig. 1. PI3K-C2β is indispensable for HCV propagation in HuH-7.5.1-8 cells. (A) Control (Con-#1 and #3), PI3K-C2α-knockdown (ΔC2α-#1, #3, and #4), and PI3K-C2β-knockdown (ΔC2β-#2, #3, and #5) cells were infected with HCV. After a 5-day culture, viral proteins (core and NS3) accumulated in cells were detected by immunoblotting. Expression of PI3K-C2α and PI3K-C2β along with actin (as a loading control) was also represented. (B) RNA fractions from Control (Con-#1) and PI3K-C2γ-knockdown (ΔC2γ-#4) cells were subjected to RT-PCR analysis to detect PI3K-C2γ and actin mRNAs. RNA extraction and RT-PCR were conducted as described under Section 2 (HCV entry assay section). (C) Control (Con-#1) and PI3K-C2γ-knockdown (ΔC2γ-#4) cells were infected with HCV. After a 5-day culture, viral proteins (core and NS3) accumulated in the cells were detected by immunoblotting. (D) In order to determine the proliferation of control (Con-#1) and PI3K-C2β-knockdown (ΔC2β-#2 and #3) cells, 2.5×10^3 cells were plated on 96-well plates and cultured for the indicated times. Proliferation was determined as described under Section 2. Data are represented as the mean ± SD from quadruplicated experiments. (E) HuH-7.5.1-8 cells were transfected with indicated siRNAs (si-Con, si-C2B-#5, and si-C2B-#6) twice with a 3-day interval. Cells were then infected with HCV. After a 5-day culture, viral proteins (core and NS3) accumulated in the cells were detected by immunoblotting. Expression of PI3K-C2β along with actin (as a loading control) was also represented. Typical data from three independent experiments are presented (A, C, E).

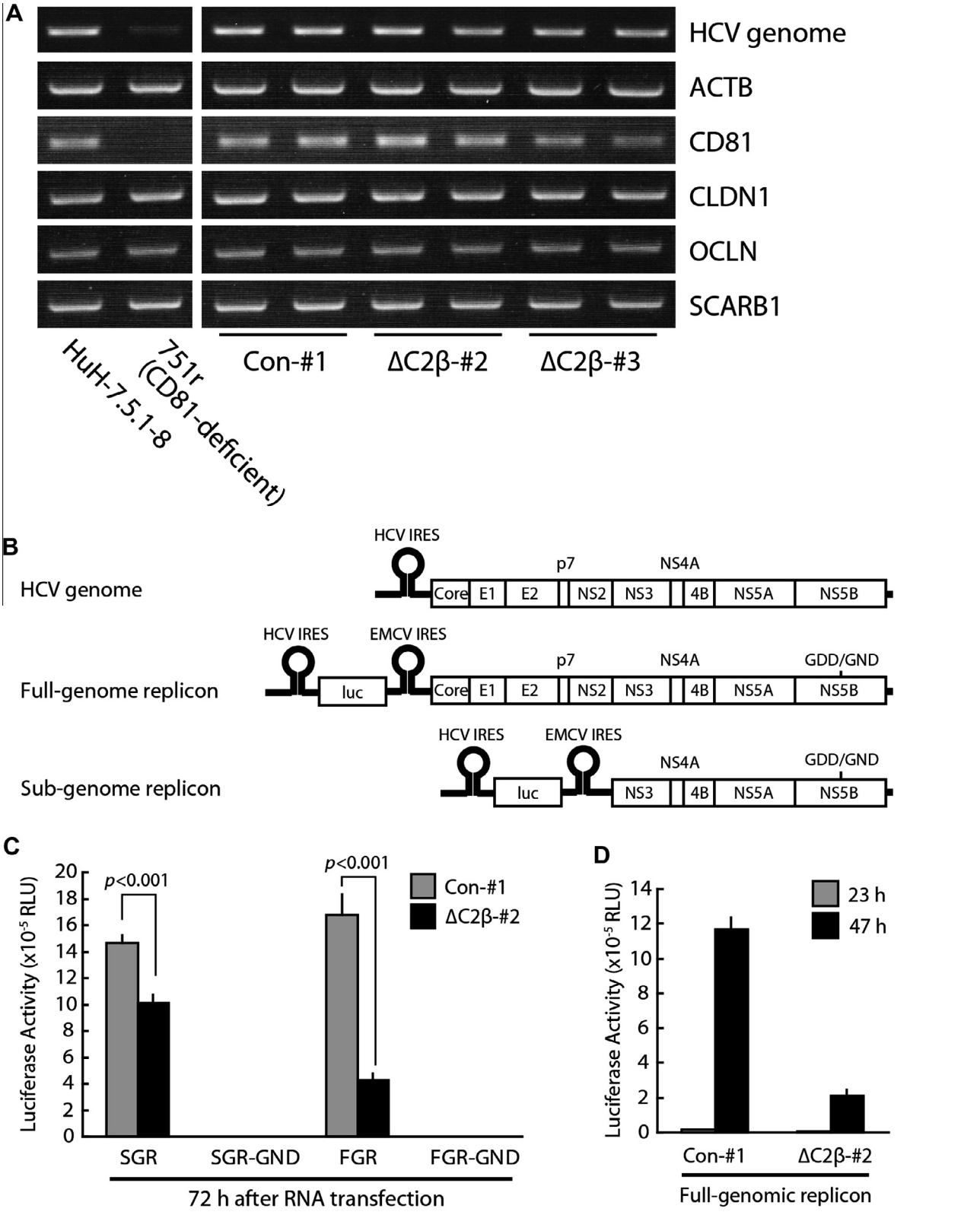

Fig. 2. PI3K-C2β knockdown represses HCV replication. (A) PI3K-C2β knockdown does not affect HCV entry. Control (Con-#1) and PI3K-C2β-knockdown (ΔC2β-#2 and #3) cells as well as HuH-7.5.1-8 (as a positive control) and CD81-deficient 751r (as a negative control) cells were subjected to HCV entry assay described under Section 2. Experiments were performed in duplicate (except positive and negative controls) and typical images from repeated experiments are presented. (B) Schematic diagram of HCV genomic and replicon RNA. (C) Control (Con-#1, gray column) and PI3K-C2β-knockdown (ΔC2β-#2, filled column) cells were transfected with RNA from either the sub-genomic replicon (SGR), replication-defective SGR (SGR-GND), full-genomic replicon (FGR), or replication-defective FGR (FGR-GND). After a 72-h culture, the luciferase activity of each sample was determined as described in Section 2. Data are represented as the mean ± SD from triplicated experiments. An unpaired Student's *t*-test was used to calculate statistical significance. (D) Control (Con-#1) and PI3K-C2β-knockdown (ΔC2β-#2) cells were transfected with the full-genomic replicon RNA. After the indicated time of culture, the luciferase activity of each sample was determined and represented as described above.

was analyzed by immunoblot analysis. The relative intensities of immunoreactive core protein bands were measured using the ImageJ Java applet.

2.3. HCV entry assay

Cells (1.4×10^5) were plated onto a well of a 12-well plate and cultured for 1 day. After removal of the medium, 0.35 ml of HCV-containing culture medium were added onto the cells and incubated for 2 h at 37 °C. Then, the medium was changed to normal growth medium supplemented by 0.1% Pluronic F-68 (Invitrogen). After the incubation at 37 °C for 2 h, the cells were rinsed with PBS containing 0.1% Pluronic F-68, followed by the incubation in 0.5 ml of AccuMax (Innovative Cell Technologies) for 5 min at room temperature in order to detach cells from the vessel and remove bound viruses but not internalized viruses [20]. The cells were then collected by centrifugation, and RNA was extracted using TRIzol Reagent (Invitrogen). The RNA fractions were transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Takara), followed by PCR analyses. In order to analyze the level of HCV genome RNA and transcripts for host cell factors, PCR was conducted in a 10-μl reaction mixture using 20 ng of cDNA as a template and Platinum PCR Super Mix (Invitrogen) according to the manufacturer's protocol. Primers used in this study were listed in Supplementary Table S1.

2.4. Supplementary methods

For cell culture, transfection, DNA construction, recombinant core protein preparation, immunoblot analysis, immunofluorescent assay and HCV infection, please consult Supplementary Data.

3. Results and discussion

3.1. PI3K-C2β is indispensable for HCV propagation in cells

Class II PI3Ks, that are relatively insensitive towards wortmannin, catalyze phosphorylation of PtdIns and PtdIns(4)*P* to produce PtdIns(3)*P* and PtdIns(3,4)P_2, respectively. Biological processes potentially controlled by these PIs (PtdIns(3)*P* for endocytosis; PtdIns(3,4)P_2 for endocytosis and proliferation) may affect HCV propagation [1,21]. In the human genome, there are three isoforms of class II PI3K, PI3K-C2α, PI3K-C2β, and PI3K-C2γ. The expression of all isoforms in HuH-7.5.1-8 cell was detected by immunoblot analysis (see Fig. 1A) and reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 1B and data not shown). Thus, we performed short hairpin RNA (shRNA)-mediated gene silencing of PI3K-C2α, PI3K-C2β, and PI3K-C2γ in HuH-7.5.1-8 cell to test their contribution to HCV propagation in the cell. We established multiple stable clones (ΔC2α-#1, #3, and #4; ΔC2β-#2, #3, and #5; ΔC2γ-#4) in which the expression of PI3K-C2α, PI3K-C2β, or PI3K-C2γ was reduced to various extents (see Fig. 1A and B) as well as stable clones harboring control constructs (Con-#1 and #3). These cells were infected with cell-cultured HCV (JFH1 strain) and incubated for 5 days to allow the virus propagation. As shown in Fig. 1A, while HCV core and NS3 proteins were accumulated in control cells, the accumulation of these viral proteins was greatly reduced in PI3K-C2β-knockdown cells. Quite severe reduction in HCV protein accumulation was observed in ΔC2β-#2, in which PI3K-C2β expression was almost undetectable; while ΔC2β-#3 and ΔC2β-#5, in which PI3K-C2β expression was modestly inhibited, showed partial reduction in HCV protein accumulation (Fig. 1A). This result suggests a dose-dependent requirement of PI3K-C2β in HCV propagation in the cell. In contrast, the effect of PI3K-C2α knockdown on viral protein accumulation was limited, although PI3K-C2α expression was almost undetectable in the cell (see ΔC2α-#1, Fig. 1A). In addition, the depletion of PI3K-C2γ (ΔC2γ-#4), as estimated by RT-PCR (Fig. 1B), did not affect viral protein accumulation (Fig. 1C). Thus we focused on PI3K-C2β functions in HCV propagation using ΔC2β-#2 and ΔC2β-#3 cells for further analyses, because these cell lines showed significant decreases in viral protein accumulation at 5 days after the infection (Fig. 1A). It should be noted that these PI3K-C2α-, PI3K-C2β-, and PI3K-C2γ-knockdown cells did not show decreased proliferation (Fig. 1D and data not shown). Further the introduction of PI3K-C2β-directed small interfering RNAs (siRNAs) (si-C2B-#5 and si-C2B-#6) into HuH-7.5.1-8 cells induced significant reduction of PI3K-C2β expression and resulted in decreases in viral protein accumulation at 5 days after the infection when compared to control cell (Fig. 1E). These non-coding small RNAs (si-C2B-#5, si-C2B-#6, and PIK3C2B-SH) target distinct sequences of PI3K-C2β mRNA, excluding the possibility that the inhibitory effect of PI3K-C2β knockdown on HCV propagation was due to the off-target effect. In addition, as shown in Fig. S1, the expression of mouse PI3K-C2β restored HCV propagation, that was repressed in PI3K-C2β-knockdown cell. We also tested virus release from PI3K-C2β-depleted cells and found that, as reflecting the reduction of HCV protein accumulation in cells (Fig. 1A), culture supernatant from HCV-infected ΔC2β-#2 and ΔC2β-#3 cells showed reduced infectivity towards naïve HuH-7.5.1-8 cells (Fig. S2). These observations taken together suggest that PI3K-C2β plays an indispensable role in HCV propagation in cells.

3.2. PI3K-C2β plays a role in HCV genome replication process

Both of PI3K-C2β-produced PIs, PtdIns(3)*P* and PtdIns(3,4)P_2, are implicated in endocytosis, the cellular process which HCV

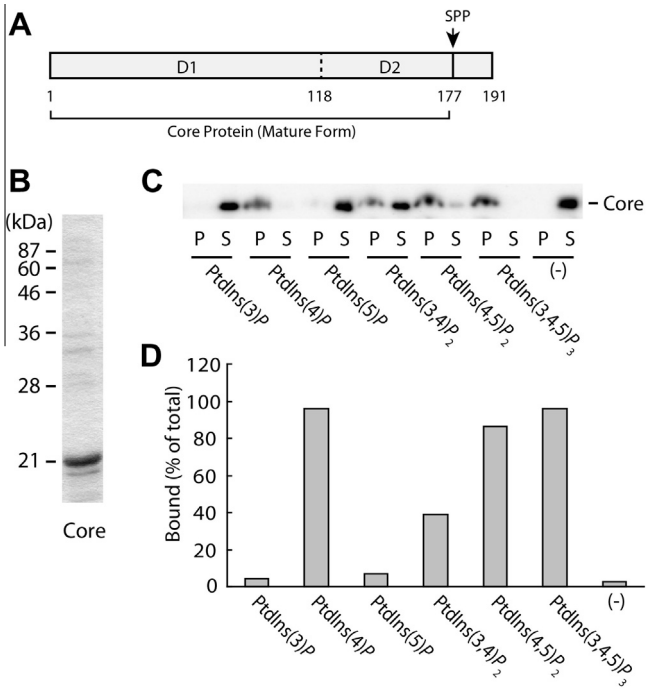

Fig. 3. HCV core protein binds to phosphoinositides *in vitro*. (A) Schematic diagram of HCV core protein. SPP, signal peptide peptidase. (B) Coomassie brilliant blue staining of recombinant core protein prepared by Sephacryl S-300 column chromatography (fraction-17). (C) Recombinant core protein bound to liposomes containing indicated PIs (P) and left unbound (S) were detected by immunoblot analysis. (D) Quantification of the bound core protein against the total amount shown in (C). Typical data from two independent experiments are presented.

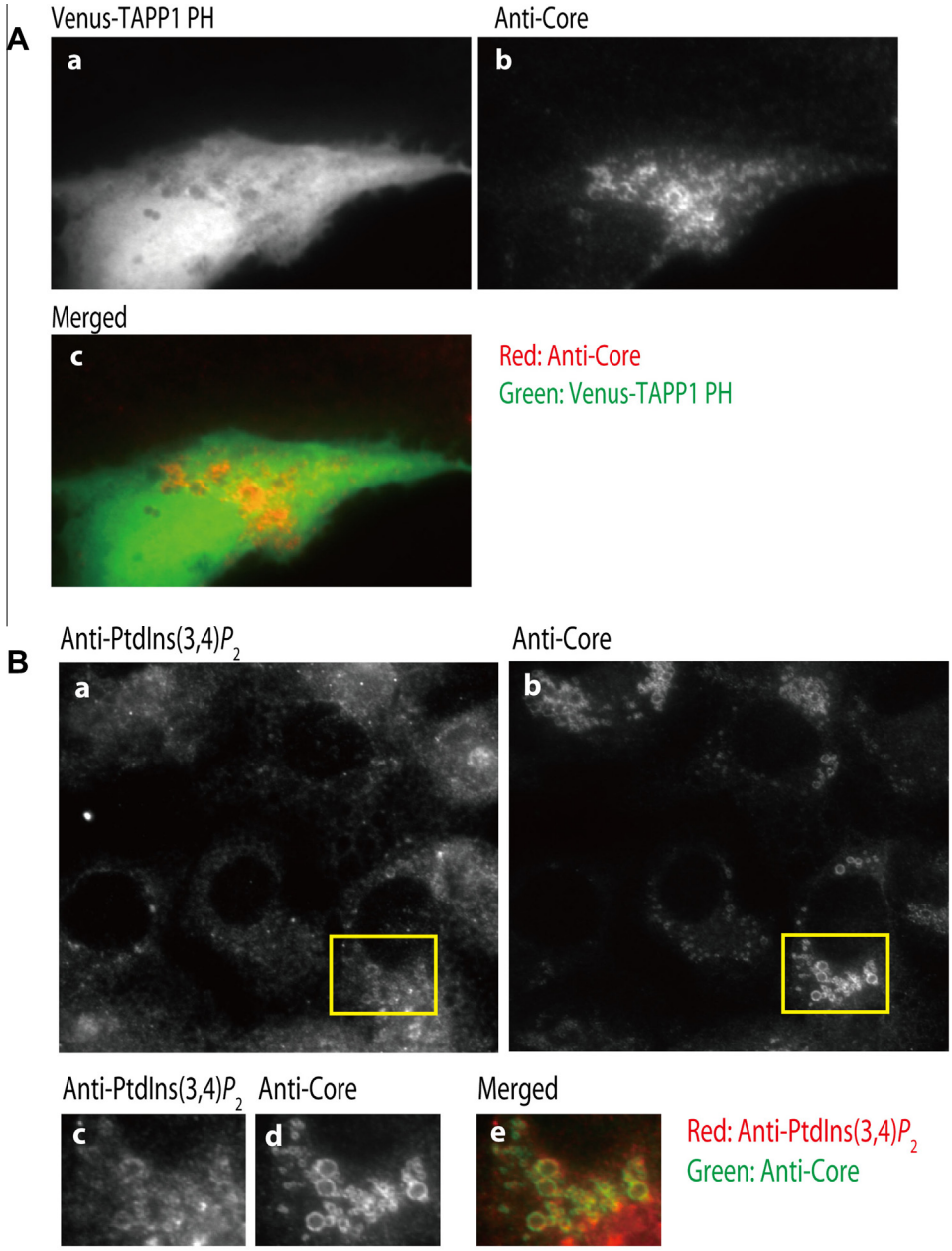

Fig. 4. PtdIns(3,4)P_2 accumulates in endomembrane structures with HCV core protein. (A) HuH-7.5.1-8 cells grown on a coverslip were transfected with TAPP1-PH/pVenus and Core-195/pEF1 expression constructs. After 2 days of culture, the cells were probed with anti-GFP (a) and anti-core (b) antibodies. Merged image is shown in (c). (B) HCV-infected HuH-7.5.1-8 cells were probed with anti-PtdIns(3,4)P_2 (a) and anti-core (b) antibodies. The selected area is shown at higher magnification in (c and d), and the merged image is shown in (e). Typical images from repeated experiments are presented.

employs to enter into the cell. Thus we first tested whether viral entry was affected by PI3K-C2β knockdown. Cells (ΔC2β-#2, ΔC2β-#3 and Con-#1) were exposed to HCV, followed by the incubation for additional 2 h to allow the virus enter into the cell. Then internalized HCV was detected by RT-PCR analysis. As shown in Fig. 2A, HCV genome RNA was detected in both PI3K-C2β-knockdown cells (ΔC2β-#2 and ΔC2β-#3) as well as control cell (Con-#1) and parental HuH-7.5.1-8 cell; while the depletion of CD81, a critical receptor for HCV, completely abrogated HCV entry (751r). This result suggests that PI3K-C2β did not contribute to entry step of HCV. It is also of note that the expression of cell surface molecules (CD81, CLDN1, OCLN, and SCARB1) that are involved in HCV entry was not affected by PI3K-C2β knockdown (Fig. 2A).

This result, together with the result shown in Fig. 1, prompts us that PI3K-C2β primarily affects intracellular HCV propagation step(s) such as genome replication process. We therefore tested the requirement of PI3K-C2β for HCV genome replication using HCV replicon systems. We used full-genomic and sub-genomic replicon systems that employ firefly luciferase as the reporter. The full-genomic replicon possesses structural proteins including core (Fig. 2B), and cells harboring the replicon potentially release infectious virus into the medium. Conversely, the sub-genomic replicon lacks structural proteins in addition to p7 and NS2 proteins (Fig. 2B). Cells were transfected with these replicon RNAs, and replication activity was determined by measuring luciferase activity. As shown in Fig. 2C, ΔC2β-#2 showed reduced replication activity of both sub-genomic (Fig. 2C, SGR) and full-genomic (Fig. 2C, FGR) replicons compared to their replication activity in control cell (Con-#1) at 72 h after the transfection. Replication-deficient mutants SGR-GND and FGR-GND exhibited no replication

activities under this condition (Fig. 2C). The transfection efficiencies of Con-#1 and ΔC2β-#2 cells were almost comparable under these conditions (data not shown). We did not use ΔC2β-#3 cell for this assay, because this cell line did not show comparable transfection efficiency for replicon RNAs (data not shown). Intriguingly, the inhibitory effect of PI3K-C2β knockdown was much larger in the full-genomic replicon system (75% reduction) than in the sub-genomic replicon system (31% reduction) (Fig. 2C). In addition, a similar magnitude of inhibition (78% reduction) was also observed in cells 47 h after the transfection of the full-genomic replicon RNA (Fig. 2D); at that time point, infectious particles for the reinfection were not sufficiently released into the culture medium. These results suggest that full genome-specific elements, including core protein, E1/E2 envelope proteins, p7 protein, and NS2 protein (Fig. 2B), were affected by PI3K-C2β itself and/or PIs generated by PI3K-C2β and involved in the replication process.

3.3. HCV core protein binds to PtdIns(3,4)P_2

HCV core protein encompasses the the D1 region (basic domain) and the D2 region (hydrophobic domain) (Fig. 3A), suggesting the interaction of the core protein with negatively charged lipids, such as PIs. Therefore we next focused on the core protein and attempted to test the binding of the core protein toward PIs. Although we have difficulties in preparing full-length core protein, recombinant core protein, encompassing the D1 region but not the D2 region was successfully produced in *Escherichia coli* (Fig. 3B), and then subjected to a liposome binding assay. As shown in Fig. 3C and D, the core protein showed binding activity toward D4-phosphorylated PIs (PtdIns(4)*P*, PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3) *in vitro*. It should be noted that recombinant core protein used for the *in vitro* binding assay lacks D2 region, thus the *in vitro* binding profile does not necessarily reflect *in vivo* function. Therefore, we next tested the localization of these D4-phosphorylated PIs with HCV core protein in cells and found overlapped localization of PtdIns(3,4)P_2 and HCV core protein in HCV-infected HuH-7.5.1-8 cells. In HuH-7.5.1-8 cells TAPP1 PH domain, a widely used PtdIns(3,4)P_2–selective probe [22], showed reticular staining pattern, which appeared to partially overlap with HCV core protein (Fig. 4A); although the overlapped staining was not so clear presumably due to relatively low binding affinity of the PH domain towards PtdIns(3,4)P_2 [23] and high background cytoplasmic staining. Therefore we next used an anti-PtdIns(3,4)P_2 antibody [24] to detect PtdIns(3,4)P_2 in HCV-infected HuH-7.5.1-8 cells. The anti-PtdIns(3,4)P_2 antibody showed, in addition to reticular staining pattern, staining of ring-like intracellular structures, which overlapped with the core protein (Fig. 4B). It is of note that other PIs, including PtdIns(4)*P*, did not show significant overlapped staining with the core protein (see Fig. S3 and data not shown). Although we are still unable to sufficiently explain why the core protein selectively associated with PtdIns(3,4)P_2 but not with other core-binding PIs in cells; these results suggest that PtdIns(3,4)P_2 accumulated in endomembrane structures through direct binding to HCV core protein.

3.4. Participation of PIs in the HCV replication cycle

The results obtained in this study show that PI3K-C2β plays an indispensable role in HCV replication in cells presumably through the production of PtdIns(3,4)P_2. Previous studies have demonstrated that PI4Ks are essential for HCV replication in cells through the interaction with NS5A protein to facilitate replication complex formation [13]. Our results raise the possibility that, in addition to playing a direct role in the replication complex formation, PI4Ks function as producers of PtdIns(4)*P*, a precursor of PtdIns(3,4)P_2. PtdIns(3,4)P_2, produced by PI3K-C2β, might play a role in the replication process reciprocally with PtdIns(4)*P*, although the precise action of PtdIns(3,4)P_2 remains elusive.

PtdIns(3,4)P_2 can be dephosphorylated by inositol polyphosphate 4-phosphatases (INPP4A and INPP4B) and potentially by PTEN to produce PtdIns(3)*P* and PtdIns(4)*P*, respectively. Knockdown of INPP4A did not affect HCV protein accumulation in cells (Fig. S4). The expression of INPP4B was not detected in HuH-7.5.1-8 cells (data not shown). These results suggest that PTEN plays a dominant role in the breakdown of PtdIns(3,4)P_2, which is required for the HCV replication cycle. Nonetheless, PTEN knockdown surprisingly resulted in complete abrogation of the HCV replication cycle in HuH-7.5.1-8 cells, although cell viability was not affected by the knockdown (Fig. S4 and data not shown). Although we can not exclude the possibility that PtdIns(3,4)P_2 is catabolized by unidentified enzyme(s); it is more likely that PTEN knockdown may increase multiple PI species, such as PtdIns(3)*P* and PtdIns(3,4,5)P_3 in addition to PtdIns(3,4)P_2, thereby disrupting a wide range of PI-regulated signals and resulting in unexpected abrogation of the HCV replication cycle. This observation suggests that multiple PIs, in addition to PtdIns(4)*P* [8–13,25] and PtdIns(3,4)P_2 (this study), are involved in the HCV replication cycle.

HCV is known to utilize endomembrane structures derived from the endoplasmic reticulum for the sites of its replication and assembly [5,7]; lipid droplets function as crucial intracellular organelles for HCV replication and assembly. HCV JFH1 core protein predominantly localizes near lipid droplets, showing a "ring"-like shape [26]. Crucial role of PI3K-C2β, as observed in Figs. 1A and 2C, prompted us to test whether PI3K-C2β knockdown affects lipid droplet formation. However, under normal growth condition and even after oleate addition, there was no difference in lipid droplet formation between PI3K-C2β-knockdown cells and control cells (Fig. S5). Furthermore, core protein localization was not altered in PI3K-C2β-knockdown cells as compared to its localization in control cells (Fig. S6). These results suggest that PI3K-C2β may not be involved in lipid droplet formation and that PI3K-C2β may be involved in the process of HCV replication after the recruitment of HCV proteins to the sites near lipid droplets. PtdIns(3,4)P_2 might be produced by PI3K-C2β, presumably at the endoplasmic reticulum, and then recruited to the core protein accumulation site through direct binding with core protein. Although further study will be required to reveal the underlying mechanism by which PIs regulate HCV replication process, results obtained in this study imply that manipulating PI signals may control HCV propagation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.048.

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