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VIRUS-CELL INTERACTIONS

# Serine 235 Is the Primary NS5A Hyperphosphorylation Site Responsible for Hepatitis C Virus Replication

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**ABSTRACT** The nonstructural protein 5A (NS5A) of the hepatitis C virus (HCV) is a phosphoprotein with two phosphorylation states: hypo- and hyperphosphorylation. Genetic mutation studies have demonstrated a cluster of serine residues responsible for NS5A hyperphosphorylation and functions in viral replication and assembly; however, the phosphorylation levels and potential interactions among the serine residues are unclear. We used three specific antibodies to measure NS5A phosphorylation at S222, S235, and S238 that were identified in our previous proteomics study. In the HCV (J6/JFH-1)-infected Huh7.5.1 cells, S222 phosphorylation was barely detected, whereas S235 phosphorylation and S238 phosphorylation were always detected in parallel in time and intracellular spaces. S235A mutation eliminated S238 phosphorylation whereas S238A mutation did not affect S235 phosphorylation, indicating that S235 phosphorylation occurs independently of S238 phosphorylation while S238 phosphorylation depends on S235 phosphorylation. In line with this, immunoprecipitation coupled with immunoblotting showed that S235 phosphorylation existed alone without S238 phosphorylation, whereas S238 phosphorylation existed only when S235 was phosphorylated on the same NS5A molecule. S235-phosphorylated NS5A constituted the primary hyperphosphorylated NS5A species. S235A mutation blunted viral replication, whereas S238A mutation did not affect replication. We concluded that S235 is the primary NS5A hyperphosphorylation site required for HCV replication. S238 is likely phosphorylated by casein kinase I$\alpha$, which requires a priming phosphorylation at S235.

**IMPORTANCE** It has been known for years that the hepatitis C virus nonstructural protein 5A (NS5A) undergoes transition between two phosphorylation states: hypo- and hyperphosphorylation. It is also known that a cluster of serine residues is responsible for NS5A hyperphosphorylation and functions; however, the primary serine residue responsible for NS5A hyperphosphorylation is not clear. Here, we show for the first time that serine 235-phosphorylated NS5A constitutes the primary hyperphosphorylated NS5A species required for viral replication. We also show that NS5A phosphorylation among the serine residues is interdependent and occurs in a directional manner, i.e., phosphorylation at serine 235 leads to phosphorylation at serine 238. Our data provide the first proof-of-principle evidence that NS5A undergoes a sequential phosphorylation cascade.

**KEYWORDS** NS5A, antibody, hepatitis C virus, protein phosphorylation, proteomics

*Hepatitis C virus* (HCV) is an enveloped virus with a 9.6-kb single-stranded positive-sense RNA genome. It infects about 185 million people worldwide and is a leading cause of liver diseases and related complications (1, 2). The HCV genome encodes a long polyprotein composed of 10 proteins from $NH_2$ to the COOH terminus: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. A complete HCV life cycle requires 3

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structural proteins (core, E1, and E2) that make up the infectious viral particles, and the remaining 7 nonstructural proteins carry out different functions (3–6). For example, NS2 and NS3/4A complex are proteases that process the polyprotein into individual functional nonstructural proteins (7, 8). NS4B induces formation of endoplasmic reticulum (ER)-derived membranous structures where HCV replication occurs (9, 10). NS5B is an RNA-dependent RNA polymerase required for HCV replication (11).

NS5A does not have apparent enzymatic activity, but it is a multifunctional protein participating in various stages of the HCV life cycle (12). Its multitasking functions are thought to be mediated by its ability to interact with viral proteins and a plethora of host proteins (12–14). NS5A consists of three domains (I, II, and III) connected by two low-complexity sequences (LCS I and II). It has been shown that NS5A forms dimers, which are critical to viral replication (15). NS5A is a phosphorylated protein with two phosphorylation states: hypo- and hyperphosphorylation that appear at 56 and 58 kDa on an SDS-PAGE gel (16). NS5A hyperphosphorylation has been associated with viral replication and assembly (17, 18). Many studies, largely based on genetic mutations, have pinpointed several serine residues in the LCS I region responsible for NS5A hyperphosphorylation and functions (17–23). For example, S225 was reported to participate in the formation of viral replication protein complex (24) as well as viral assembly (18). S229 seems an irreplaceable amino acid critical to the viral life cycle (17, 18), as both alanine (phosphorylation-ablated) and aspartate (phosphorylation-mimicking) mutations abolished viral replication (17, 18). S232 is frequently identified in adaptive mutations (25). Isoleucine mutation at S232 enhances viral replication in HCV genotype 1 (26); however, alanine mutation at the same site reduces viral replication or assembly in genotype 2 (17, 18), leaving S232 a mysterious site for functions in different genotypes. Recently, S235 was shown to be a critical amino acid for HCV replication, likely via regulating replication complex formation (22, 23). Thus, genetic mutations that either remove or mimic phosphorylation have been very instrumental in demonstrating the functions of the above-described serine residues. However, whether phosphorylation at these serine residues really occurs in the infected cells and whether there is a predominant serine residue responsible for NS5A hyperphosphorylation are not completely clear.

A number of studies have indicated that phosphorylation among the above-mentioned serine residues is interdependent. For example, *in vitro* kinase assays seem to suggest that S229 phosphorylation facilitates S232 phosphorylation in a synthetic NS5A peptide (27). Aspartate mutations suggest that NS5A phosphorylation occurs in a sequential cascade where phosphorylation of one serine residue leads to phosphorylation of the other serine residues (12, 21). In our previous proteomics study (22), we identified phosphorylation at S222, S235, and S238 with high confidence scores. Using genetic mutations, we found that alanine mutation at S235 blunted NS5A hyperphosphorylation and viral replication. Single-alanine mutation at S222 or S238 did not have apparent effects; however, double mutations at S222 and S238 reduced NS5A hyperphosphorylation and viral replication (22), suggesting that phosphorylation events among these sites are interdependent. In the previous work, we generated an antibody specific to NS5A S235 phosphorylation and used it to show that S235 phosphorylation correlated with viral replication in time and intracellular locations (22). Here, we generated two additional antibodies specific to NS5A S222 and S238 phosphorylation to measure their levels of phosphorylation and to study interdependence among S222, S235, and S238 phosphorylation.

## RESULTS

**NS5A S235 and S238 phosphorylation occurred in parallel.** Figure 1A lists the serine residues that are highly conserved in the LCS I region of NS5A from six major HCV genotypes. Figure 1B shows the dot blot results characterizing the specificity of the three antibodies to NS5A S222, S235, and S238 phosphorylation. Each antibody recognized its designated phosphorylation site in the synthetic peptides in a dose-dependent manner. None of the antibodies recognized the peptides when the desig-

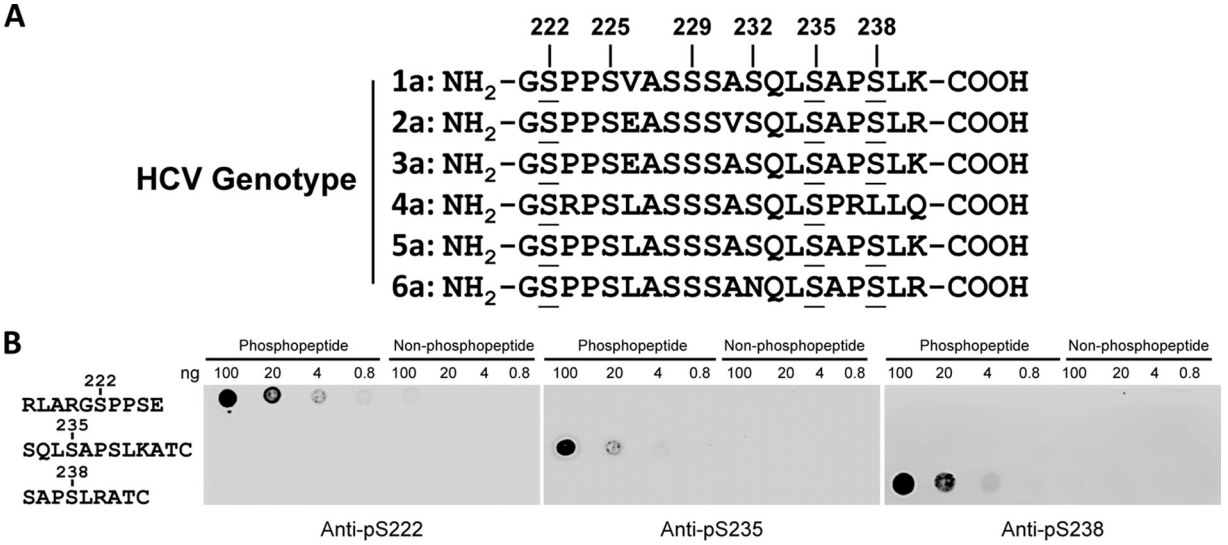


**FIG 1** Three antibodies specific to NS5A phosphorylation at S222, S235, and S238 were characterized. (A) Partial peptide sequences of the LCS I region of NS5A from six major HCV genotypes. The UniProt accession numbers are the following: 1a, P27958; 2a, Q99IB8; 3a, Q81258; 4a, O39929; 5a, O91936; and 6a, O39927. Phosphorylation events at S222, S235, and S238 were identified with high confidence scores in a previous study (22). (B) Dot blot tests for the specificity of the three antibodies. Synthetic peptides with or without phosphorylation at the designated serine residues (numbered) were dotted on nitrocellulose membranes before detection with the antibodies.

nated site was not phosphorylated. None of the antibodies cross-reacted with the nondesignated sites. On the immunoblots (Fig. 2A), S222 phosphorylation was barely detected in the HCV (J6/JFH-1)-infected Huh7.5.1 cells (multiplicity of infection [MOI] of 0.001), although the level of total NS5A protein gradually increased with time and plateaued on the fourth day after the infection (Fig. 2G). Note that the detection of S222 phosphorylation by the antibody was interfered with when both S225 and S229 were phosphorylated. As shown in the dot blot analysis (Fig. 2J), while the S222 phosphorylation-specific antibody detected a synthetic peptide with a single phosphorylation at S222, it failed to detect a synthetic peptide with triple phosphorylations at S222, S225, and S229. In contrast to S222 phosphorylation, S235 phosphorylation was apparent on the second day after the infection and corresponded to the hyperphosphorylated band of NS5A (Fig. 2B, p58). Its phosphorylation level continued to increase with time and plateaued on the fourth day after the infection (Fig. 2H). Similar to S235 phosphorylation, S238 phosphorylation also corresponded to NS5A hyperphosphorylation (Fig. 2C), and its phosphorylation level showed a time-dependent increase similar to that of S235 phosphorylation (Fig. 2H). Infection at a higher MOI (0.01) yielded similar results (Fig. 2D to F). Figure 2I shows that S235 phosphorylation is positively correlated with S238 phosphorylation with a high Pearson's correlation coefficient (0.95).

**NS5A S235 phosphorylation was a prerequisite to S238 phosphorylation.** The apparent positive correlation between NS5A S235 and S238 phosphorylation suggested interdependence among the phosphorylation sites. To test the interdependence, alanine mutation was made in one site and phosphorylation at the other sites was measured. To avoid interference of the HCV life cycle on the protein levels, transfection-friendly HEK293T cells that do not support the HCV life cycle were used for the study (28). In the wild-type NS3-NS5A polyprotein-expressing HEK293T cells, NS5A appeared as two characteristic hypo- and hyperphosphorylated bands (Fig. 3A to C). The ratio of hyper- to hypophosphorylated NS5A in the wild-type NS3-NS5A-transfected HEK293T cells was similar to that in the HCV-infected Huh7.5.1 cells (Fig. 2A). S222 phosphorylation was barely detected in the NS3-NS5A polyprotein-expressing HEK293T cells (Fig. 3A), while S235 and S238 phosphorylation were apparent (Fig. 3B and C), like those observed in the HCV-infected Huh7.5.1 cells (Fig. 2A to C), indicating that the HEK293T

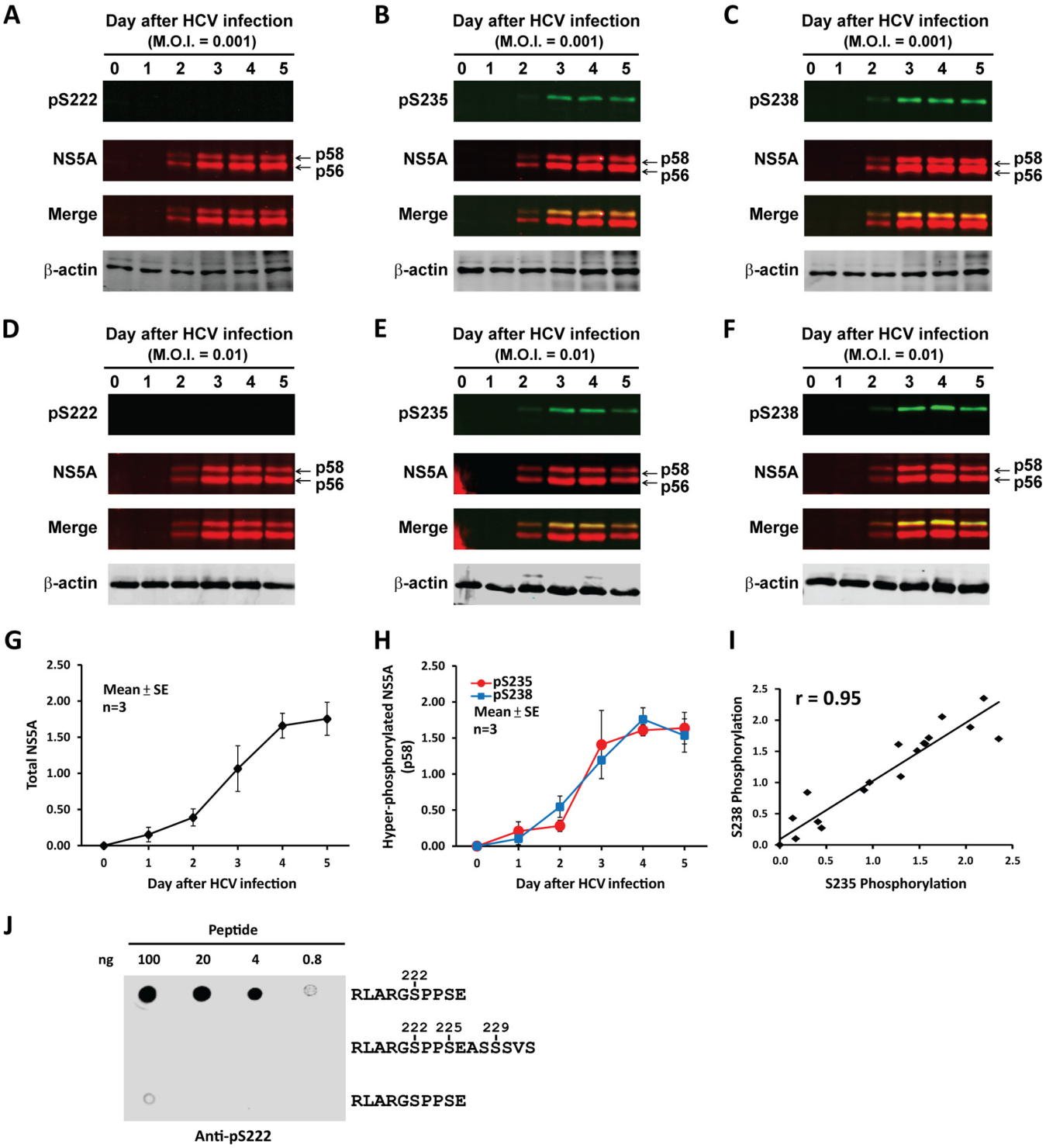


**FIG 2** NS5A S235 phosphorylation and S238 phosphorylation showed parallel time-dependent increases in the infected cells. (A to F) Immunoblots for NS5A phosphorylation at S222, S235, and S238 in the HCV-infected Huh7.5.1 cells at an MOI of 0.001 (A to C) or 0.01 (D to F). ß-Actin served as a loading control. (G and H) Line plots summarizing total NS5A and NS5A phosphorylation at S235 and S238 from three independent experiments. Relative protein abundance was quantified with the Li-Core Odyssey scanner and software. Values are means ± standard errors (SE). (I) Pearson's correlation analysis for S235 and S238 phosphorylation. (J) Dot blot analysis of the S222 phosphorylation-specific antibody for detection interference by phosphorylation at S225 and S229. Phosphorylated serine residues are numbered.

cells host kinases and phosphatases similar to those of the Huh7.5.1 cells and, hence, are a reasonable model for studying NS5A phosphorylation.

In the NS3-NS5A polyprotein-expressing HEK293T cells, S222 alanine mutation (S222A) did not affect S235 (Fig. 3B) or S238 (Fig. 3C) phosphorylation. In contrast, the

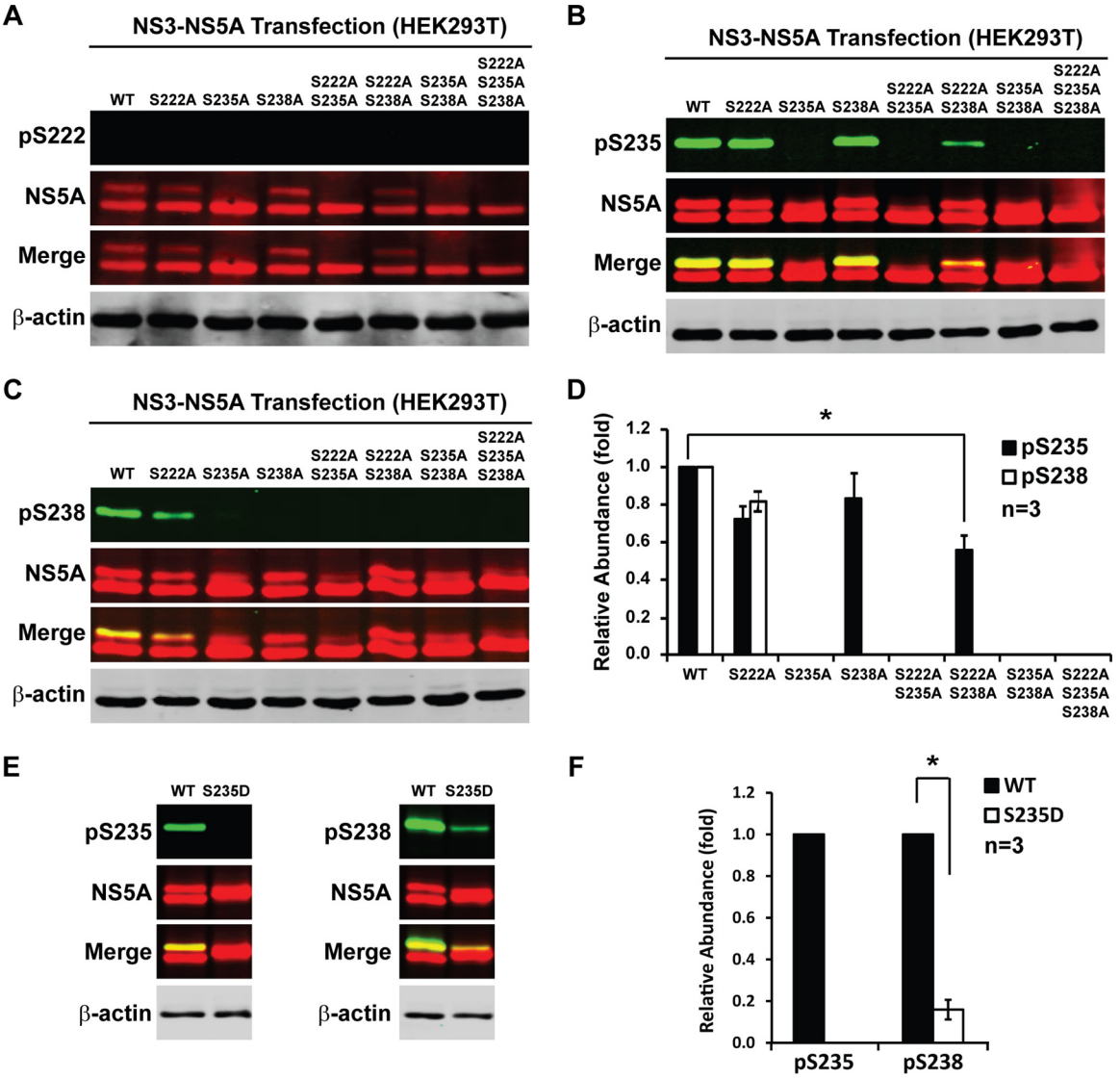


**FIG 3** NS5A S235 phosphorylation was a prerequisite to S238 phosphorylation. Immunoblots for NS5A phosphorylation at S222 (A), S235 (B), and S238 (C) in the HEK293T cells. The HEK293T cells were transfected with various NS3-NS5A expression constructs: the wild types (WT) and single-, double-, or triple-alanine mutations at S222, S235, or S238 for 2 days before the cell lysates were collected for immunoblotting. ß-Actin staining served as a loading control. (D) A bar diagram summarizing the immunoblotting results from three experiments. Values are means ± SE ($n = 3$). The asterisk indicates significance: $P$ value of $<0.05$ by Student's $t$ test. (E and F) Representative immunoblot images (E) and summary (F) for NS5A phosphorylation at S235 and S238 in the HEK293T cells transfected with an S235D mutant NS3-NS5A expression construct.

S235 alanine mutation (S235A) abolished S235 phosphorylation (Fig. 3B) as well as S238 phosphorylation (Fig. 3C), indicating that S238 phosphorylation requires S235 phosphorylation. S238 alanine mutation (S238A) abolished S238 phosphorylation (Fig. 3C); however, S238 alanine mutation did not affect S235 phosphorylation (Fig. 3B). Thus, S235 phosphorylation can occur independently of S238 phosphorylation. Figure 3D summarizes the results from three independent experiments. Compared to the wild-type NS5A, single-alanine mutation at S222 or S238 did not affect S235 phosphorylation (Fig. 3D), while double-alanine mutations at S222 and S238 (S222A/S238A) reduced S235 phosphorylation by 50% (Fig. 3D). Thus, a full S235 phosphorylation level requires that either S222 or S238 remains wild type.

Given the dependence of S238 phosphorylation on S235 phosphorylation (Fig. 3C), we examined whether S238 phosphorylation could be detected when S235 was mutated to aspartate (S235D), which was commonly used to mimic phosphorylation

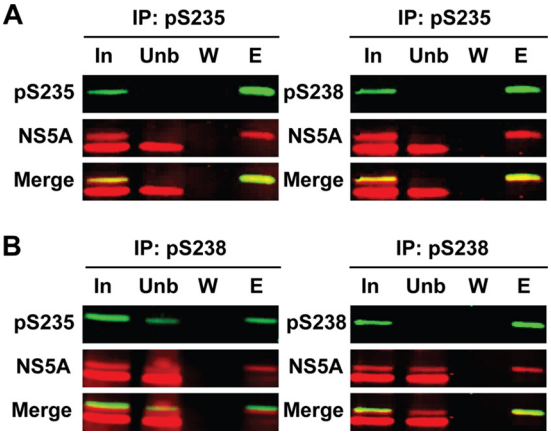


**FIG 4** NS5A S235 phosphorylation could occur alone, while S238 phosphorylation occurred only with S235 phosphorylation on the same NS5A molecule. (A) Immunoblots for NS5A and NS5A phosphorylation at S235 (left) or S238 (right) in the immunoprecipitate (IP) of the S235 phosphorylation-specific antibody. (B) Immunoblots for NS5A and NS5A phosphorylation at S235 (left) or S238 (right) in the immunoprecipitate of the S238 phosphorylation-specific antibody. In, input, i.e., HCV (J6/JFH1)-infected Huh7.5.1 cell lysate; Unb, unbound; W, wash; E, eluate.

and to rescue viral replication (18, 22). In the HEK293T cells transfected with the wild-type NS3-NS5A construct, S235 and S238 phosphorylation were both apparent (Fig. 3E). However, in the S235D mutant NS3-NS5A-transfected cells, S235 phosphorylation was not detected by the S235 phosphorylation-specific antibody, consistent with our previous results (22). The level of S238 phosphorylation of the S235D mutant NS5A was reduced to about 20% of that of wild-type NS5A (Fig. 3F). Thus, S235D mutation partially represented S235 phosphorylation and resulted in partial S238 phosphorylation.

**NS5A S235 phosphorylation could exist alone, whereas S238 phosphorylation existed only when S235 was phosphorylated on the same NS5A molecule.** One mechanism that can explain the dependence of NS5A S238 phosphorylation on S235 phosphorylation is casein kinase I$\alpha$ (CKI$\alpha$), which phosphorylates serine/threonine when the serine at the $-3$ position (i.e., S235) is phosphorylated first (pS-X-X-S/T) (29). This would predict that S235 phosphorylation occurs independently of S238 phosphorylation and that S238 phosphorylation occurs with S235 phosphorylation on the same NS5A molecule. To test these predictions, we immunoprecipitated NS5A from the HCV-infected Huh7.5.1 cell lysate using either S235 or S238 phosphorylation-specific antibody followed by immunoblotting for S235 and S238 phosphorylation. As shown in Fig. 4A (left), we were able to remove all S235-phosphorylated NS5A from the HCV-infected cell lysate (pS235 blot, lane In) with the S235 phosphorylation-specific antibody because there was no detectable S235-phosphorylated NS5A in the unbound fraction (lane Unb). The removal of S235-phosphorylated NS5A from the input resulted in a sharp reduction of the hyperphosphorylated NS5A band in the unbound fraction (Fig. 4A, NS5A blot, lane Unb), indicating that S235-phosphorylated NS5A is the major species of the hyperphosphorylated NS5A. In the unbound fraction after S235-phosphorylated NS5A was immunoprecipitated (Fig. 4A, right, lane Unb), S238 phosphorylation was not detected, indicating that most S238 phosphorylation does not exist alone independently of S235 phosphorylation. In fact, all S238-phosphorylated NS5A in the input was detected in the immunoprecipitate of the S235 phosphorylation-specific antibody (Fig. 4A, right, lane E), indicating that S238 phosphorylation exists only when S235 is phosphorylated on the same NS5A molecule. In contrast, when all S238-phosphorylated NS5A was immunoprecipitated from the input by the S238 phosphorylation-specific antibody (Fig. 4B, right, pS238 blot, lane Unb), there was still detectable hyperphosphorylated NS5A in the unbound fraction (NS5A blot, lane Unb), and the hyperphosphorylated NS5A was phosphorylated at S235 (Fig. 4B, left, pS235

blot, lane Unb), supporting that S235 phosphorylation occurs independently of S238 phosphorylation.

**S235- and S238-phosphorylated NS5A colocalized with double-stranded RNA.** To dissect functions of NS5A phosphorylated at S222, S235, and S238, their intracellular localizations were examined in the HCV-infected Huh7.5.1 cells by confocal immunofluorescence microscopy. S222-phosphorylated NS5A was not detected in the cells (Fig. 5A, top row), whereas S235 (Fig. 5A, middle row)- and S238 (Fig. 5A, bottom row)-phosphorylated NS5A distributed throughout the cytoplasm and colocalized with the double-stranded RNA (dsRNA) virus replication marker. Smaller amounts of S235- and S238-phosphorylated NS5A colocalized with the viral assembly marker lipid droplet (Fig. 5B, middle and bottom rows). Based on the methods of Manders et al. (30), the colocalization coefficient for S235-phosphorylated NS5A and dsRNA was 0.82 (Fig. 5C), 2.3-fold higher than that (0.35) for S235-phosphorylated NS5A and lipid droplet (Fig. 5D). Similarly, the colocalization coefficient for S238-phosphorylated NS5A and dsRNA was 0.78, 1.6-fold higher than that (0.49) for S238-phosphorylated NS5A and lipid droplet. Thus, more S235- and S238-phosphorylated NS5A is associated with the replication marker than the assembly marker. Similar observations were made in the wild-type (WT) JFH1 replicon-transfected Huh7.5.1 cells (Fig. 6A and B). These results are consistent with a major role of S235- and S238-phosphorylated NS5A in HCV replication.

To differentiate the functions of NS5A phosphorylation at S235 versus S238 in HCV replication, dsRNA was examined in the Huh7.5.1 cells transfected with S235A or S238A mutant JFH1 replicon using confocal immunofluorescence microscopy. As seen in Fig. 6C, there was no detectable dsRNA or NS5A phosphorylation at S235 or S238 in the S235A replicon-transfected cells, consistent with a key role of S235 phosphorylation in viral replication. In contrast, in the S238A replicon-transfected cells (Fig. 6D), the staining for dsRNA and S235 phosphorylation was apparent despite the lack of S238 phosphorylation. Thus, NS5A S235 phosphorylation is a critical phosphorylation event for viral replication while S238 phosphorylation is probably dispensable for viral replication.

**Casein kinase I$\alpha$ was responsible for S235 and S238 phosphorylation.** The lipid kinase PI4KIII$\alpha$ (23, 31–34) and the protein kinase CKI$\alpha$ (18, 22, 27, 28, 35) are the two most-studied kinases involved in NS5A hyperphosphorylation. To investigate their roles in S235 and S238 phosphorylation, we measured S235 and S238 phosphorylation in the T7 polymerase-expressing Huh7 cells (T7-Huh7) whose PI4KIII$\alpha$ or CKI$\alpha$ was knocked down prior to NS3-NS5B expression. As shown in the immunoblotting results, PI4KIII$\alpha$ knockdown (Fig. 7A) did not affect either S235 or S238 phosphorylation (Fig. 7B and C). In contrast, CKI$\alpha$ knockdown (Fig. 7D) significantly reduced both S235 and S238 phosphorylation (Fig. 7E and F). It was interesting that when the CKI$\alpha$ protein level was reduced by about 50%, there was a parallel 50% reduction in S235 and S238 phosphorylation. Similar results were obtained with the CKI$\alpha$-specific inhibitor D4476. D4476 reduced S235 (Fig. 8A) as well as S238 (Fig. 8B) phosphorylation in a dose-dependent manner (Fig. 8C), consistent with the idea that CKI$\alpha$ is responsible for both S235 and S238 phosphorylation.

## DISCUSSION

A cluster of serine residues is responsible for NS5A hyperphosphorylation and functions (12); however, direct measurements of their phosphorylation levels were made possible only recently with phosphorylation-specific antibodies (21–23). In the present work, we simultaneously measured NS5A phosphorylation at S222, S235, and S238 in the HCV (J6/JFH1)-infected Huh7.5.1 cells with three high-quality antibodies (Fig. 1B). At this stage, we did not make antibodies to S225, S229, or S232 phosphorylation. Although these sites are reported to be responsible for NS5A hyperphosphorylation and functions (17, 18), they were identified with much lower identification confidence scores in our own study (22).

S222 is a major phosphorylation site identified in several studies (19–23); however,

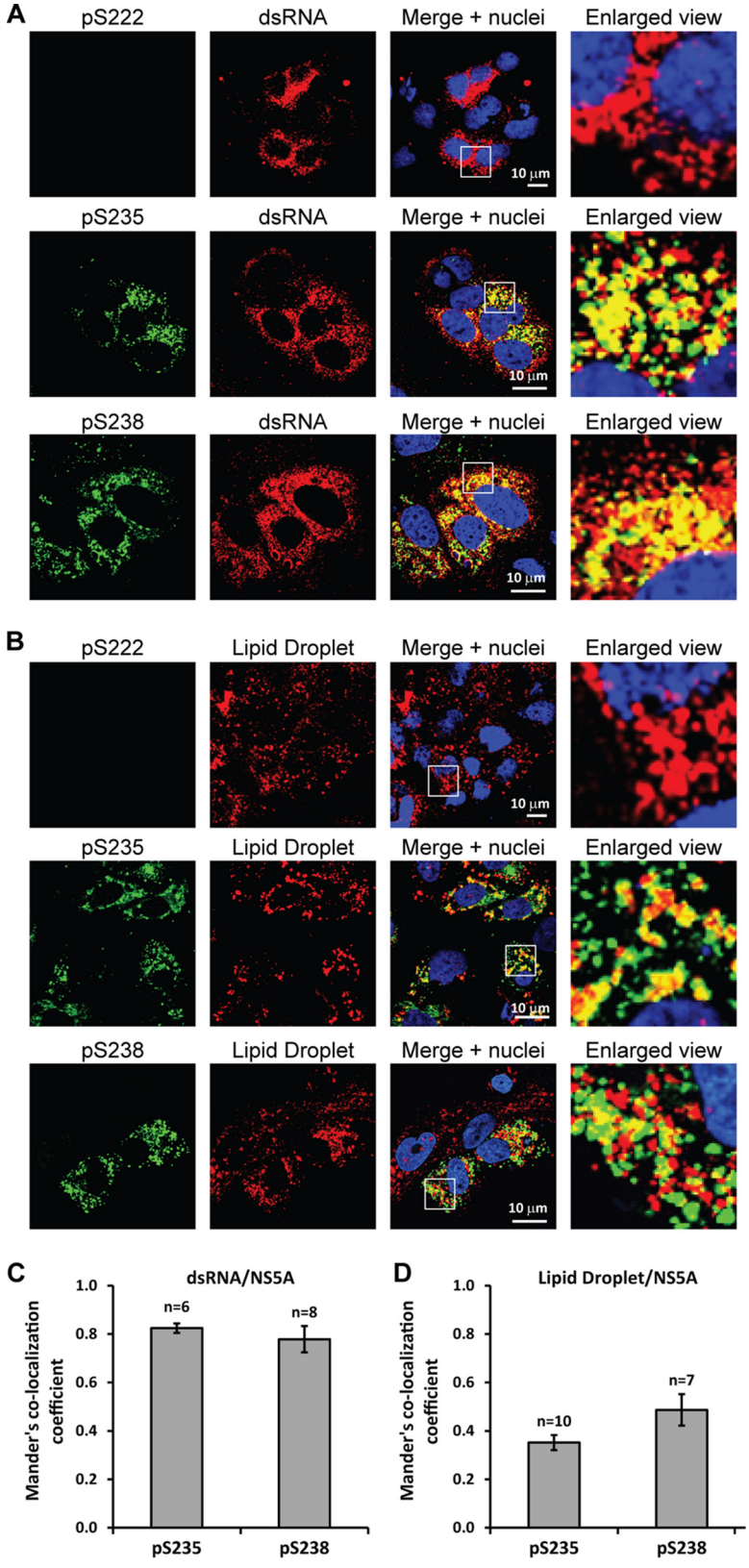


**FIG 5** S235- and S238-phosphorylated NS5A colocalized with double-stranded RNA. Confocal immunofluorescence micrographs of NS5A phosphorylated at S222, S235, or S238 costained for dsRNA (A) or lipid droplet (B) in the Huh7.5.1 cells infected with HCV for 6 days. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (in blue). Boxes indicate the areas enlarged. Mander's colocalization coefficient for S235 and S238 phosphorylated NS5A with dsRNA (C) and lipid droplet (D). Numbers of cells analyzed are indicated.

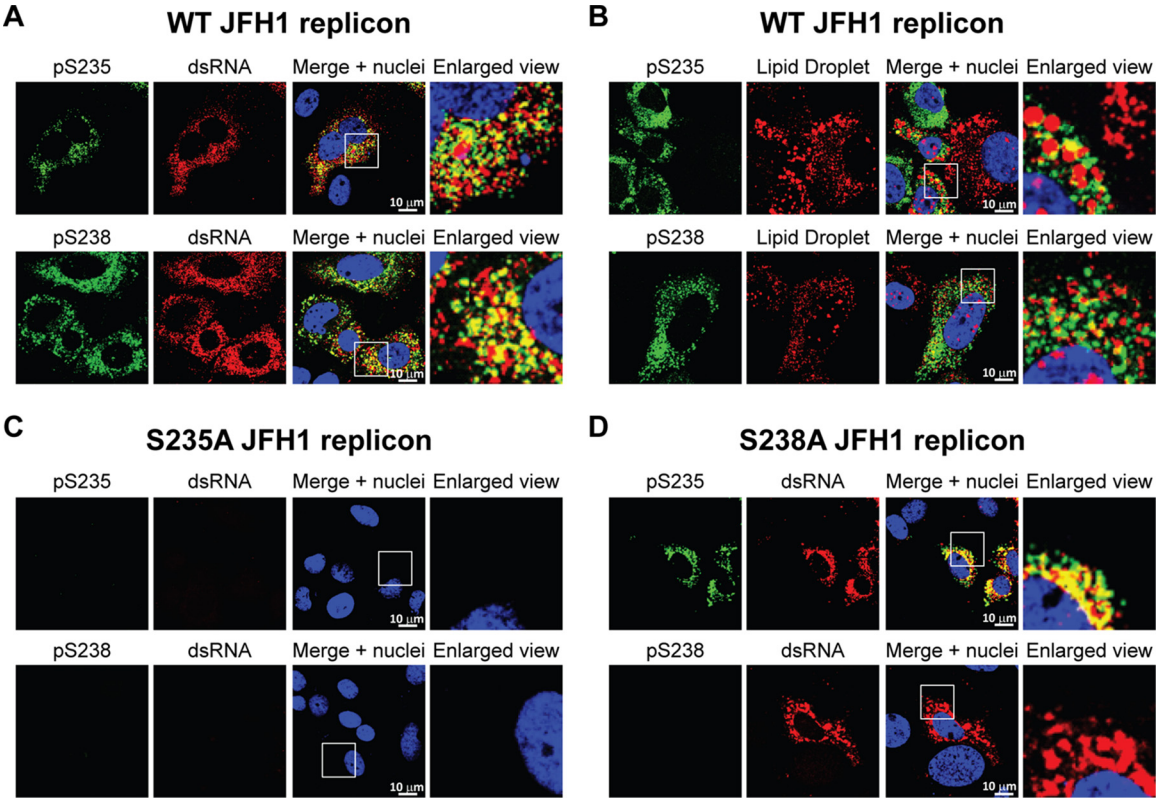


**FIG 6** NS5A S235 phosphorylation is essential for HCV replication. Confocal immunofluorescence micrographs of NS5A phosphorylated at S235 or S238 costained for dsRNA (A) or lipid droplet (B) in the wild-type (WT) JFH1 replicon-transfected Huh7.5.1 cells. (C and D) Confocal immunofluorescence micrographs of NS5A phosphorylated at S235 or S238 costained for dsRNA in the Huh7.5.1 cells transfected with S235A (C) or S238A (D) mutant replicon. Nuclei were stained with DAPI (in blue). Boxes indicates the areas enlarged.

we could hardly detect its phosphorylation in HCV-infected cells with our S222 phosphorylation-specific antibody (Fig. 2A and D). This is likely due to the fact that S225 and S229 phosphorylation blocked the detection of S222 phosphorylation by the antibody (Fig. 2J). Another possibility is that the S222-phosphorylated full-length NS5A assumes a particular structure different from that of the immunizing synthetic peptide and thus could not be detected by the antibody. Therefore, we are not sure of the phosphorylation status of S222 in the infected cells. These observations signified the need to characterize each phosphorylation-specific antibody. In our previous study (22), we showed that the detection of S235 phosphorylation by the S235 phosphorylation-specific antibody was not interfered with by S238 phosphorylation. The case is completely different for the S222 phosphorylation-specific antibody (Fig. 2J). The detection of S238 phosphorylation by the S238 phosphorylation-specific antibody apparently was not interfered with by S235 phosphorylation, since S238 phosphorylation was detected in the immunoprecipitate pulled down by the S235 phosphorylation-specific antibody (Fig. 4A, right, lane E).

In contrast to S222 phosphorylation, S235 and S238 phosphorylation always occurred in parallel and increased with time of infection (Fig. 2H and I). Both S235 and S238 phosphorylation occurred in intracellular locations of viral replication (Fig. 5A and 6A), suggesting their roles in viral replication. However, S238 phosphorylation seems dispensable in the HCV life cycle, because S238A mutation did not affect the reporter viral activity or viral replication (Fig. 6D) (17, 18, 22). In contrast to S238 phosphorylation, S235 phosphorylation is responsible for NS5A hyperphosphorylation, as S235A mutation eliminated the hyperphosphorylated (p58) band (Fig. 3A to C). Immunoprecipitation that removed the S235-phosphorylated NS5A species greatly reduced the hyperphosphorylated (p58) band (Fig. 4A). Thus, S235-

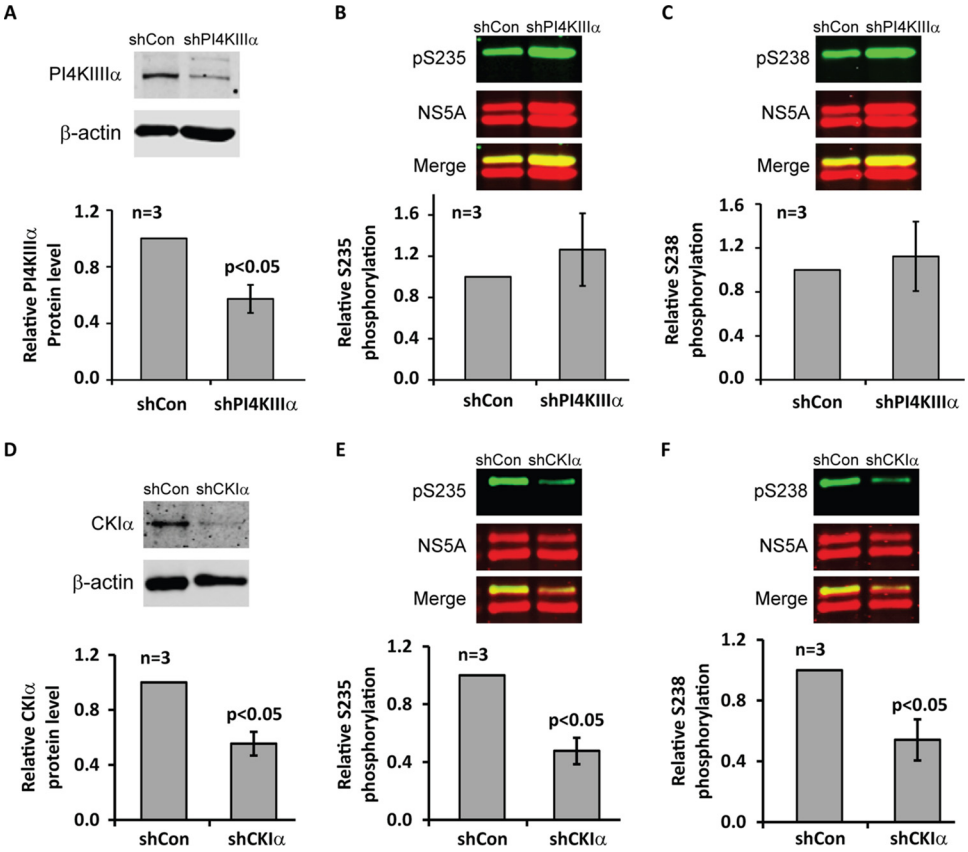


**FIG 7** CKIα was responsible for NS5A S235 and S238 phosphorylation. Immunoblots for PI4KIIIα (A), CKIα (D), NS5A S235 phosphorylation (B and E), and NS5A S238 phosphorylation (C and F) in the T7-Huh7 cells. Control (shCon), PI4KIIIα knockdown (shPI4KIIIα), or CKIα (shCKIα) knockdown T7-Huh7 cells were transfected with the NS3-NS5B expression construct for 1 day before the immunoblot analysis. The bar diagrams summarize results from three experiments. Values are means ± SE normalized against values under the control conditions.

phosphorylated NS5A constitutes the primary hyperphosphorylated NS5A species. S235-phosphorylated NS5A colocalized with the replication marker dsRNA (Fig. 5A and 6A). S235A mutation abolished viral replication (Fig. 6C) (17, 18, 22). S235-phosphorylated NS5A is the primary hyperphosphorylated NS5A species required for HCV replication.

Since the discovery of the two phosphorylation states of NS5A (16), it has been shown that NS5A undergoes maturation from the hypophosphorylated state to the hyperphosphorylated state (36). Later studies based on *in vitro* kinase assays (27) and aspartate mutations (21) have alluded to a sequential phosphorylation cascade among the serine residues in the LCS I region for NS5A hyperphosphorylation (12). Here, we used two phosphorylation-specific antibodies to show for the first time that NS5A phosphorylation occurred in a directional manner from S235 to S238. S235 and S238 (Fig. 1A) fall within the priming phosphorylation preference of CKIα (29). Following this preference, it is expected that S235 phosphorylation can occur independently of S238 phosphorylation. Consistent with this, we found that S235A mutation eliminated S238 phosphorylation (Fig. 3C), whereas S238A mutation did not affect S235 phosphorylation (Fig. 3B). In addition, we detected S235 phosphorylation in the absence of S238 phosphorylation (Fig. 4B, left, lane Unb). The priming phosphorylation preference also predicts that S238 phosphorylation depends on S235 phosphorylation. In line with this, we found that S235D partially permitted S238 phosphorylation (Fig. 3E) while S235A mutation eliminated S238 phosphorylation (Fig. 3C). Moreover, there was no S238 phosphorylation in the absence of S235 phosphorylation (Fig. 4A, right, lane Unb). If S238 phosphorylation depends on S235 phosphorylation, S238 phosphorylation should

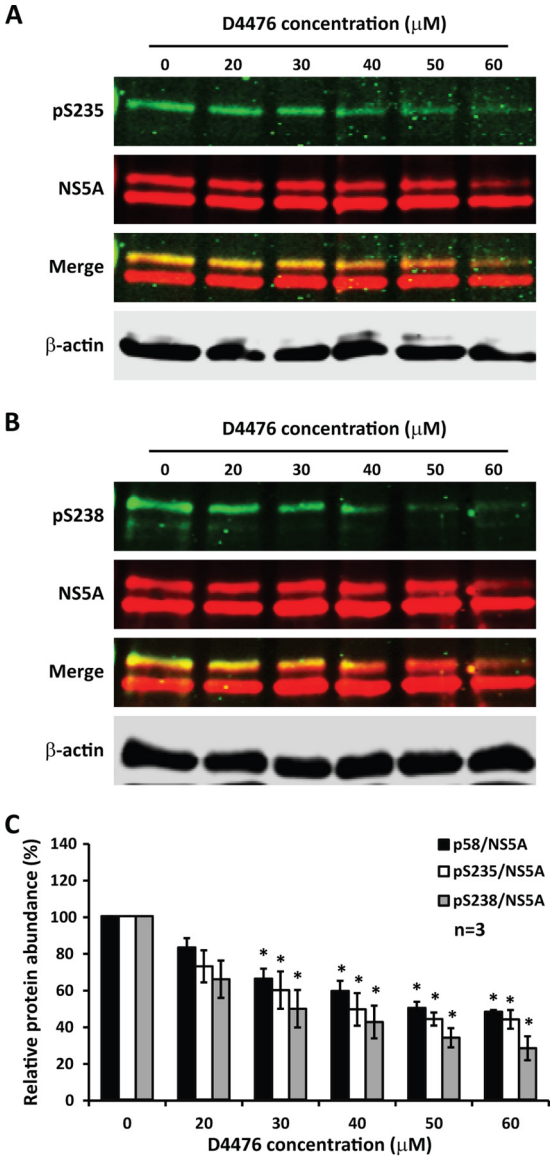


**FIG 8** CKI$\alpha$ inhibition reduced NS5A phosphorylation at S235 and S238. Immunoblots for NS5A phosphorylated at S235 (A) and S238 (B) in T7-Huh7 cells. The cells were transfected with an NS3-NS5B expression construct and treated with the CKI$\alpha$ inhibitor D4476 for 1 day prior to immunoblotting. $\beta$-Actin served as a loading control. (C) Summary for three independent experiments. Values are means $\pm$ SE, normalized against values of the vehicle control (0 $\mu$M). Asterisks indicate significance: *P* value of $<0.05$ by Student's *t* test against values of the vehicle control.

be on the same NS5A molecule as S235 phosphorylation. Indeed, we found that the S238-phosphorylated NS5A was in the immunoprecipitate pulled down by the S235 phosphorylation-specific antibody (Fig. 4A, right, eluate). Thus, NS5A S235 phosphorylation is a prerequisite to S238 phosphorylation.

It has been shown that NS5A S232 phosphorylation by CKI$\alpha$ was facilitated by S229 phosphorylation (27). Since S229, S232, S235, and S238 (Fig. 1A) fit the priming phosphorylation preference of CKI$\alpha$, whether S229 serves as the initial phosphorylation event for the sequential phosphorylation cascade of NS5A requires additional phosphorylation-specific antibodies for further study. Which kinase initiates the first priming phosphorylation event is another intriguing question. Between PI4KIII$\alpha$ and CKI$\alpha$, the two frequently studied kinases responsible for NS5A hyperphosphorylation, our data show that CKI$\alpha$ is the kinase responsible for S235 and S238 phosphorylation (Fig. 7 and 8) (22). Hypothetically, CKI$\alpha$ (18), PI4KIII$\alpha$ (31), or Plk1 (37)

could participate in S229 or S232 phosphorylation, thereby initiating the phosphorylation cascade. Sequential phosphorylation cascade is a ubiquitous posttranslational modification that regulates protein functions (38). A good example is $\beta$-catenin, which is phosphorylated by CKI at S45, which in turn serves to prime subsequent phosphorylation at S41, S37, and S33 by glycogen synthase kinase 3. When S45 is mutated to alanine, which terminates the sequential phosphorylation cascade, underphosphorylated $\beta$-catenin is not degraded by the proteasome and results in inappropriate activation of the Wnt signaling pathway found in a variety of cancer malignancies (39). Similar regulatory mechanisms via sequential phosphorylation cascade were reported for vesicular stomatitis virus (40) and rotavirus (41). In the case of HCV, the complete NS5A phosphorylation cascade and its functions remain to be elucidated.

## MATERIALS AND METHODS

**Cell culture.** Huh7.5.1 human hepatocarcinoma cells, T7 polymerase-expressing Huh7 cells (T7-Huh7, courtesy of Steve S.-L. Chen from the Academia Sinica, Taiwan), or HEK293T kidney cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries). The cells were cultured in a 5% $CO_2$ incubator (Astec Inc.) at 37°C.

**Antibodies, immunoblotting and dot blotting.** Commercially available primary antibodies were PI4KIII$\alpha$ (4902; Cell Signaling), NS5A (7B5; BioFront Technologies Inc.), dsRNA (J2; English and Scientific Consulting), and casein kinase I$\alpha$ (SC-6477; Santa Cruz Biotechnology). Lipid droplet fluorescent dye BODIPY 493/503 was purchased from Invitrogen (D-3922). Anti-mouse IgG IRDye680 (P/N 926-68070) and anti-rabbit IgG IRDye800 (P/N 926-32213) secondary antibodies were from Li-Core (Nebraska, USA). The NS5A S235 phosphorylation-specific antibody was characterized previously (22). The NS5A S222 and S238 phosphorylation-specific antibodies were custom-made by GeneTex Corporation, Taiwan, with an S222 phosphopeptide (RLARGpSPPSE) and an S238 phosphopeptide (SAPpSLRATC). Specificity of the phosphorylation-specific antibodies was examined with dot blot assay using phospho- and nonphosphopeptide spotted onto nitrocellulose membranes prior to immunoblotting. Immunoblotting was carried out as described previously (22).

**HCV infection.** Infectious J6/JFH-1 (genotype 2a HCV) viral particles were produced as described previously (22) and used to infect the Huh7.5.1 cells at various multiplicities of infection (MOIs).

**Plasmids and transfection.** The NS3-NS5A expression construct (pcDNA3.1-NS3-5A) was made in the first core facility, National Taiwan University College of Medicine. The T7-driven NS3-NS5B expression construct was a gift from Steve S.-L. Chen (Academia Sinica, Taiwan). The JFH1 HCV replicon (JFH1/SG-Neo) was a gift from Charles M. Rice (University of Rockefeller). Single-, double-, and triple-alanine mutations at S222, S235, and S238 in the NS3-NS5A expression construct were made with site-directed mutagenesis using the following primer sequences: S222A, 5′-GGCGCGGCGCTTGGCACGGGGAGCACCTCCATCTGAGGCGAGCTC-3′; S235A, 5′-GAGCTCCTCAGTGAGCCAGCTAGCAGCACCGTCGCTGCGGGCCAC-3′; S238A, 5′-AGTGAGCCAGCTATCAGCACCGGCGCTGCGGGCCACCTGCACCAC-3′; and S235A/S238A, 5′-AGTGAGCCAGCTAGCAGCACCGGCGCTGCGGGCCACCTGCACCAC-3′. Similar mutations were made in the JFH1 replicon. Plasmid transfection was done with the Lipofectamine 2000 reagent (11668-027; Invitrogen) by following the manufacturer's instructions.

**Immunoprecipitation.** Huh7.5.1 cells were infected with HCV for 6 days before the cell lysates were subjected to immunoprecipitation with the phosphorylation site-specific NS5A antibodies. Proteins that bound to NS5A were coimmunoprecipitated with protein G by following the manufacturer's instructions (28-9440-08; GE Healthcare Life Sciences) prior to immunoblotting.

**Replicon transfection.** The JFH1 replicon plasmid was linearized with XbaI digestion, purified, and transcribed into RNA using a MEGAscript T7 transcription kit (AM1334; Invitrogen). The *in vitro*-transcribed RNA was then transfected into the Huh7.5.1 cells using a 4D-Nucleofector system (Lonza) by following the manufacturer's manual.

**Gene-specific knockdown.** Plasmids carrying gene-specific targeting short hairpin RNA (shRNA) sequences (PI4KIII$\alpha$, 5′-ACGACATGATCCAGTACTATC-3′; CKI$\alpha$, 5′-GCCACAGTTGTGATGGTTGTT-3′) and nontargeting shRNA sequence (shCon, 5′-CAAATCACAGAATCGTCGTAT-3′) were purchased from the RNAi core facility at the Academia Sinica, Taiwan. The plasmid was packaged into lentivirus as described previously (28). The lentivirus was used to infect the cells for gene-specific knockdown.

**CKI$\alpha$ inhibition.** CKI$\alpha$-specific inhibitor D4476 (D1944; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; D8418; Sigma-Aldrich) before use. One day prior to the experiment, the T7-Huh7 cells were seeded in a 6-well plate ($9 \times 10^5$/well) before transfection with the NS3-NS5B expression construct and exposure to 0, 20, 30, 40, 50, and 60 $\mu$M D4476 for 1 day. The cell lysates were collected for immunoblotting for NS5A and NS5A phosphorylation.

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