Sequential S232/S235/S238 Phosphorylation of the Hepatitis C Virus Nonstructural Protein 5A

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ABSTRACT The hepatitis C virus (HCV) protein NS5A is a phosphorylated protein with crucial roles in viral replication and assembly. NS5A was thought to undergo sequential phosphorylation on a series of conserved serine residues; however, the phosphorylation cascade remained obscure. Using three phosphorylation-specific antibodies, we found that phosphorylation at S232, S235, and S238 occurred in parallel in HCV-infected Huh7.5.1 cells, suggestive of intramolecular sequential NS5A phosphorylation from S232 through S235 to S238 by casein kinase I α (CKI α). In line with this, alanine mutation at S225, S229, or S232 reduced, whereas aspartate mutation at the same sites rescued, NS5A phosphorylation at S232, S235, and S238. In contrast, alanine or aspartate mutation at S235 or S238 had little or no effect on S232 or S235 phosphorylation. Consistent with an intramolecular sequential phosphorylation cascade, S232, S235, and S238 phosphorylation coexisted on one single NS5A molecule. Phosphorylation of NH₂-terminal serine residues in one NS5A molecule did not rescue phosphorylation of COOH-terminal serine residues in another NS5A molecule. CKI α inhibition reduced NS5A phosphorylation at S232, S235, and S238. In summary, our results are indicative of a CKI α -mediated intramolecular, sequential phosphorylation cascade from S232 through S235 to S238 of the HCV NS5A protein. S225 and S229 also contribute substantially to the above sequential phosphorylation cascade of NS5A.

IMPORTANCE The nonstructural protein 5A (NS5A) of the hepatitis C virus was thought to undergo sequential intramolecular phosphorylation on a series of serine residues; however, direct evidence was missing. We offer the first direct evidence of a CKI α -mediated intramolecular sequential NS5A phosphorylation cascade from serine 232 through 235 to 238. This sequential phosphorylation cascade occurs in the disordered low-complexity sequence I region, which together with the domain I region forms an RNA-binding groove in an NS5A dimer. Sequential phosphorylation in the disordered region adds charge-charge repulsion to the RNA-binding groove and probably thereby regulates NS5A's RNA-binding ability and functions in viral RNA replication and assembly.

KEYWORDS antibody, HCV, NS5A, phosphorylation, S232

The hepatitis C virus (HCV) is an RNA virus affecting a large portion of the human population (1). The nonstructural protein 5A (NS5A) is an HCV protein critical to the viral life cycle (2, 3). It has three structural domains (I, II, and III) connected by two low-complexity sequence (LCS) regions (2). Except for domain I, whose structure has been solved with crystallography (4, 5), the rest of the NS5A protein is thought to be highly disordered. NS5A interacts with many host cell proteins and alters host cell signaling and the immune system in favor of HCV propagation (2, 3, 6). In addition, NS5A interacts with other viral proteins to form an RNA replication complex indispensable to viral replication (2, 3). It has been shown that NS5A forms a dimer via interaction

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between two domain I regions (7) and that domain I and LCS I constitute the minimal protein portion in the NS5A dimer involved in RNA binding (3, 8, 9).

When analyzed with SDS-PAGE plus immunoblotting, NS5A appears as two bands at 56 and 58 kDa, which represent, respectively, hypo- and hyperphosphorylated forms of NS5A (10). Numerous studies have pinpointed a cluster of eight highly conserved serine residues (S222, S225, S228, S229, S230, S232, S235, and S238) in the LCS I region responsible for NS5A hyperphosphorylation (11–18). Mutations in some of these serine residues reduce NS5A hyperphosphorylation in association with effects on viral replication and assembly (11, 12). For years, NS5A was thought to transit from a hypo- to hyperphosphorylated state via phosphorylation of these serine residues (2, 19). Many of the serine residues are spaced two or three amino acids apart and fit nicely to the phosphorylation preference of casein kinase I α (CKI α) (20) and glycogen synthase kinase 3 β (GSK3 β) (21). Both kinases have been implicated in NS5A phosphorylation (13, 22, 23). CKI α phosphorylates a serine residue at position 0 when an upstream serine or threonine residue at position –3 is phosphorylated or primed [(pS/pT)XXS] (20). GSK3 β phosphorylates a serine residue when a downstream serine residue at position 4 is phosphorylated (SXXXpS) (21). Thus, NS5A transition from a hypo- to hyperphosphorylated state can be anticipated via a sequential intramolecular phosphorylation cascade from the NH₂ to COOH terminus or in the reverse direction, depending on the first priming phosphorylation event and the participating kinase. This will result in a number of differently phosphorylated NS5A species, which could potentially interact with the vast number of host and viral proteins, hence conferring different functions (3).

In a recent phosphoproteomics study (13), we identified in the LCS I region six phosphorylated serine residues: S222, S225, S229, S232, S235, and S238. Using two phosphorylation-specific antibodies, we showed that S238 phosphorylation depends on S235 phosphorylation, in line with the idea of sequential NS5A phosphorylation by CKI α (16). Both S235 and S238 are phosphorylated by CKI α (16), indicative of a priming phosphorylation event at S232. In fact, an earlier study based on an *in vitro* kinase assay has shown that S229 phosphorylation primes S232 phosphorylation by CKI α (24), suggesting that S229 phosphorylation initiates sequential NS5A phosphorylation by CKI α . However, NS5A hyperphosphorylation persists even when S229 is mutated to alanine (11, 12). Moreover, a phosphorylation-ablated alanine mutation and phosphorylation-mimicking aspartate mutation at S229 both sabotage HCV replication (11, 12), leaving the functions of S229 phosphorylation mysterious. Thus, the initiating phosphorylation event and the subsequent phosphorylation cascade remain obscure for NS5A hyperphosphorylation.

In the present study, we made an NS5A S232 phosphorylation-specific antibody and used it to show that S232 phosphorylation primes CKI α -mediated phosphorylation at S235 followed by phosphorylation at S238. This sequential phosphorylation cascade results in NS5A hyperphosphorylation, a necessary condition for viral replication and assembly.

RESULTS

NS5A phosphorylation at serines 232, 235, and 238 occurred in HCV-infected Huh7.5.1 cells. To study the sequential NS5A phosphorylation cascade from S232 through S235 to S238, an antibody specific to S232 phosphorylation was generated and characterized alongside two antibodies specific to S235 and S238 phosphorylation (13, 16). On dot blots (Fig. 1A), all three antibodies detected their own designated phosphorylation sites in a dose-dependent manner without cross-reactivity. All three antibodies detected hyperphosphorylated NS5A in HEK293T cells transfected with an NS3-NS5A construct (Fig. 1B). In HCV (J6/JFH1 genotype 2a)-infected Huh7.5.1 cells (Fig. 2), NS5A phosphorylation at S232, S235, and S238 was detected on the second day after infection. Thereafter, the phosphorylation levels continued to increase in parallel with time up to 6 days.

Alanine mutation at S225, S229, or S232 reduced NS5A sequential phosphorylation from S232 through S235 to S238. If NS5A phosphorylation occurs in the order

FIG 1 Characterization of NS5A S232, S235, and S238 phosphorylation-specific antibodies. (A) Dot blot analysis. Synthetic phosphopeptides and nonphosphopeptides were diluted 5-fold and dotted on nitrocellulose membranes before detection with the antibodies. The peptide sequences are shown with the serine residue of interest indicated in red. (B) Immunoblotting (IB) for NS5A and NS5A phosphorylation at S232 (pS232), S235 (pS235), and S238 (pS238) in NS3-NS5A-transfected HEK293T cells. NS5A phosphorylation at each serine residue was detected with a phosphorylation-specific antibody (rabbit) followed by detection for total NS5A with a general NS5A antibody (mouse) on the same membrane. Proteins were visualized with a Li-Cor Odyssey scanner and software. Arrowheads indicate hypo- and hyperphosphorylated NS5A at 56 (p56) and 58 (p58) kDa. M and 3-5A, respectively, represent mock- and NS3-NS5A construct-transfected cells. Note that when the red band merges with the green band, it changes to yellow.

of S232, S235, and S238, alanine mutation at S232 should ablate phosphorylation at all three sites. Alanine mutation at S235 should ablate phosphorylation at S235 and S238 but not at S232. Alanine mutation at S238 would ablate only S238 phosphorylation. Indeed, in HEK293T cells transfected with various alanine mutant NS3-NS5A constructs, alanine mutation at S232 ablated phosphorylation at S232 (Fig. 3A, lane S232A), S235 (Fig. 3C, lane S232A), and S238 (Fig. 3E, lane S232A). Alanine mutation at S235 (S235A) ablated S235 and S238 phosphorylation (Fig. 3C and E, lanes S235A), but not S232 phosphorylation (Fig. 3A, lane S235A). Alanine mutation at S238 ablated only S238

FIG 2 Immunoblotting showed paralleled increases in NS5A phosphorylation at S232 (A), S235 (B), and S238 (C) in HCV-infected Huh7.5.1 cells. Protein samples were collected at 1 to 6 days after infection and subjected to immunoblotting for total NS5A and NS5A phosphorylation at S232 (pS232), S235 (pS235), and S238 (pS238). β-Actin was used as a loading control. (D to F) Summary of three independent immunoblotting results. Values are phosphorylation intensities divided by total NS5A intensities for each day and normalized against values on day 1. Values are means ± standard errors of the means (n = 3). *, P < 0.05 (t test against values on day 1).

FIG 3 Sequential phosphorylation from S232 through S235 to S238 was reduced when S225, S229, or S232 was mutated to alanine in HEK293T cells. Immunoblotting was performed for NS5A and NS5A phosphorylation at S232 (A), S235 (C), and S238 (E) in HEK293T cells transfected with the wild type (WT) or various serine-to-alanine mutant NS3-NS5A constructs. The bar graphs summarize the immunoblotting results from three independent experiments (B, D, and F). Values are phosphorylation intensities divided by total NS5A intensities and normalized against the values in the wild-type (WT)-transfected cells. Values are means $\pm$ standard errors of the means ($n = 3$). *, $P < 0.05$ (t test against the values in the wild-type-construct-transfected cells). ND, not detected.

phosphorylation (Fig. 3E, lane S238A) and not S232 and S235 phosphorylation (Fig. 3A and C, lanes S238A). Three independent experiments showed similar results (Fig. 3B, D, and F). Thus, alanine mutation at S232 eliminated NS5A phosphorylation at S232, S235, and S238, in line with intramolecular sequential NS5A phosphorylation from S232 through S235 to S238.

In addition to S232A, S225A and S229A also reduced NS5A phosphorylation at S232, S235, and S238 in HEK293T cells (Fig. 3, lanes S225A and S229A). Thus, alanine mutation at S225, S229, and S232 reduced NS5A phosphorylation at S232, S235, and S238.

Similar results were observed in NS3-NS5A-transfected Huh7 cells expressing T7 polymerase (T7-Huh7) with subtle differences. While S232A completely eliminated hyperphosphorylation (Fig. 3A, lane S232) as well as phosphorylation at S235 (Fig. 3C, lane S232A) and S238 (Fig. 3E, lane S232A) in HEK293T cells, S232A did not completely eliminate hyperphosphorylation (Fig. 4A, lane S232) or phosphorylation at S235 (Fig. 4C, lane S232) and S238 (Fig. 4E, lane S232A) in T7-Huh7 cells. While S235A reduced

FIG 4 Sequential phosphorylation from S232 through S235 to S238 was reduced when S225, S229, or S232 was mutated to alanine in T7-Huh7 cells. Immunoblotting was performed for NS5A and NS5A phosphorylation at S232 (A), S235 (C), and S238 (E) in the T7-Huh cells transfected with wild type (WT) or various serine-to-alanine mutant NS3-NS5A constructs. The bar graphs summarize the immunoblotting results from three independent experiments (B, D, and F), with values determined as described in the legend of Fig. 3. *, $P < 0.05$ (t test against the values in the wild-type-construct-transfected cells). ND, not detected.

S232 phosphorylation in HEK293T cells (Fig. 3A), S235A did not affect S232 phosphorylation in T7-Huh7 cells (Fig. 4A). S238A did not affect phosphorylation at S232 (Fig. 3A) and S235 (Fig. 3C) in HEK293T cells but elevated S232 phosphorylation in T7-Huh7 cells (Fig. 4A).

NS5B has been implicated in NS5A hyperphosphorylation (25). To evaluate this, we compared NS5A phosphorylation at S232, S235, and S238 in T7-Huh7 cells transfected with an NS3-NS5A (Fig. 4) or NS3-NS5B (Fig. 5) construct. We did the comparison only for the S225A, S229A, S232A, S235A, and S238A mutant NS5As because alanine mutations at the other serine residues had no or little effect on NS5A phosphorylation (Fig. 4). As seen, NS5A phosphorylation at S232, S235, and S238 are grossly similar for the wild type and the various alanine mutant NS5As in the absence (Fig. 4) or presence (Fig. 5) of NS5B. S225A, S229A, and S232A profoundly reduced NS5A phosphorylation at S232, S235, and S238. In contrast, S235A did not affect phosphorylation at S232 but significantly reduced S235 and S238 phosphorylation. S238A significantly increased

FIG 5 Alanine mutation at the NH₂ terminus of S232 reduced NS5A sequential phosphorylation from S232 to S238. Immunoblotting was performed for NS5A and NS5A phosphorylation at S232 (A), S235 (C), and S238 (E) in NS3-NS5B-transfected T7-Huh7 cells. The bar graphs summarize the immunoblotting results from three independent experiments (B, D, and F), with values determined as described in the legend of Fig. 3. *, $P < 0.05$ (t test against the values in the wild-type-construct-transfected cells). ND, not detected.

S232 phosphorylation without affecting S235 phosphorylation. Thus, without or with NS5B, alanine mutation of the serine residues at the NH₂ terminus of S232 (including S232) reduced sequential NS5A phosphorylation from S232 through S235 to S238. Alanine mutation of the serine residues at the COOH terminus of S232 did not reduce NS5A phosphorylation at S232 or S235.

There were subtle effects of NS5B on NS5A phosphorylation. While S232 phosphorylation was barely detected in the S229A mutant NS5A in the absence of NS5B (Fig. 4A, lane S229A), it was readily detected in the same NS5A mutant in the presence of NS5B (Fig. 5A, lane S229A). This S232 phosphorylation was accompanied with phosphorylation at S235 and S238 in the S229A mutant NS5A in the presence of NS5B (Fig. 5C and

E, lanes S229A), consistent with sequential NS5A phosphorylation from S232 to S238. While S235 phosphorylation was barely detected in the S225A and S229A mutant NS5As in the presence of NS5B (Fig. 5C, lanes S225A and S229A), it was readily detected in the absence of NS5B (Fig. 4C, lanes S225A and S229A). This S235 phosphorylation occurred with S238 phosphorylation in the S225A and S229A mutant NS5As in the absence of NS5B (Fig. 4E, lanes S225A and S229A), again in line with the sequential phosphorylation cascade of NS5A.

Aspartate mutation rescued sequential NS5A phosphorylation from S232 through S235 to S238. Despite structural differences, aspartate is often used to mimic serine phosphorylation, perhaps due to similarity in their charges (11–14, 26). We hereby tested whether aspartate mutation could rescue intramolecular NS5A sequential phosphorylation from S232 to S238. The T7-Huh7 cells were transfected with a wild-type, S225D, S229D, S232D, S235D, or S238D NS3-NS5B construct before being examined for NS5A phosphorylation at S232, S235, and S238. As summarized in Fig. 6, S225D, S229D, and S232D could completely or partially rescued NS5A phosphorylation at S232, S235, and S238. S229D had the best overall rescuing effects. Thus, aspartate mutation of the serine residues at the NH₂ terminus of S232 (including S232) could rescue sequential NS5A phosphorylation from S232 to S238. S235D partially rescued S238 phosphorylation while reducing S232 phosphorylation. S238D did not affect S232 or S235 phosphorylation. Thus, aspartate mutation of the serine residues at the COOH terminus of S232 has no or little effect on phosphorylation at S232 and S235.

Sequential NS5A phosphorylation could not be rescued in *trans*. If sequential NS5A phosphorylation occurs in the order of S232, S235, and S238 within one single molecule, phosphorylation of an NH₂-terminal serine residue in one NS5A molecule should not rescue phosphorylation of a COOH-terminal serine residue in another NS5A molecule. To test this, we examined whether S232 phosphorylation in the S235A mutant NS5A could rescue S235 phosphorylation in the S232A mutant NS5A in T7-Huh7 cells doubly transfected with the S235A and S232A mutant NS3-NS5B constructs. When S232A NS5A was expressed alone, it was barely phosphorylated at S232, S235, or S238 (Fig. 7A, C, and E, lanes 2). When S235A NS5A was expressed alone, it was phosphorylated at S232 but not at S235 or S238 (Fig. 7A, C, and E, lanes 3). When S232A and S235A NS5A were coexpressed in the same cells, there was barely any detectable S235 phosphorylation (Fig. 7C, pS235, lane 5), despite apparent S232 phosphorylation (Fig. 7A, pS232, lane 5). Thus, S232 phosphorylation in S235A NS5A cannot rescue S235 phosphorylation in S232A NS5A. The level of S232 phosphorylation in the doubly transfected cells (Fig. 7A, pS232, lane 5) was about half of that in the singly transfected cells (Fig. 7A, pS232, lane 3) because only half the amount of the plasmid was used in the doubly transfected cells. In line with the intramolecular phosphorylation cascade, S235 phosphorylation in one NS5A molecule also did not rescue S238 phosphorylation in another NS5A molecule. When S238A NS5A was expressed alone, it was phosphorylated at S232 and S235 (Fig. 7A and C, lanes 4). However, S235 phosphorylation on the S238A mutant NS5A did not rescue S238 phosphorylation in the S232A mutant NS5A (Fig. 7E, pS238, lane 6). Likewise, S235 phosphorylation in the S238A mutant NS5A (Fig. 7C, pS235, lane 4) did not rescue S238 phosphorylation in the S235A mutant NS5A (Fig. 7E, pS238, lane 7). The above results support an intramolecular sequential phosphorylation cascade from S232 through S235 to S238 on the same NS5A molecule.

Phosphorylation at serine 232, 235, and 238 occurred on the same NS5A molecule. The above results (Fig. 3 to 7) are indicative of a sequential intramolecular NS5A phosphorylation cascade from S232 through S235 to S238. If this is true, S232 phosphorylation could occur alone independently of S235 and S238 phosphorylation, while S235 and S238 phosphorylation should coexist with S232 phosphorylation on the same NS5A molecule. To test this, we took advantage of an S235 phosphorylation-specific antibody that is capable of pulling down most hyperphosphorylated NS5A from HCV-infected Huh7.5.1 cell lysate (16). As shown in Fig. 8B, almost all NS5A containing S235 phosphorylation was immunoprecipitated with the S235 phosphorylation-specific

FIG 6 Aspartate mutation at S225, S229, and S232 rescued NS5A sequential phosphorylation from S232 through S235 to S238. T7-Huh7 cells were transfected with various NS3-NS5B expression constructs, including the wild type (WT) and S225D, S229D, S232D, S235D, and S238D mutants, before immunoblotting for NS5A and NS5A phosphorylation at S232 (A), S235 (C), and S238 (E). (B, D, and F) Graphs summarize immunoblotting results from three independent experiments, with values determined as described in the legend of Fig. 3. *, $P < 0.05$ (t test against the values in the wild-type-construct-transfected cells). ND, not detected.

antibody as there was no more detectable S235 phosphorylation in the unbound fraction (Fig. 8B, pS235, lane Ub). The removal of NS5A with phosphorylated S235 (S235-phosphorylated NS5A) from the cell lysate left a small but detectable amount of hyperphosphorylated NS5A in the unbound fraction (Fig. 8B, NS5A, lane Ub) that was phosphorylated at S232 (Fig. 8A, pS232, lane Ub), indicating that S232 phosphorylation

FIG 7 Phosphorylation in one NS5A molecule did not rescue phosphorylation in another NS5A molecule. T7-Huh7 cells were transfected with various NS3-NS5B expression constructs, wild type (WT), S232A, S235A, and S238A, before immunoblotting for NS5A phosphorylation at S232 (A), S235 (C), and S238 (E). For the rescue experiments, some cells were doubly transfected with any two of the three alanine mutant NS3-NS5B constructs before immunoblotting. (B, D, and F) Graphs summarize immunoblotting results from three independent experiments, with values determined as described in the legend of Fig. 3. *, $P < 0.05$ (t test against the values in the wild-type-construct-transfected cells). ND, not detected.

can occur alone independently of S235 phosphorylation. In the S235-phosphorylated NS5A, S232 phosphorylation was detected (Fig. 8A, pS232, lane E). Thus, S232 phosphorylation coexists with S235 phosphorylation on the same NS5A molecule. Similarly, S238 phosphorylation was also detected in the S235-phosphorylated NS5A (Fig. 8C, pS238, lane E), indicating that S238 phosphorylation coexists with S235 phosphorylation on the same NS5A molecule. There was barely any detectable S238 phosphoryla-

FIG 8 Immunoprecipitation coupled with immunoblotting showed coexistence of S232, S235, and S238 phosphorylation on the same NS5A molecule. HCV-infected Huh7.5.1 cells were subjected to immunoprecipitation (IP) using an S235 phosphorylation-specific antibody before being blotted for NS5A phosphorylation at S232 (A), S235 (B), and S238 (C). In, input (total cell lysate); Ub, unbound fraction; W, wash; E, eluate. (D) Summary of three independent experiments. Values are calculated as the difference in the phosphorylation intensities of a serine residue in the input (pSXI_{in}) minus that in the unbound fraction (pSXUb) divided by pSX_{in} and are presented as percentages. Thus, the value represents the percentage of S235 phosphorylation in S232- or S238-phosphorylated NS5A.

tion in the unbound fraction when the S235-phosphorylated NS5A was immunoprecipitated (Fig. 8C, pS238, lane Ub). Thus, almost all S238 phosphorylation occurs with S235 phosphorylation on the same NS5A molecule. Results of three independent experiments are summarized in Fig. 8D. On average, about 5% of S232-phosphorylated NS5A contains S235 phosphorylation. Most S232-phosphorylated NS5A occurs independently of S235 phosphorylation. Nearly all (96%) S238-phosphorylated NS5A contains S235 phosphorylation.

Casein kinase I α -mediated NS5A phosphorylation at serines 232, 235, and 238.

The fact that S232, S235, and S238 fall within the phosphorylation preference of CKI α (20, 24) prompted us to test whether CKI α inhibition would reduce phosphorylation at S232, S235, and S238 in T7-Huh7 cells transfected with the NS3-NS5B construct. We have previously shown that CKI α knockdown or inhibition reduced phosphorylation at S235 and S238 (16). In the present study, we found that CKI α knockdown did not affect S232 phosphorylation but reduced S235 and S238 phosphorylation (Fig. 9A and B). Given multiple CKI α isoforms with redundant functions (27), the CKI α inhibitor D4476 was used to repeat the test. As seen in Fig. 9C and D, D4476 significantly reduced phosphorylation at S232, S235, and S238, in line with sequential intracellular NS5A phosphorylation from S232 through S235 to S238 by CKI α .

DISCUSSION

In the present study, we used site-directed mutagenesis and three phosphorylation-specific antibodies (Fig. 1) to show that NS5A undergoes CKI α -mediated sequential intramolecular phosphorylation from S232 through S235 to S238. Six lines of evidence support this. One, S232, S235, and S238 phosphorylation occurs in parallel in HCV-infected Huh7.5.1 cells (Fig. 2). Two, alanine mutation at upstream serine residues reduces phosphorylation of downstream serine residues whereas alanine mutation at downstream serine residues does not affect phosphorylation of upstream serine residues (Fig. 3 to 5). Three, aspartate mutation at upstream serine residues rescues phosphorylation of downstream serine residues whereas aspartate mutation at downstream serine residues does not affect phosphorylation of upstream serine residues (Fig. 6). Four, phosphorylation of upstream serine residues occurs independently of phosphorylation of downstream serine residues (Fig. 8). Phosphorylation of downstream serine residues coexists with phosphorylation of upstream serine residues on the same NS5A molecule (Fig. 8). Five, phosphorylation of upstream serine residues does not transcomplement phosphorylation of downstream serine residues (Fig. 7). Six, CKI α mediates NS5A phosphorylation at S232, S235, and S238 (Fig. 9). This sequential intramolecular NS5A phosphorylation cascade likely occurs instantaneously in the HCV-infected Huh7.5.1 cells because most NS5A exists as either a hypo- or hyperphosphorylated form. There is barely any visible protein band between hypo- and hyperphosphorylated NS5A.

The above-described intramolecular phosphorylation cascade occurs in the intrinsically disordered LCS I region of NS5A (2, 28). Based on crystal structures (4, 5) and

FIG 9 Casein kinase α -mediated NS5A sequential phosphorylation from S232 through S235 to S238. (A) Immunoblotting for CKI α , NS5A, and NS5A phosphorylation at S232, S235, and S238 in NS3-NS5B construct-transfected T7-Huh7 cells. The cells were infected with lentivirus carrying a nontargeting control shRNA (shCon) or CKI α -specific shRNA sequence for 2 days before being transfected with the NS3-NS5B construct for 1 day prior to immunoblotting. (B) A bar graph summarizing the immunoblotting results from three independent experiments. Values are means $\pm$ standard errors of the means. *, $P < 0.05$ (t test against the values in the control cells). (C) Effects of CKI α inhibitor (D4476) on NS5A phosphorylation at S232. T7-Huh7 cells were transfected with NS3-NS5B construct and exposed to D4476 for 1 day before immunoblotting. (D) Summary of three independent experiments. *, $P < 0.05$.

biochemical characterization (8), this intrinsically disordered LCS I region together with the domain I region of NS5A constitutes an RNA-binding groove in an NS5A dimer (7). Previously, phosphorylation of S146 at the interface between two domain I regions was shown to switch the NS5A dimer structure from an open configuration to a closed configuration and negatively regulates NS5A hyperphosphorylation (29). To this, we add the notion that perhaps sequential phosphorylation in the LCS I region brings in multiple negative charges to this region, resulting in charge-charge repulsion that opens up the RNA-binding groove to facilitate RNA binding and hence viral replication and assembly (11–13). Another potential function of sequential NS5A phosphorylation in the disordered LCS I region is that it alters the structure of the LCS I region for protein-protein interaction (30, 31). Because NS5A interacts with a plethora of viral and host proteins (3), it is tempting to speculate that perhaps sequential NS5A phosphorylation in the LCS I region fine-tunes the disordered structure for interactions with different proteins and, hence, NS5A-mediated functions (18).

Although NS5A phosphorylation levels were similar in the HEK293T cells and the T7-Huh7 cells, there were differences. For example, S235 phosphorylation was barely detected in the S232A mutant NS3-NS5A-transfected HEK293T cells (Fig. 3), but it was readily detected in the S232 mutant NS3-NS5A-transfected T7-Huh7 cells (Fig. 4), suggesting that the HEK293T and T7-Huh7 cells probably host different kinase/phosphatase repertoires for S235 phosphorylation, a necessary condition for HCV replication (16, 32). The differences in the kinase/phosphatase repertoires may explain why HEK293T kidney cells could support the HCV life cycle but only to some extent (33–35).

In addition to S232, S225 and S229 at the NH₂ terminus of S232 also influence sequential NS5A phosphorylation from S232 to S238. Alanine mutation at S225 or S229 greatly reduced NS5A phosphorylation at S232, S235, and S238 (Fig. 3 to 5), which can be rescued with an aspartate mutation at S225 or S229 (Fig. 6). S229 phosphorylation has been shown to prime S232 phosphorylation by CKI α (24). Thus, S229 phosphorylation could probably initiate the sequential intramolecular NS5A phosphorylation cascade from S232 to S238. However, this sequential phosphorylation cascade is not sufficient for the HCV life cycle because both a phosphorylation-ablated mutation and a phosphorylation-mimicking mutation at S229 severely reduce HCV replication (11, 12). Thus, S229 must have additional functions, independent of phosphorylation and critical to the HCV life cycle. Alternatively, S229 may undergo rapid phosphorylation-dephosphorylation cycles to fire signals for sequential NS5A phosphorylation. Because S229 does not fall within the phosphorylation preference of CKI α , it is probably phosphorylated by other kinases. It has been demonstrated that NS5A hyperphosphorylation requires NS3, NS4A, NS4B, and NS5A to be encoded on the same polyprotein molecule (36) and that an active NS3 protease function is required for NS5A hyperphosphorylation (37). Perhaps during the NS3-mediated *cis* cleavage of the NS3-NS5A polyprotein, a certain structural change occurs in the NS3-NS5A polyprotein and recruits a kinase to phosphorylate S229. Protein kinases, including polo-like kinase 1 (38), calmodulin-dependent kinase II (35), and GSK3 β (22) reported to affect NS5A phosphorylation, may be recruited for S229 phosphorylation. In addition, the lipid phosphatidylinositol-4 kinase III α (25) may potentially alter the lipid composition to recruit kinases for S229 phosphorylation. Without bona fide measurements of S229 phosphorylation, all of the above speculations remain only speculations. How S225 phosphorylation affects sequential NS5A phosphorylation from S232 to S238 is another question that requires further study.

If S232 phosphorylation primes S235 and S238 phosphorylation, one less intuitive observation is that only a small percentage of S232-phosphorylated NS5A is phosphorylated at S235 (Fig. 8). One simple explanation is that S235 might be phosphorylated by kinases (35) other than CKI α that requires priming phosphorylation at S232. For example, an *in vitro* kinase assay shows that calmodulin-dependent kinase II can directly phosphorylate S235 in a synthetic peptide that is not phosphorylated at S232 (13). This possibility is less likely in that an alanine mutation at S232 nearly eliminates S235 phosphorylation (Fig. 4C). Another possibility is interference in the detection of phosphorylation with antibodies. If S235 phosphorylation interferes with antibody detection of S232 phosphorylation, the amount of S232 phosphorylation will be low. This possibility is also less likely in that S232 was detected in the S235-phosphorylated NS5A (Fig. 8A). Another possibility is that S232 is dephosphorylated after its phosphorylation primes S235 and S238 phosphorylation. This leaves S235- and S238-doubly phosphorylated NS5A as the major hyperphosphorylated NS5A species (Fig. 8C), consistent with our protein mass spectrometry results (13).

One interesting observation is that alanine mutation at S238 elevated S232 phosphorylation (Fig. 4), suggestive of a feedback mechanism from the end of the sequential phosphorylation cascade. In other words, if the sequential phosphorylation cascade does not propagate to S238, a mechanism kicks in to reiterate signals for sequential phosphorylation at S232. Casein kinase II might be a candidate for such a feedback mechanism. Casein kinase II phosphorylates serine (i.e., S232) or threonine at position 0 when position +3 is a negatively charged aspartate or glutamate that resembles phosphorylated serine (i.e., S235) or threonine (39). We do not know the phosphorylation status of S229 in the S238A mutant NS5A without an antibody to S229 phosphorylation.

In summary, our results support a sequential intramolecular NS5A phosphorylation cascade from S232 through S235 to S238. Both S225 and S229 phosphorylations influence the sequential phosphorylation cascade. Finally, S238 phosphorylation may provide a feedback mechanism to regulate the sequential cascade. Our results really

have just begun to unravel an intricate regulatory network for NS5A phosphorylation by kinases and phosphatases.

MATERIALS AND METHODS

Cell culture. Human hepatocellular carcinoma cells (Huh7.5.1), T7 polymerase-harboring human hepatocellular carcinoma cells (T7-Huh7), and human kidney cells HEK293T were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Biological Industries) in a 5% CO₂ incubator (Astec, Inc.) at 37°C.

Reagents. β -Actin antibody was purchased from Sigma-Aldrich (catalog number A5441). Anti-NS5A domain I antibody was purchased from BioFront Technologies Inc. (7B5). Casein kinase I α antibody was purchased from Santa Cruz Biotechnology (SC-6477). Serine 235 and 238 phosphorylation-specific antibodies were characterized previously (13, 16). A rabbit serine 232 phosphorylation-specific polyclonal antibody was generated against a synthetic peptide (SSSVpSQLSAPSLRATC) by GeneTex, Inc., Taiwan. The antibody was purified with two affinity chromatography assays, with an immunizing phosphopeptide followed by a nonphosphopeptide with the same sequence. Dot blotting was performed to characterize the antibody (13). IRDye 680-labeled anti-mouse IgG (product no. 926-68070) and IRDye 800-labeled anti-rabbit IgG (926-32213) secondary antibodies were purchased from Li-Cor (Nebraska, USA). The CKI α inhibitor D4476 was purchased from Sigma-Aldrich (catalog no. D1944).

The expression constructs for NS3-NS5A and NS3-NS5B were made in pcDNA3.1 (16) and pTM (40) plasmids and transfected into cells with Lipofectamine 2000 reagent (11668-027; Invitrogen). Alanine mutations at S225, S228, S229, S230, and S232 in the NS3-NS5A and NS3-NS5B constructs were made with site-directed mutagenesis using the following primer sequences: S225A, 5'-CTTGGCACGGGGATCACTCCAGCTGAGGCGAGCTCCTCAGTGAG-3'; S228A, 5'-GGATCACCTCCATCTGAGGCGGCTCCTCAGTGAGCCAGCTATCA-3'; S229A, 5'-ATCACCTCCATCTGAGGCGAGCGCTCAGTGAGCCAGCTATCAGC-3'; S230A, 5'-ACCTCCATCTGAGGCGAGCTCCGAGTGAGCCAGCTATCAGCACC-3'; S232A, 5'-ATCTGAGGCGAGCTCCTCAGTGGCCAGCTATCAGCACCCTGCT-3'. Primer sequences for alanine mutations at S222, S235, and S238 were reported previously (16).

HCV infection. Infectious viral particles were collected from the culture medium of Huh7.5.1 cells transfected with a full-length HCV genome construct (genotype 2a HCV, J6/JFH1) (41) as described previously (35). The viral particles were used to infect Huh7.5.1 cells at a multiplicity of infection of 0.001.

Immunoblotting. A detailed immunoblotting procedure was described previously (13). Briefly, HCV-infected or expression construct-transfected cells were washed with phosphate-buffered saline (PBS) and lysed in a lysis buffer. After a brief centrifugation, the supernatant was collected, and the protein concentrations were quantified. In general, 20 μ g of protein was separated by SDS-PAGE before the protein was transferred to a nitrocellulose membrane for blocking and subsequent detection with primary antibodies and secondary antibodies. Protein was visualized and quantified with a Li-Cor Odyssey scanner and software.

Immunoprecipitation. HCV-infected Huh7.5.1 cell lysate (180 μ g) was mixed with 8 μ g of phosphorylation-specific antibody (16) in 200 μ l of PBS and incubated at 4°C overnight. Protein G-coated magnetic beads (80 μ l) (catalog no. 28-9440-08; GE Healthcare Life Sciences) was washed with 500 μ l of PBS before being added to the above mixture and incubated at 4°C for 4 h. The unbound fraction was collected before the beads were washed with 200 μ l of PBS twice. Proteins bound to the beads were eluted in 40 μ l of 2% SDS solution with boiling (15 min) before immunoblotting analysis.

shRNA-mediated gene knockdown. Plasmid containing a small hairpin RNA (shRNA) targeting CKI α (5'-GCCACAGTTGTGATGGTTGT-3') or nonspecific shRNA (5'-CAAATCACAGAATCGTCGTAT-3') was purchased from the RNA interference (RNAi) core facility of the Academia Sinica, Taiwan. The plasmids were packaged into lentivirus using T-Pro NTR II reagent (JT97-N002M; T-Pro Biotechnology) according to the manufacturer's instructions before the lentivirus was used to infect cells for gene-specific knockdown (35).

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