Hepatitis C Virus Particle Assembly Involves Phosphorylation of NS5A by the c-Abl Tyrosine Kinase*

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Background: HCV NS5A regulates viral RNA replication and virus particle assembly.

Results: Phosphorylation of NS5A by c-Abl is required for efficient production of infectious HCV particles but not for viral RNA replication.

Conclusion: HCV particle assembly involves tyrosine phosphorylation of NS5A.

Significance: This study provides the first evidence for the importance of NS5A tyrosine phosphorylation in the HCV life cycle.

Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is thought to regulate the replication of viral RNA and the assembly of virus particles in a serine/threonine phosphorylation-dependent manner. However, the host kinases that phosphorylate NS5A have not been fully identified. Here, we show that HCV particle assembly involves the phosphorylation of NS5A by the c-Abl tyrosine kinase. Pharmacological inhibition or knockdown of c-Abl reduces the production of infectious HCV particles but does not affect the replication of the virus. The nonstructural proteins, on the other hand, form RNA replication complexes on ER-derived membranous compartments (4). Many of the nonstructural proteins also facilitate the assembly of virus particles.

Recent evidence suggests that HCV particle assembly is initiated near the interface between the ER membrane and cytosolic lipid droplets (LDs), organelles for the storage of neutral lipids (5–7). After proteolytic maturation at the ER membrane, the capsid protein core traffics to the surface of LDs (8) where it binds to viral RNA. The resulting nucleocapsids acquire envelopes by budding into the ER lumen and mature into virus particles. The virus particles are released from the host cell through the secretory pathway.

NS5A is a non-enzymatic protein that plays a critical but as yet undefined role in RNA replication and particle assembly (9). NS5A consists of an amino-terminal amphipathic α-helix and three structural domains (domains I–III). The amphipathic α-helix anchors NS5A to the cytosolic side of the ER membrane (10, 11). Domains I and II are required at least for RNA replication (12, 13), whereas domain III is required for particle assembly (14). NS5A binds to viral RNA in vitro (15, 16) and has therefore been proposed to transport viral RNA from replication complexes to LD-resident core (6, 7).

NS5A is a phosphoprotein that exists in basally phosphorylated (p56) and hyperphosphorylated (p58) forms (17, 18). Studies using HCV subgenomic replicons have proposed that NS5A hyperphosphorylation negatively regulates RNA replication (19–21). Although multiple serine and threonine phosphorylation sites have been identified in NS5A (21, 22), their roles in particle assembly are unknown, except for a serine residue at the carboxyl terminus of domain III. This residue, Ser457, is phosphorylated by casein kinase II in vitro (23) and is required for efficient particle assembly (23, 24). A recent study has shown that casein kinase I is also involved in HCV particle assembly through NS5A hyperphosphorylation (25). In addi-

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2 The abbreviations used are: HCV, hepatitis C virus; HCVcc, cell culture-grown HCV; ER, endoplasmic reticulum; NS5A, nonstructural protein 5A; LD, lipid droplet; CML, chronic myeloid leukemia.
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tion to serine and threonine phosphorylation, we have reported that overexpressed NS5A is tyrosine-phosphorylated in response to treatment with peroxanate, an inhibitor of protein tyrosine phosphatases (26). Moreover, we and others have reported that NS5A binds to non-receptor protein-tyrosine kinases such as c-Src, Fyn, Syk, and c-Abl in vitro (26–28). However, whether NS5A is tyrosine-phosphorylated in HCV-infected cells is yet to be determined.

The c-Abl tyrosine kinase was discovered as the cellular homolog of v-Abl, the oncoprotein of the Abelson murine leukemia virus (29), and has since been implicated in human cancers (30). In patients with chronic myeloid leukemia (CML), the gene for c-Abl has been translocated from chromosome 9 to chromosome 22 (31). The derivative chromosome 22, known as the Philadelphia chromosome, directs the expression of the fusion protein Bcr-Abl, the cause of CML. The Abl family kinase inhibitor imatinib has proved effective and well tolerated in the treatment of CML and is considered a paradigm of targeted therapeutics.

The Abl family of non-receptor tyrosine kinases consists of c-Abl and Arg (30). Viruses such as coxsackie B virus (32), vaccinia virus (33), and Ebola virus (34) exploit c-Abl and Arg for their entry or egress (35). Although imatinib has been identified as a potential anti-HCV agent in an unbiased compound screen (36), the role of Abl family kinases in the HCV life cycle is unknown.

In this study, we show that c-Abl is involved in HCV particle assembly. We also show that c-Abl phosphorylates NS5A at Tyr330 both in vitro and in the context of HCV infection and that this residue is required for efficient particle assembly. Our results suggest that c-Abl promotes HCV particle assembly by phosphorylating NS5A at Tyr330.

Experimental Procedures

Cell Culture—Huh-7.5 human hepatoma cells were kindly provided by Charles M. Rice (The Rockefeller University) and were cultured in DMEM (Wako) supplemented with 10% FBS (Sigma-Aldrich) and 0.1 mM nonessential amino acids (Wako). COS-7 cells and HEK293T cells were cultured in DMEM (Wako) supplemented with 10% FBS provided by Charles M. Rice (37). Site-directed mutagenesis was used to replace conserved tyrosine residues in NS5A with phenylalanine. pFL-J6/JFH1 was kindly provided by Charles M. Rice (37). Site-directed mutagenesis was used to replace NS5A Tyr330 with phenylalanine. EcoRI/Bsp1407I and Clal/Bsp1407I fragments were cloned into pFL-J6/JFH1 and pSGR-JFH1 (39), respectively. pSGR-JFH1 was kindly provided by Takaji Wakita (National Institute of Infectious Diseases, Japan). The sequence of NS5A domain II (amino acids 250–338) was amplified by PCR from pFL-J6/JFH1 and cloned into pGEX-4T-3.

HCV Infection—HCVcc (J6/JFH1) with adaptive mutations (P-47) was described previously (40). Huh-7.5 cells were infected with HCVcc at a multiplicity of infection of 3. The media were replaced with fresh media 4–6 h after infection.

Focus-forming Assay—To evaluate extracellular infectivity, culture supernatants were collected. To evaluate intracellular infectivity, cells were trypsinized and suspended in culture media. Cells were centrifuged at 2000 × g for 2 min, suspended in culture media, and subjected to three cycles of freezing and thawing. Samples were centrifuged at 2000 × g for 2 min. Supernatants were used to infect naive Huh-7.5 cells. Cells were incubated for 72 h, fixed with methanol for 20 min at −20 °C, and stained with anti-core antibody. The foci of infected cells were counted manually with a fluorescence microscope (IX70, Olympus).

Real-time PCR—Cells were washed three times with PBS. Total RNA was extracted using a High Pure RNA isolation kit (Roche Life Science). One hundred ng of total RNA was subjected to reverse transcription. RNA in culture supernatants was extracted using a QIAamp viral RNA mini kit (Qiagen). Real-time PCR was performed using a SYBR FAST qPCR kit (KAPA Biosystems) and a StepOne Plus real-time PCR system (Life Technologies). Reactions contained HCV-specific forward and reverse primers (5′-CTTCACGGCAAAAGGCT-TCA-3′ and 5′-CAAGCACCCTATCAGGCAGT-3′, respectively). The standard curve was constructed using HCV RNA transcribed in vitro. To quantify c-Abl and Arg mRNA levels, real-time PCR was performed with c-Abl-specific forward and reverse primers (5′-GGCTGGGTGCTCCAAGCAA-3′ and 5′-ACACAGGCCATGTTACCA-3′, respectively) or Arg-specific forward and reverse primers (5′-CTGCAGATGGCAAGG- TGTATGT-3′ and 5′-TGTTGTACGAGCCATCAG-3′, respectively). The standard curves were constructed using c-Abl and Arg RNA transcribed in vitro.

Retroviral Infection—Retroviral packaging was performed using HEK293T cells. Retrovirus-containing culture supernatants were filtered through a 0.45-μm-pore size filter and used to infect Huh-7.5 cells in the presence of 8 μg/ml Polybrene for 8 h. After fresh media were added, cells were incubated for 48 h.
**Immunoblotting**—Cells were lysed in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 2 μg/ml aprotinin) and centrifuged at 15,000 x g for 20 min at 4 °C. Immunoprecipitation was performed using protein A-Sepharose (GE Healthcare). The immunoprecipitates were subjected to SDS-PAGE. Blots were quantified using ImageJ software.

**Purification of GST Fusion Proteins**—Competent cells (DH5α) were transformed with pGEX-4T-3-NS5A domain II wild type (WT) or Y330F and cultured in LB broth. The expression of the GST fusion proteins was induced by adding IPTG to a final concentration of 0.5 mM. LB cultures were incubated for 2 h at 37 °C and centrifuged at 5000 x g for 15 min at 4 °C. After sonication in PBS containing 1 mM PMSF and 2 μg/ml aprotinin, Triton X-100 was added to a final concentration of 1%. Lysates were cleared by centrifugation and incubated with glutathione-Sepharose 4B (GE Healthcare) for 30 min at room temperature. GST fusion proteins were eluted with elution buffer (50 mM Tris (pH 8.0), 10 mM reduced glutathione).

**In Vitro Kinase Assay**—HEK293T cells were transfected with pcDNA3-c-Abl-FLAG, lysed in lysis buffer, and centrifuged at 15,000 x g for 20 min. Immunoprecipitation was performed using anti-FLAG antibody-agarose (Medical and Biological Laboratories). Immunoprecipitates were incubated with GST-NS5A domain II WT or Y330F in kinase buffer (20 mM Tris (pH 7.4), 1 mM DTT, 10 mM MgCl<sub>2</sub>, 10 mM ATP) for 15 min at room temperature. GST fusion proteins were eluted with elution buffer (50 mM Tris (pH 8.0), 10 mM reduced glutathione).

**In Vitro Transcription**—In vitro transcription of HCV RNA was performed as described elsewhere (41). Briefly, DNA templates were linearized with Xbal and treated with mung bean nuclease. Linearized templates were treated with proteinase K and purified by ethanol-chloroform extraction. RNA was transcribed in vitro using a MEGAscript kit (Ambion) and purified using a High Pure RNA isolation kit. RNA stocks (1 μg/μl) were stored at −80 °C. In vitro transcriptions of c-Abl and Arg RNA were performed similarly, except that linearized pcDNA3-c-Abl and pcDNA3-Arg were not treated with mung bean nuclease.

**Electroporation**—Cells were trypsinized, suspended in culture media, and centrifuged at 2000 x g for 2 min. After washing with PBS, cells were suspended at 1 x 10<sup>7</sup> cells/ml in PBS. A cell suspension (400 μl) was mixed with 5 μg of RNA in a 4-mm-gap cuvette and electroporated at 950 microfarads and 260 V using a Gene Pulser Xcell electroporation system (Bio-Rad). After a 10-min recovery time, cells were suspended in culture media and plated.

**Immunofluorescence**—Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 50 μg/ml digitonin for 5 min, and incubated with primary antibodies overnight at 4 °C. Alexa Fluor 488- and 555-conjugated anti-mouse IgG (Cell Signaling Technology) and Alexa Fluor 647-conjugated anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. LDs were stained with 5 μg/ml BODIPY 493/503 (Molecular Probes). Nuclei were stained with Hoechst 33258 (Wako). Cells were observed with a confocal microscope (TCS SP1, Leica).

**Immunoprecipitation-Real-time PCR**—Immunoprecipitation-real-time PCR was performed as described elsewhere (24), with minor modifications. Cells were washed with PBS and incubated in hypotonic buffer (10 mM HEPS (pH 7.6), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM PMSF) for 5 min on ice. Nonidet P-40 was added to a final concentration of 1%. Lysates were incubated for 10 min on ice and cleared by centrifuging at 4000 x g for 15 min. Glycerol was added to a final concentration of 5%. Lysates were precleared by incubation with protein A-Sepharose for 30 min at room temperature and then were incubated with anti-FLAG or anti-core monoclonal antibody for 1 h. Immunoprecipitation was performed using protein A-Sepharose. Immunoprecipitates were washed three times with wash buffer (10 mM Tris (pH 7.6), 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT). The immunoprecipitated core was eluted by incubating it with elution buffer (50 mM Tris (pH 8.0), 1% SDS, 10 mM EDTA) for 10 min at 65 °C. After incubation with 100 μg of proteinase K for 30 min at 37 °C, coprecipitated HCV RNA was extracted using TRIzol LS reagent (Life Technologies) and subjected to reverse transcription and real-time PCR.

**Statistical Analysis**—Data were analyzed by unpaired Student’s t test.

**Results**

**c-Abl Is Involved in HCV Particle Assembly**—Treatment with the Abl inhibitor imatinib prevents the spread of HCV infection in cell culture (36) but does not appear to affect virus entry (42). To determine which step in the HCV life cycle is blocked by treatment with imatinib, Huh-7.5 cells were infected with cell culture-grown HCV (HCVcc) of the J6/JFH1 chimeric genome (37, 40) and incubated in the presence or absence of imatinib. Extracellular and intracellular virus particles were harvested and quantified by a focus-forming assay. Imatinib treatment markedly reduced both the extracellular and intracellular infectivity (Fig. 1, A and B), whereas it only marginally reduced the intracellular HCV RNA levels (Fig. 1C) and core and NS5A expression (Fig. 1D). Imatinib treatment did not significantly affect cell viability (Fig. 1E), consistent with a previous study (36). These results indicate that imatinib treatment reduces HCV particle assembly with a minimal effect on viral RNA translation and replication.

Imatinib inhibits c-Abl, Arg, and several other tyrosine kinases (31, 43). To examine whether c-Abl or Arg is required for HCV particle assembly, Huh-7.5 cells stably expressing an shRNA against c-Abl or Arg, or both were established and infected with HCVcc. Knockdown of c-Abl and Arg was confirmed by immunoblotting (Fig. 2A). Similar to treatment with imatinib, knockdown of c-Abl reduced extracellular and intracellular infectivity (Fig. 2, B and C) but did not significantly affect intracellular HCV RNA levels (Fig. 2D) or core expression (Fig. 2E). These results indicate that c-Abl is involved in HCV particle assembly. In contrast, the knockdown of Arg had no significant effect on extracellular or intracellular infectivity (Fig. 2, B and C). However, because Arg mRNA levels were more than 10 times lower than c-Abl mRNA levels in Huh-7.5

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from the lysates of HCVcc-infected Huh-7.5 cells, and its tyrosine phosphorylation was evaluated by immunoblotting. The p58 form of NS5A was preferentially tyrosine-phosphorylated (Fig. 3A). This phosphorylation was reduced by knockdown of c-Abl (Fig. 3A). In addition, NS5A of the Con1 isolate was tyrosine-phosphorylated in COS-7 cells when coexpressed with WT but not kinase-dead c-Abl (Fig. 3B, KD). These results suggest that c-Abl is involved in the tyrosine phosphorylation of NS5A.

To identify a putative phosphorylation site for c-Abl, tyrosine residues conserved between the Con1 (genotype 1b) and JFH1 (genotype 2a) isolates were mutated to phenylalanine in Con1 NS5A. Of the eight mutations, the Y334F mutation significantly reduced NS5A tyrosine phosphorylation in COS-7 cells (Fig. 3, C and D). This residue is at the carboxyl terminus of domain II and corresponds to Tyr<sup>330</sup> of JFH1 NS5A (Fig. 3E). c-Abl phosphorylated the NS5A domain II (JFH1) in vitro (Fig. 3F). This phosphorylation was reduced by the Y330F mutation (Fig. 3F). These results suggest that c-Abl phosphorylates NS5A at Tyr<sup>330</sup>.

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**FIGURE 1. Treatment with imatinib reduces HCV particle assembly.** A–D, Huh-7.5 cells were infected with HCVcc (J6/JFH1) and treated with dimethyl sulfoxide (DMSO, vehicle) or imatinib (10 μM) for 72 h. A, extracellular and intracellular virus particles were harvested and used to infect naive Huh-7.5 cells. Cells were stained for core (green) and nuclei (blue). Scale bar, 200 μm. B, extracellular and intracellular infectivity (focus-forming units/ml) was quantified by real-time PCR and normalized to control values. Data represent the mean ± S.D. (n = 3), *p < 0.05. D, the expression levels of core and NS5A were evaluated by immunoblotting (IB). The intensity ratio of core or NS5A to GAPDH was calculated from blots and normalized to the control value. E, Huh-7.5 cells were treated with dimethyl sulfoxide or imatinib (10 μM) for 72 h. Cell viability was assessed by trypan blue assay. Data represent the mean ± S.D. (n = 3). NS, not significant.

**FIGURE 2. Knockdown of c-Abl reduces HCV particle assembly.** A–E, Huh-7.5 cells were infected with a control or c-Abl shRNA-encoding retrovirus together with a control or Arg shRNA-encoding retrovirus. A, the expression levels of c-Abl and Arg were evaluated by immunoblotting (IB). B–E, cells were infected with HCVcc (J6/JFH1) and incubated for 48 h. B, extracellular and intracellular virus particles were harvested and used to infect naive Huh-7.5 cells. Cells were stained for core (green) and nuclei (blue). Scale bar, 200 μm. C, extracellular and intracellular infectivity (focus-forming units/ml) was quantified by counting core-positive foci and normalized to control values. Data represent the mean ± S.D. (n = 3). *, p < 0.05. D, the expression levels of core and NS5A were evaluated by immunoblotting (IB). The intensity ratio of core or NS5A to GAPDH was calculated from blots and normalized to the control value. E, the same mutation did not significantly affect the replication of the JFH1 subgenomic replicon (SGR-JFH1) (Fig. 4A). The Y330F mutation reduced this phosphorylation (Fig. 4A) as well as the extracellular and intracellular infectivity (Fig. 4, B and C). Although the Y330F mutation also slightly reduced intracellular HCV (J6/JFH1) RNA levels (Fig. 4D) and NS5A expression (Fig. 4, A and E), the same mutation did not significantly affect the replication of the JFH1 subgenomic replicon (SGR-JFH1) (Fig. 4, F and G). These results suggest that Tyr<sup>330</sup> of NS5A is required for efficient HCV particle assembly.

**c-Abl Phosphorylates NS5A at Tyr<sup>330</sup>**—We have previously reported that the SH3 domain of c-Abl binds to NS5A in vitro and that overexpressed NS5A is tyrosine-phosphorylated in response to pervanadate treatment (26). We therefore investigated the possibility that c-Abl promotes HCV particle assembly by phosphorylating NS5A. NS5A was immunoprecipitiated cells (Fig. 2F), the possibility cannot be excluded that Arg is also involved in HCV particle assembly.

**c-Abl Is Dispensable for the Binding of Core to Viral RNA**—NS5A has been proposed to transport viral RNA from replication complexes to the LD-resident core, thereby promoting nucleocapsid formation (6, 7). We examined whether tyrosine phosphorylation by c-Abl regulates the subcellular localization...
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Tyrosine Tyr330 of NS5A (Tyr2306 in polyprotein numbering) is conserved among all genotypes of HCV (13) and conforms to the preferred c-Abl substrate sequence YXXP (44). We identified this tyrosine residue as a putative phosphorylation site for c-Abl by coexpressing NS5A mutants with c-Abl in COS-7 cells and in vitro kinase assay. Although these systems do not fully represent the situation for NS5A in HCV-infected cells, our results using J6/JFH1 also indicate that NS5A tyrosine phosphorylation is dependent, at least in part, on Tyr330. Tyr330 of NS5A is therefore likely to be a direct target of c-Abl in HCV-infected cells. The SH2 domains of Crk and Nck are known to recognize phosphorylated YXXP sequences (45) but do not appear to bind to NS5A in HCV-infected cells (data not shown).

Recent mass spectrometric analyses of NS5A identified multiple serine and threonine but not tyrosine phosphorylation sites (21, 22). Given that NS5A was purified from subgenomic replicon-harboring cells in these analyses, it is conceivable that the phosphorylation of NS5A at Tyr330 may occur only in virus-particle-producing cells.

We showed that the Y330F mutation did not significantly affect SGR-JFH1 replication (Fig. 4, F and G). In contrast, a previous study showed that the Y330A mutation abolishes SGR-JFH1 replication (13). This difference may indicate that although NS5A Tyr330 phosphorylation is dispensable for RNA replication, the structure of the carboxyl terminus of domain II influences the efficiency of RNA replication.

HCV exploits various host kinases to complete its life cycle (46). For example, HCV RNA replication requires c-Src activity (47). We showed that imatinib treatment reduced intracellular viral RNA levels by ~20% (Fig. 1C). This may be because imatinib weakly inhibits c-Src (48) or other kinases involved in HCV RNA replication.

The identification of c-Abl as a host factor for HCV particle assembly may have clinical implications. HCV has infected ~3% of the world’s population and is a major cause of liver cirrhosis and hepatocellular carcinoma (49). This has prompted the development of numerous anti-HCV agents that target viral proteins such as the NS3–4A protease and NS5A (50) or host proteins such as cyclophilin A (51). Imatinib and more potent Abl inhibitors are already in clinical use as targeted therapeutic agents for CML (31). Although the spread of HCV infection may not necessarily require particle assembly (52),
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**FIGURE 4.** The NS5A Y330F mutation reduces HCV particle assembly. A–E, Huh-7.5 cells were electroporated with HCV genomic RNAs encoding NS5A WT and Y330F (J6/JFH1 WT and Y330F, respectively) and incubated for 48 h. A, cells were lysed and subjected to immunoprecipitation (IP) with anti-NS5A antibody. The level of phosphorylated NS5A was evaluated by anti-phosphotyrosine (p-Tyr) immunoblotting (IB). The black arrowheads indicate the positions of NS5A. B, extracellular and intracellular virus particles were harvested and used to infect naive Huh-7.5 cells. Cells were stained for core (green) and nuclei (blue). Scale bar, 200 μm. C, extracellular and intracellular infectivity (focus-forming units/ml) was quantified by counting core-positive foci and normalized to control values. Data represent the mean ± S.D. (n = 3). *, p < 0.01. D, intracellular HCV RNA levels were quantified by real-time PCR and normalized to control values. Data represent the mean ± S.D. (n = 3). *, p < 0.01. E, the level of NS5A expression was evaluated by immunoblotting. F and G, Huh-7.5 cells were electroporated with HCV subgenomic replicon RNAs encoding NS5A WT and Y330F (SGR-JFH1 WT and Y330F, respectively) and incubated for 48 h. F, intracellular HCV RNA levels were quantified as described in D. NS, not significant. G, the level of NS5A expression was evaluated by immunoblotting.

**FIGURE 5.** Knockdown of c-Abl does not affect the association of NS5A with LDs. A and B, Huh-7.5 cells were infected with a control or c-Abl shRNA-encoding retrovirus. Cells were infected with HCVcc (J6/JFH1) and incubated for 48 h. A, cells were stained for NS5A (red), LDs (green), and nuclei (blue). The boxed regions are shown enlarged in the right panels. Scale bar, 10 μm. B, the percentage of LDs associated with NS5A in each cell was calculated. Data represent the mean ± S.D. Fifteen cells were analyzed. NS, not significant. C and D, Huh-7.5 cells were electroporated with HCV genomic RNAs encoding NS5A WT and Y330F (J6/JFH1 WT and Y330F, respectively) and incubated for 48 h. The subcellular localization of NS5A was analyzed as described in A and B.
our results suggest the potential of Abl inhibitors as therapeutic agents for HCV infection.

c-Abl can contribute to liver tumorigenesis when activated by oncogenic receptor tyrosine kinases (53). However, it is unknown whether c-Abl is activated in HCV-induced hepatocellular carcinoma.

Serine/threonine phosphorylation of the NS5A/NS5 proteins has been found not only in HCV but also in other members of the Flaviviridae family such as bovine viral diarrhea virus, yellow fever virus (54), and dengue virus (55). In addition, several members of the Flaviviridae family require Src family kinases for the assembly and maturation of the virus particles (56, 57). It would be interesting to determine whether tyrosine phosphorylation of the NS5A/NS5 proteins is a conserved feature of the Flaviviridae family.

Author Contributions—S. Y. conceived, designed, and performed the experiments, analyzed the data, and wrote the paper. K. T. performed the experiments and analyzed the data. K. C., C. H., and H. Y. analyzed the data. X. S. and H. H. contributed reagents/materials/analysis tools. K. S. conceived and designed the experiments and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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