Protein Kinase C-related Kinase 2 Regulates Hepatitis C Virus RNA Polymerase Function by Phosphorylation*

Seong-Jun Kim‡, Jung-Hee Kim‡, Yeon-Gu Kim, Ho-Soo Lim, and Jong-Won Oh§

From the Department of Biotechnology, Yonsei University, Seoul 120-749, Korea

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The hepatitis C virus (HCV) NS5B protein is the viral RNA-dependent RNA polymerase required for replication of the HCV RNA genome. We have identified a peptide that most closely resembles a short region of the protein kinase C-related kinase 2 (PRK2) by screening of a random 12-mer peptide library displayed on the surface of the M13 bacteriophage with NS5B proteins immobilized on microwell plates. Competitive phage enzyme-linked immunosorbent assay with a synthetic peptide showed that the phage clone displaying this peptide could bind HCV RNA polymerase with a high affinity. Coimmunoprecipitation and colocalization studies demonstrated in vivo interaction of NS5B with PRK2. In vitro kinase assays demonstrated that PRK2 specifically phosphorylates NS5B by interaction with the N-terminal finger domain of NS5B (amino acids 1–187). Consistent with the in vitro NS5B-phosphorylating activity of PRK2, we detected the phosphorylated form of NS5B by metabolic cell labeling. Furthermore, HCV NS5B immunoprecipitated from HCV subgenomic replicon cells was specifically recognized by an antiphosphoserine antibody. Knock-down of the endogenous PRK2 expression using a PRK2-specific small interfering RNA inhibited HCV RNA replication. In contrast, PRK2 overexpression, which was accompanied by an increase of in the level of its active form, dramatically enhanced HCV RNA replication. Altogether, our results indicate that HCV RNA replication is regulated by NS5B phosphorylation by PRK2.

The hepatitis C virus (HCV) is a major cause of non-A and non-B hepatitis, leading to liver cirrhosis and hepatocellular carcinoma (1, 2). HCV is an enveloped virus with a positive stranded RNA genome of ~9.6 kb belonging to the Flaviviridae family (3). The HCV viral genome encodes a single polyprotein of ~3,010 amino acids, which is proteolytically processed by a combination of host and viral proteases into at least 10 distinct structural and nonstructural proteins. The structural proteins include C, E1, E2, and p7, and the nonstructural (NS) proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B (4, 5). Among the nonstructural proteins, HCV NS5B is an RNA-dependent RNA polymerase (RdRp) that is important for replication of the HCV RNA genome (6–8). This protein contains motifs shared by all RdRps and possesses the finger, palm, and thumb subdomains (9–12). HCV NS5B is anchored to the endoplasmic reticulum through the C-terminal domain of 21 hydrophobic amino acids (13–15) and forms a putative HCV RNA replicase complex with other viral NS proteins (16–19).

Many cellular enzymes involved in DNA and RNA metabolism, such as DNA polymerase α, topoisomerase IIα, and DNA-dependent RNA polymerase I and II, are phosphoproteins, and their functions are known to be regulated by cellular kinase-mediated phosphorylation (20–26). Several viral RdRps are also modified by phosphorylation. Dengue virus type-2 RNA polymerase is phosphorylated at a serine residue by casein kinase II. Phosphorylation of this polymerase regulates interaction with other viral proteins and the function of viral RNA replicase (27, 28). Yellow fever virus NS5 is also phosphorylated by serine/threonine protein kinases (29). The RdRp of turnip yellow mosaic virus is phosphorylated in a PEST region residue, and the phosphorylation of the PEST-rich sequences in turnip yellow mosaic virus RdRp may be involved in selective processing by the ubiquitin/proteasome degradation system (30). Recently, phosphorylation of the N-terminal 126-amino acid region of cucumber mosaic virus RNA polymerase 2a protein by a 60-kDa tobacco plant origin protein kinase was demonstrated to inhibit interaction of 2a RNA polymerase with the 1a protein, which is essential for replication of cucumber mosaic virus (31). The phosphorylated form of HCV NS5B was observed when expressed in insect cells using a recombinant baculovirus (32). However, phosphorylation of HCV NS5B in human liver cells has not been demonstrated, and the cellular kinase that phosphorylates HCV NS5B has not been yet identified.

In this work, we identified one peptide with amino acid sequences homologous to protein kinase C-related kinase 2 (PRK2) by screening a phage-displayed 12-mer random peptide library with HCV NS5B protein as bait. We characterized the interaction of NS5B and PRK2 both in vitro and in vivo and demonstrated that PRK2 is the specific cellular kinase phosphorylating HCV NS5B. Furthermore, we show that phosphorylation of NS5B is involved in the regulation of HCV RNA replication in an HCV subgenomic replicon cell line.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and a Random Peptide Library—Plasmid pThNS5B used for expression of full-length HCV NS5B in Escherichia coli has been described previously (7). Sequences corresponding to the finger-palm domain (amino acids 1–371), the palm-thumb domain (amino acids 188–591), and the thumb domain (amino acids 372–591) of

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HCV NS5B was amplified by PCR using pThNS5B as a template and cloned into the NheI-EcoRI site of pET-28a(+) (Novagen) to obtain pET-28a (+)/NS5B(1–371), pET-28a (+)/NS5B(188–591), and pET-28a (+)/NS5B(372–591), respectively. The Nhel and EcoRI sites were included at the N and the C termini of oligonucleotides, respectively, to facilitate cloning. Stop codons were introduced at the end of each of the cloned sequences from 5′ to the EcoRI site.

pcDNA4.0-HCVNs was constructed to obtain a Tet-ON inducible system for Huh7 human hepatoma cells expressing the HCV proteins NS3 to NS5B. The HCV nonstructural gene for NS3 to NS5B was amplified by PCR with the region-specific primers HCV-NS3 to NS5B. The HCV nonstructural gene for NS3 to NS5B was amplified by PCR using pThNS5B as a template and cloned into the NheI-EcoRI site of pET-28a(+) (Kangnung National University, Korea) and used for competitive phage ELISA. Each well of a Ni-NTA HisSorb plate (Qiagen) was coated with 10 ng of phosphate-buffered saline (PBS) and the phosphorylated form of PRK2 were determined by immunoblotting with anti-PRK2 (clone PSR-45; Zymed Laboratories) antibodies. For the sake of clarity, the phosphorylated forms of PRK2 were indicated with an asterisk (*).

Establishment of a Tetracycline-inducible Stable Cell Line Expressing HCV Nonstructural Proteins (NS3–5B)—The human hepatoma stable cell line Huh7TR expressing the tetracycline repressor was obtained by transfection of pcDNA6/TR (Invitrogen) carrying a gene encoding the selectable marker blasticidin and a gene coding for the tet operon repressor protein (TetR) and by selection of stably transfected cell lines resistant to 0.1 μg/ml of blasticidin S (Invitrogen). Clonal colonies expanded under blasticidin selection was transfected with either pcDNA4.0-HCVNs (for the Huh7TR-NS cell line) or the pcDNA4.0/T0/myc-hisB vector alone (for the Huh7TR-4 cell line). Cell lines transfected with each plasmid were selected by using 100 ng/ml Zeocin (Invitrogen) and 10 μg/ml blasticidin S for 3 weeks. Stable Huh7TR-NS and Huh7TR-4 cell lines were maintained under the selection conditions (5% CO2, 37 °C).

Establishment of an HCV Replicon Cell Line—An HCV subgenomic replicon, pRSZ2 (36), derived from the parental HCV Con-1 replicon L77–NS3–3′ (AJ242652) was kindly provided by Dr. Christoph Seeger (Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia). pRSZ2 linearized with Scal was used for in vitro transcription as described previously (7). An HCV subgenomic replicon expressing NS5A and the eukaryotic marker neomycin phosphotransferase (NPTII), HCV RNA, R-1, was established by transfection of in vitro transcribed HCV subgenomic RNA using a Gene Pulser system (Bio-Rad) followed by selection with 1 mg/ml G418 as described previously (37, 38).

Commonnucprecipitation and Immunoblotting—For the PKR2-NS5B commonnucprecipitation, Huh7/TR-NS or Huh7 R-1 cells grown in a 10-cm plate were suspended in 0.1 ml of lysis buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM glycerol, 10 mM tetradsodium pyrophosphate, 100 mM NaF, 17.5 mM Mg-glycophosphate) supplemented with an EDTA-free protease inhibitor mixture (Roche Applied Science). The resuspended cells were incubated for 10 min on ice and clarified by centrifugation at 21,000 × g for 20 min at 4 °C. PKR2 was immunoprecipitated with anti-PKR2 antibodies (Cell Signaling Technology). Immunoblottings were performed using anti-PKR2, anti-NS5B (Santa Cruz Biotechnology), or anti-NS5A antibodies. For immunoprecipitation of the phosphorylated form of NS5B, Huh7 or HCV R-1 subgenomic replicon cells grown in a 3 × 10-cm plate were either untransfected or transfected with pSUPER-PKR2 or pcDNA3.1-PKR2 using FuGENE 6 (Roche Applied Science) before lysis and immunoprecipitation with anti-phosphoserine (clone PSR-45; Zymed Laboratories) antibodies. Immunoblottings were performed with anti-NS5B (Santa Cruz Biotechnology), anti-phospho-tyrosine (clones PY-7E1 and PY20; Zymed Laboratories) antibodies. Translation levels of PKR2 and the phosphorylated form of PKR2 were determined by immunoblotting with anti-PKR2 or anti-phospho-PKR2 antibodies (Cell Signaling Technology).
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**Immunoprecipitation and in Vitro Kinase Assays**—For the PRK2-NS5B communoprecipitation, HEK 293T or Huh7 (for transient expression by transfection of pFLAG-PRK2) cells grown in a 10-cm plate were harvested, washed with cold PBS, and suspended in 0.5 M of lysis buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>) supplemented with an EDTA-free protease inhibitor mixture. The resuspended cells were stored for 10 min on ice and clarified by centrifugation at 21,000 × g for 20 min at 4 °C to obtain cell lysates. Proteins in the cell lysates were immunoprecipitated with polyclonal anti-PRK2 antibodies, polyclonal antibodies against a consensus sequence present in the three PKC isoforms (α, β, γ) (anti-PKC; Zymed Laboratories), or rabbit anti-FLAG polyclonal antibodies (Sigma) for 1 h at 4 °C in binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5% Triton X-100, 100 mM NaCl) containing an EDTA-free protease inhibitor mixture (Roche Applied Science). The immunocomplexes from HEK 293T cells were washed three times with binding buffer, subjected to SDS-PAGE (8% gel), and analyzed by immunoblotting with anti-PRK2, anti-PKC, or anti-pana-His antibodies. For the *in vitro* PRK2 kinase assay, the PRK2-immunocomplexes were washed three times with binding buffer and once with ice-cold 1 × kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM dithiothreitol). An *in vitro* kinase reaction was performed in 20 μl of kinase buffer containing 0.2 μg of full-length HCV NS5B deletion derivatives, 100 μM ATP, and 10 μCi of γ-<sup>32</sup>P-ATP (Amersham Biosciences) for 30 min at 30 °C. When indicated, *in vitro* kinase reactions were performed in the presence of various kinase inhibitors at IC<sub>50</sub> (39, 40). The inhibitors were 20 μM HA1077 (PRK2 inhibitor; Upstate Biotechnology, Inc.), 50 μM LY294002 (PI3-kinase inhibitor; Calbiochem), 1 μM wortmannin (PI3-kinase inhibitor; Sigma), 10 μM SB203580 (p38 mitogen-activated protein kinase inhibitor; Calbiochem), 50 μM PD98059 (extracellular signal-regulated kinase-1/2 inhibitor; Calbiochem), and 10 μM GF109203X (PKC inhibitor, Calbiochem). Reactions were stopped by the addition of 6 × SDS sample buffer, followed by heating at 95 °C. The supernatants containing phosphorylated proteins were pooled by centrifugation, subjected to SDS-PAGE (8% gel), and analyzed by autoradiography.

**Cell Labeling and PRK2 Knock-down by siRNA**—Huh7TR-4 and Huh7TR-NS stable cell lines cultured in 10-cm-diameter plates were preincubated for 30 min in phosphate-free Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% dialyzed fetal bovine serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were then labeled with 80 μCi of [γ-<sup>32</sup>P]-ATP (Amersham Biosciences, carrier-free, 10 μCi/ml/ml) of culture medium for 4 h. Cells were washed three times with PBS and lysed in 1 ml of cold lysis buffer C (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM tetrasodium pyrophosphate, 17.5 mM β-glycerophosphate, 1% Triton X-100) containing an EDTA-free protease inhibitor mixture by incubation on ice for 10 min. Cleared cell lysates were immunoprecipitated with a monoclonal anti-NS5B antibody, washed three times with lysis buffer using SDS-PAGE (8% gel), and analyzed by autoradiography. To test the effect of PRK2 knock-down on NS5B phosphorylation, Huh7TR-NS stable cell lines seeded in a 10-cm-diameter plate were induced with 1 μg/ml tetracycline and transfected with 5 μg of either pSUPER or pSUPER-PRK2 using FuGENE 6. After further growth for 48 h, cell labeling with [γ-<sup>32</sup>P]-ATP was performed as described above. Expression levels of PRK2 were determined by immunoblotting with anti-PRK2 antibodies.

**Immunofluorescence Staining**—Huh7TR-NS cells were cultured in 8-well chamber slides (Nunc) to ~60% confluence. At 48 h postinduction with 1 μg/ml tetracycline, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at -20 °C, and permeabilized with PBS containing 0.2% Triton X-100 for 30 min at room temperature. After washing five times with PBS, the cells were then treated with a blocking solution (PBS containing 1% BSA, 0.1% gelatin, and 5% goat serum) for 30 min at room temperature, incubated with rabbit anti-PRK2 antibodies and a monoclonal anti-NS5B antibody overnight at 4 °C, and washed five times with PBS containing 1% BSA and 0.1% gelatin. The cells were incubated further with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) (Vector Laboratory) or rhodamine-conjugated horseshoe anti-mouse IgG (Jackson Immuno Research Laboratories) antibodies for 2 h and washed five times with PBS. Nuclei were visualized using 1 μM 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Confocal images were obtained with a Bio-Rad Radiance 2000 multiphoton laser scanning confocal microscope.

**TaqMan Real Time Quantitative Reverse Transcription-PCR**—R1 cells grown to 80% confluence were transfected with pSUPER-PRK2 or pcDNA3-PRK2 (41) (kindly provided by Dr. Vincent L. Cryns, Northwestern University) using FuGENE 6 reagent. After 48 h, total RNA was extracted with TRIzol reagent (Invitrogen) and purified according to the manufacturer’s recommendations. HCV replicon RNA levels were quantified with the ABI PRISM 7700 sequence detection system. Triplicate RNA samples (1 μg each) were amplified with the TaqMan EZ reverse transcription-PCR kit (Applied Biosystems). The primer and probe sequences specific for the HCV 5′-untranslated region (UTR) were as follows: sense primer 5′-UUT-R, 5′-GCCGCTCAACGATCCG- TAGTATGGATGTC-3′; antisense primer 5′-UUT-R, 5′-ACCAAGGCCCTTTGGGAGCCGACTCAGCTACAT-5′; and a dual fluorophore-labeled probe, 5′-BHQ1 (6-carboxyfluorescein)-CTCGGAAACCGGTGACTACA- C-TAMRA (6-carboxytetramethylrhodamine)-3′. The target was reverse transcribed with recombinant T7 RNA polymerase at 50 °C for 2 min and 65 °C for 10 min followed by 40 cycles of amplification at 95 °C for 20 s and 62 °C for 1 min. Cellular glyceraldehyde-3-phosphate dehydrogenase mRNA from the same RNA extract was used as an internal control. RNA standards (HCV 5′-UTR), run in triplicate in every reaction, were prepared by *in vitro* transcription with T7 RNA polymerase and purified by electrophoresis on a 5% polyacrylamide gel containing 8% urea as described previously (35).

**RESULTS**

**Identification of PRK2 as a Protein Interacting with HCV NS5B**—To search for peptides interacting with HCV NS5B, the viral RdRp, phage-displayed 12-mer random peptide library was screened with the N-terminal His<sub>6</sub>-tagged NS5B protein that was immobilized on Ni<sup>2+</sup>-coupled microwell plates. The phages were engineered to display up to five copies of a random peptide on their surfaces by fusing at the N terminus of the gene III protein of the M13 phage (42, 43). The library had a diversity of 1.5 × 10<sup>6</sup> independent transformants. Sequencing the DNA in the random peptide regions of 20 randomly selected phages revealed diversity of library (data not shown). Full-length HCV RNA polymerase was purified to near homogeneity by three successive column chromatographies as described previously (35). For biopanning, 800 ng of NS5B, which saturated each 96-well of the microtiter plates under our binding conditions, was immobilized, and screening was performed in the presence of salt, detergent, and a blocking agent such as BSA or skimmed milk to prevent nonspecific binding of phages. As a control, the N-terminal His<sub>6</sub>-tagged core protein, an HCV structural protein, was used to confirm that no phages nonspecifically binding to the Ni-NTA HisSorb plate or the His<sub>6</sub> tags of the recombinant proteins were enriched during three rounds of biopanning. After three rounds of biopanning of two independent screenings, a total of 80 phage clones that selectively bound to HCV NS5B at the peptide fused to the M13 phage gene III protein were randomly isolated, and the DNA sequences in their random peptide regions were analyzed. Among the 80 phages, one clone was isolated with the highest frequency of 29/80 from two independent screenings. The amino acid sequence deduced from the DNA sequence coding for a short peptide, named the C1 peptide, was N-TSTAGRIVRRAI-C. All 29 clones had identical nucleotide sequences, indicating that they originated from a single enriched phage. A total of 20 phage clones isolated using the HCV core protein as bait exhibited peptide sequences not related to the sequences screened with NS5B (the peptide sequences selected with the HCV core protein will be reported elsewhere), indicating that no nonspecifically bound phage clones were enriched by random peptide display screening.

To confirm the binding specificity of the selected phage, we performed a competitive phage ELISA using the synthetic HCV NS5B-binding peptide SC1. The relative ability of the free synthetic SC1 peptide to inhibit interaction of NS5B with the selected phage was determined. The free peptide inhibited binding of the selected phage to HCV NS5B in a dose-dependent manner (Fig. 1B), indicating that phage C1 and the synthetic peptide compete for binding to NS5B. Because M13 phages display several copies of the peptide on their surfaces,
the exact dissociation constant cannot be determined. However, 40 nm SC1 completely blocked the binding of phage C1 to NS5B, indicating a high affinity PRK2-NS5B binding through this peptide interface.

**In Vivo Interaction of PRK2 with HCV NS5B**—Because the C1 peptide was selectively enriched with HCV NS5B from two independent screenings with a high efficiency and bound to NS5B with a high affinity, we analyzed this interaction in detail. We searched the sequence data base with the C1 peptide sequence to identify potential NS5B-binding cellular proteins. We found that PRK2 contains the sequence N-GRLVRRAI-C (7/8 of amino acids were identical to the sequence of the peptide selected) in the 518–525 amino acid region of PRK2 (GenBank accession number NM_006256) (Fig. 1A). We then wanted to determine whether endogenous PRK2 is able to interact with NS5B in the presence of the HCV viral nonstructural proteins NS3–NS5B, which are known to interact with NS5B either directly or indirectly (16–18). To this end, we established a tetracycline-inducible human hepatoma cell line Huh7TR-NS expressing HCV nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B. After 48 h postinduction of Huh7TR-NS cells with tetracycline, expression of HCV nonstructural proteins was verified by immunoblotting analyses for NS3 (68 kDa), NS4A (56 kDa), and NS5B (66 kDa) with appropriate specific monoclonal antibodies (Fig. 2A). When endogenous PRK2 was immunoprecipitated from Huh7TR-NS cell lysates with anti-PRK2 antibodies, HCV NS5B protein coimmunoprecipitated with the PRK2 was detected (Fig. 2B), indicating that endogenous PRK2 is able to bind HCV NS5B in vivo. A similar experiment using an HCV subgenomic replicon cell line R-1 also demonstrated the interaction of PRK2 with NS5B, and no interaction with NS5A that is known as a phosphoprotein (44) was observed (Fig. 2C, compare middle and bottom panels).

To demonstrate further the interaction between NS5B and PRK2 in vivo, colocalization of these proteins was examined in Huh7TR-NS. Immunofluorescent staining of PRK2 exhibited a distinct cytoplasmic and perinuclear localization (Fig. 3A). Previous reports have demonstrated that HCV NS5B mainly resides on the endoplasmic reticulum around perinuclear regions with other nonstructural proteins (14, 45). We verified that NS5B localizes predominantly in speckle-like structures around perinuclear region (Fig. 3B). There was an extensive but not complete colocalization of PRK2 and NS5B in all cells expressing HCV nonstructural proteins in several independent experiments (Fig. 3C).

**Phosphorylation of HCV NS5B by PRK2 in Vitro**—The phosphorylated form of HCV NS5B has been observed when expressed in insect cells using a recombinant baculovirus (32), but the cellular kinase responsible for the phosphorylation of NS5B has not been identified. Having demonstrated that PRK2 interacts with NS5B both in vitro and in vivo, we tested whether PRK2 phosphorylates NS5B in vitro because PRK2 has a Ser/Thr protein kinase activity (46). First, we confirmed the specific interaction of NS5B with PRK2 but not with PKC isozymes PKC-α, -β, and -γ (47) using a coimmunoprecipitation assay. Even though PKC and PRK2 have 87% amino acid sequence identity in the kinase domain and 48% identity in the N-terminal regulatory region (48), endogenous PRK2, but not the conventional PKC isoforms PKC-α, -β, and -γ, could pull down the His6-tagged full-length NS5B protein added to HEK 293T cell lysates (Fig. 4B, third panel, compare lanes 3 and 6). Immunoblotting analysis with an anti-NS5B antibody indicated a constant amount of NS5B used in the analyses (bottom panel).

We next performed an in vitro kinase assay to determine whether PRK2 can phosphorylate NS5B. Endogenous PRK2 in HEK 293T cells was immunoprecipitated with anti-PRK2 antibodies and reacted with purified recombinant full-length NS5B that was expressed in E. coli. 32P-Labeled proteins in the immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography. NS5B was phosphorylated successfully by immunoprecipitated PRK2 (Fig. 5A, lane 2), and no autophosphorylation activity of NS5B was observed (lane 3). In contrast, no NS5B protein was phosphorylated by cPKC (Fig. 5B, lane 2), even though cPKC did undergo autophosphorylation under the same conditions (lanes 1 and 2). These results indicate that inability of cPKC (Fig. 4B) to bind NS5B led no phosphorylation of NS5B, even though PRK2 and cPKCs have a similar kinase domain. Lack of the N-GRLVRRAI-C homologous region in all of the 11 known PKC isoforms (Fig. 4A) probably accounts for this specificity.

To demonstrate further that NS5B phosphorylation is mediated by PRK2, we performed in vitro kinase assays in the presence of various protein kinase inhibitors as a final concentration by which each kinase inhibitor was known to inhibit the corresponding kinase activity by 50% (39, 40). When the assays were performed with either exogenous (Fig. 6A) or endogenous (Fig. 6B) PRK2, autophosphorylation of immunoprecipitated PRK2 was significantly inhibited only by a PRK2-specific inhibitor HA1077 with a concomitant inhibition of NS5B phos-
phorylation. Other kinase inhibitors including a PKC-specific inhibitor GF109203X had no significant inhibitory effect. These results clearly indicate that phosphorylation of NS5B is mediated specifically by PRK2 kinase activity.

The Finger Domain of Amino Acids 1–187 of NS5B Is Phosphorylated by PRK2—To determine the NS5B domain interacting with and phosphorylated by PRK2, three truncated forms of NS5B were prepared (Fig. 7A). Because amino acids 188–371 of NS5B form a finger domain along with the N-terminal 187 amino acids, as determined from the three-dimensional structure of NS5B (9–12), this middle part of NS5B (amino acids 188–371) was not expressed separately to avoid NS5B structural alteration. Preparation of the N-terminal finger region (amino acids 1–187) was not successful because the protein expression level and the purification yield were low under various experimental conditions. Using the NS5B derivatives shown in Fig. 7A, we first mapped the PRK2 binding site on NS5B by determining which NS5B derivatives can be coimmunoprecipitated with endogenous PRK2 in HEK 293T cell lysates using anti-PRK2 antibodies. NS5B(1–371) was coimmunoprecipitated with PRK2 (Fig. 7B, lane 2), but NS5B(188–371) and NS5B(372–591) were not (lanes 1 and 3), indicating NS5B by determining which NS5B derivatives can be coimmunoprecipitated with endogenous PRK2 in HEK 293T cell lysates using anti-PRK2 antibodies. NS5B(1–371) was coimmunoprecipitated with PRK2 (Fig. 7B, lane 2), but NS5B(188–371) and NS5B(372–591) were not (lanes 1 and 3), indicating NS5B by determining which NS5B derivatives can be coimmunoprecipitated with endogenous PRK2 in HEK 293T cell lysates using anti-PRK2 antibodies. NS5B(1–371) was coimmunoprecipitated with PRK2 (Fig. 7B, lane 2), but NS5B(188–371) and NS5B(372–591) were not (lanes 1 and 3), indicating NS5B by determining which NS5B derivatives can be coimmunoprecipitated with endogenous PRK2 in HEK 293T cell lysates using anti-PRK2 antibodies. 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that binding of PRK2 is directed to the N-terminal finger domain (amino acids 1–187) of NS5B. In addition, in vitro PRK2 kinase assays with the three purified domains of NS5B used in the binding assay revealed that NS5B phosphorylation occurs in the N-terminal finger domain (Fig. 7C, lane 2).

In Vivo Phosphorylation of HCV NS5B by PRK2—We determined whether there is a phosphorylated form of NS5B in Huh7TR-NS cells. Huh7TR-NS cells were induced with tetracycline for 48 h and further grown in the presence of 32P for 4 h. Metabolically labeled HCV NS5B was immunoprecipitated with a monoclonal anti-NS5B antibody. The phosphorylated form NS5B was detected by SDS-PAGE followed by autoradiography (Fig. 8A, lane 2). In contrast, immunoprecipitation using lysates from Huh7TR-4 cells was negative (lane 1), demonstrating presence of the phosphorylated form of NS5B in human hepatoma cells.

To determine whether PRK2 phosphorylates NS5B in vivo, expression of the endogenous PRK2 gene was knocked down by transfection of the pSUPER-PRK2 vector directing the synthesis of the 19-bp double-stranded PRK2 target sequence by the polymerase III H1-RNA gene promoter (34). Transfection of the pSUPER-PRK2 vector, but not the expression vector itself, into Huh7TR-NS reduced the endogenous PRK2 level by 86%, based on quantification of the blot by densitometric scanning and normalization by using the respective tubulin protein bands (Fig. 8B, top panel). NS5B phosphorylation was inhibited by 84%, as determined from image analysis (Fig. 8C, compare lanes 2 and 3). Levels of NS5B in the total lysates were uniform (Fig. 8B, middle panel), demonstrating that reduction in the amount of the phosphorylated form of NS5B was not the result of a decreased NS5B level. These results indicate that suppression of PRK2 expression is sufficient to affect phosphorylation of NS5B significantly, and thus PRK2 is the cellular kinase phosphorylating NS5B, considering that the transfection efficiency of the GFP expression vector in Huh7TR-NS was ~60% by the transfection method used in this analysis.

Consistent with the result shown in Fig. 8A, we could detect the phosphorylated form of NS5B in the R-1 HCV replicon cells by immunoprecipitation using an anti-phosphoserine-specific antibody followed by immunoblotting with polyclonal anti-NS5B antibodies (Fig. 9A, lane 6). Anti-phosphothreonine and anti-phosphotyrosine antibodies did not immunoprecipitate the phosphorylated form of NS5B but successfully immunoprecipitated the activated form of Akt phosphorylated at Ser-473.
and Thr-308 (49) (bottom panel) and the activated form of PI3-kinase p85 phosphorylated at Tyr-508 (50) (middle panel), respectively. The phosphorylation level of NS5B was comparable with that of Akt and PI3-kinase p85, which are present in their active forms in the absence of particular extracellular stimuli. These results indicate that a significant amount of NS5B in the R-1 cells is indeed in a phosphorylated form and that serine residues are potential targets for PRK2-mediated phosphorylation. In R-1 cells transfected with pSUPER-PRK2, the serine-phosphorylated NS5B level decreased (Fig. 9B, compare lanes 5 and 6). In addition, the total NS5B level also decreased slightly (compare lanes 2 and 3), which is likely because of inhibition of HCV RNA replication by prevention of NS5B phosphorylation (Fig. 10A). In contrast, PRK2 overexpression appreciably augmented the NS5B phosphorylation (Fig. 9C, compare lanes 5 and 6). The substantial increase of NS5B level (compare lanes 2 and 3) is well correlated with the 5-fold increase in HCV replicon RNA level in the PRK2-overexpressing R-1 cells as shown in Fig. 10C. These results support the model that NS5B phosphorylation by PRK2 plays an important role in HCV RNA replication.

**Effect of Knock-down and Overexpression of PRK2 on HCV RNA Replication**—Having demonstrated that PRK2 specifically phosphorylates NS5B both in vitro and in vivo, we wanted to study whether PRK2 knock-down and overexpression in R-1 cells can regulate HCV subgenomic RNA replication. Introduction of pSUPER-PRK2 led to a significant decrease in PRK2 protein levels and a consequent reduction in the level of phosphorylated form of PRK2 (Fig. 10B, top and middle panels). Transfection of R-1 cells with 2 μg of pSUPER-PRK2 inhibited HCV subgenomic RNA replication by ~40%, and the inhibitory effect of PRK2 knock-down increased slightly with 6 μg of pSUPER-PRK2 (Fig. 10A). Furthermore, PRK2 overexpression with a concomitant increase of the phosphorylated form of PRK2 (Fig. 10D) enhanced HCV RNA replication in a dose-dependent manner (Fig. 10C). In the R-1 cells transfected with 6 μg of pcDNA3.1-PRK2, the HCV subgenomic RNA level increased by 5-fold. Our demonstration of a decrease and increase of HCV RNA replication in PRK2 knock-down and overexpression R-1 cells, respectively, underscores the physiological importance of NS5B phosphorylation in HCV RNA replication.

**DISCUSSION**

The HCV NS5B protein is the viral RdRp required for replication of the HCV genome (6–8). Even though NS5B is phosphorylated in insect cells (32), the cellular kinase responsible for its phosphorylation has not been yet identified. A random peptide phage display has been used to map protein-protein interactions (51, 52) and applied successfully to identification of cellular proteins interacting with the p85 subunit of PI3-kinase (53), transportin 1 (54), Erbin (55), and the SH3 domain of Eps8 (56). Using this phage display system, we identified an HCV NS5B-binding peptide that is homologous to the region of amino acids 518–525 of PRK2. We performed competitive phage ELISA to determine whether interaction between NS5B and the C1 phage selected by biopanning is inhibited by the phage-derived peptide SC1. The result shown in Fig. 1B indi-

![Fig. 7](image.png)

**Fig. 7. PRK2 binds and phosphorylates the N-terminal region of NS5B (amino acids 1–187).** A, schematic representation of full-length HCV NS5B and its derivatives. The indicated amino acid positions of NS5B were assigned to processed NS5B from the polyprotein, as predicted from the nucleotide sequence of the full-length HCV genotype 1b genome (33). The finger (black box), palm (white box), thumb (gray box), and C-terminal (hatched box) domains of NS5B are indicated. Numbers in parentheses indicate the number of amino acids in each NS5B derivative. B, coimmunoprecipitation was performed as in Fig. 4, using the indicated purified NS5B proteins. Immunoprecipitates (IP) were separated by SDS-PAGE (8% gel), transferred to a nitrocellulose membrane, and probed for PRK2 (B panel) or coimmunoprecipitated NS5B (middle panel) with the antibodies indicated at the right of each panel. The bottom panel shows equivalent amounts of NS5B added to the cell lysates in coimmunoprecipitation and in vitro kinase assays. IB, immunoblotted. C, immunoprecipitated PRK2 proteins were used for in vitro kinase assays, as in Fig. 5, using the indicated purified NS5B proteins. Arrowheads indicate the positions of NS5B(1-371) phosphorylated by the immunoprecipitated PRK2. Molecular mass standards are shown on the left.
cates a high affinity interaction between NS5B and PRK2. Recently, a similar approach has been used to screen peptides interacting with NS5B (57). Several 7-mer disulfide-constrained peptides containing a core motif of FPWG were found which inhibit the enzymatic activity of NS5B in a conformation-dependent manner. However, the 12-mer linear SC1 peptide identified in this work did not significantly inhibit the enzymatic function of NS5B, even with a 5-fold molar excess over NS5B added to an in vitro RdRp assay (15–20% inhibition with a poly(A)(U)_{20} primer substrate). This result suggests that the SC1 binding site does not overlap with the active site and that binding of SC1 neither alters conformation of NS5B nor blocks

\[ \text{S.-J. Kim, J.-H. Kim, Y.-G. Kim, H.-S. Lim, and J.-W. Oh, unpublished observation.} \]
The in vitro kinase assays revealed that NS5B is phosphorylated specifically by PRK2, but the related kinase cPKC could not phosphorylate NS5B (Fig. 5). This specificity can be explained by lack of a C1 peptide homologous region in cPKC and other isoforms of PKC (Fig. 4A). Specific phosphorylation of NS5B by PRK2 was demonstrated further by showing that the phosphorylation is inhibited only by HA1077, a specific PRK2 inhibitor, but not by other kinase inhibitors tested (Fig. 6). PRK2 is known to be activated in vitro by lipids, such as arachidonic acid, oleic acid, and other unsaturated acids (60, 61). However, we demonstrated that even a basal level of the active form of PRK2 in the immunoprecipitates from untreated cells is capable of phosphorylating NS5B efficiently. Phosphorylation of recombinant NS5B by PRK2 in vitro indicates that other viral and/or cellular proteins are not required to expose phosphorylation sites on NS5B by conformational change via interaction. PRK2 phosphorylated the N-terminal region of NS5B (amino acids 1–187) as shown in Fig. 7C. This finger domain of NS5B contains several possible phosphorylation sites predicted by the NetPhos 2.0 Prediction Program (www.cbs.dtu.dk/services/NetPhos/) (62), including serine residues at positions 46, 76, 84, 96, 99, and 112; threonine residues at 12, 41, 77, and 132; and tyrosine residues at 64 and 103. The three-dimensional structure of HCV NS5B protein (Protein Data Bank 1OS5) shows that all of these putative phosphorylation sites reside on the surface of the N-terminal NS5B finger domain, suggesting that these sites could be accessed by PRK2 for phosphorylation of the NS5B. Because the phosphorylated form of NS5B was only immunoprecipitated by an anti-phosphoserine-specific antibody but not by anti-phosphothreonine- and anti-phosphotyrosine-specific antibodies (Fig. 8B), phosphorylation may occur on the serine residue(s) described above. We are currently mapping the phosphorylation site(s) in NS5B to verify this prediction.

The in vitro PRK2-NS5B interaction was demonstrated both in Huh7TR-NS cells expressing HCV nonstructural proteins and in R-1 cells supporting HCV subgenomic RNA replication (Figs. 2 and 3). Consistent with an in vitro NS5B phosphorylation activity of PRK2, we detected the phosphorylated form of NS5B in Huh7TR-NS cells by metabolic cell labeling with 32P (Fig. 8A). More importantly, physiological relevance of the PRK2-NS5B interaction in vivo was demonstrated by showing that reduction of the PRK2 level by a PRK2-specific siRNA is correlated with suppression of NS5B phosphorylation (Fig. 8C). Because down-regulation of the endogenous PRK2 level significantly reduced NS5B phosphorylation, and a specific PRK2 inhibitor HA1077 strongly inhibited the phosphorylation of NS5B in vitro as shown in Fig. 6, we concluded that PRK2 is responsible for the phosphorylating NS5B. Furthermore, our data shown in Fig. 10 demonstrate a role of PRK2-mediated NS5B phosphorylation in HCV RNA replication. Introduction of a PRK2-specific siRNA into R-1 cells induced more than 50% silencing of PRK2 expression (Fig. 10, top panel) and decreased the level of PRK2 active form (middle panel) as evaluated by immunoblotting analyses and consequently suppressed HCV RNA replication (Fig. 10A). In addition, PRK2 overexpression significantly increased HCV RNA replication (Fig. 10C), indicating a positive effect of NS5B phosphorylation in HCV RNA replication.

PRK2 is an effector of the small GTPases Rho and Rac (46, 63). It binds the middle SH3 domain of Nck, the SH2-SH3 adaptor protein, and thus is predicted to be targeted to the activated receptor protein-tyrosine kinases via the SH2 domain of Nck (46). PRK2 is also known to be activated by 3’-phosphoinositide-dependent protein kinase-1 (64) and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-ni-2 (65). Therefore, HCV NS5B phosphorylation is likely to be regulated by multiple signaling pathways. Furthermore, PRK2 is known to be cleaved by caspase-3 during apoptosis (66), and HCV infection is associated with liver infiltration of cytotoxic T lymphocytes producing TNF-α (67, 68). For HCV-infected cells to continue RNA replication in a state of continuous exposure to death signals, we speculate that the infected cells may find a way that prevents inactivation of PRK2 by TNF-α-mediated cleavage. In this context, it is of particular interest that an HCV subgenomic replicon cell line R-1, and
even Huh7TR-NS cells, in which there is no HCV RNA replication, are resistant to TNF-α (data not shown). This result suggests that HCV nonstructural protein(s) is probably involved in conferring resistance to TNF-α and is in agreement with the previous finding that HCV replicon cells are resistant to TNF-α-mediated apoptosis (69). Direct physical interaction of PRK2 with NS5B may be implicated in the regulation of apoptosis through prevention of PRK2 cleavage. The C-terminal region of PRK2 produced by caspase-3 cleavage of PRK2 binds Akt and down-regulates its kinase activity and thus inhibits Akt downstream pro-survival signaling, such as BAD phosphorylation (61). Therefore, prevention of PRK2 cleavage by a direct interaction between NS5B and PRK2 or other mechanisms may contribute to the HCV-infected cells escaping from the host immune response mediated by liver-infiltrating cytotoxic T lymphocytes and sustaining the NS5B phosphorylation state.

In summary, we have identified PRK2 as the cellular kinase that binds and phosphorylates HCV NS5B both in vitro and in vivo. Our results indicate that NS5B phosphorylation by PRK2 has a positive effect on HCV RNA replication. What might be possible mechanisms for regulation of HCV replication by phosphorylation of NS5B? Phosphorylation of NS5B can lead to changes in its properties. First, as in the case of host DNA-dependent RNA polymerase II (70), phosphorylation of NS5B may play an important role for NS5B to be in the processive elongation form. Second, interaction of NS5B with other viral/cellular proteins (16–19, 71, 72) or homopolymeric oligomerization of NS5B (73) can be influenced by NS5B phosphorylation and/or by phosphorylation-induced conformation change of NS5B. Mapping of specific phosphorylation site(s) on NS5B and understanding how the phosphorylated form of NS5B regulates HCV RNA replication would certainly help us define further the role of NS5B phosphorylation in the life cycle of HCV. Because PRK2 is involved in the NS5B phosphorylation and HCV replication, knock-down of PRK2 expression by using a siRNA as shown in this work, or specific inactivation of PRK2 activity will provide an opportunity to interfere with HCV RNA replication.

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